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RCS MEDDH-288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

ANNUAL PROGRESS REPORT

1 July 1974 – 30 June 1975

VOLUME II

WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D.C. 20012

UNCLASSIFIED

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report.

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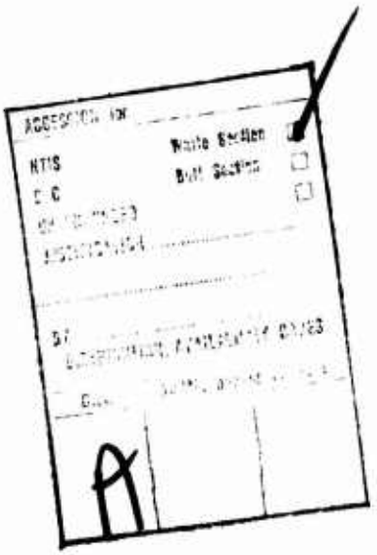

AD

RCS MEDDH - 288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

(Projects, tasks, and work units are listed in Table of Contents)

Annual Progress Report
1 July 1974 - 30 June 1975

Volume II

Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Researches, National Academy of Sciences - National Research Council.

SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.

TABLE OF CONTENTS

VOLUME II

PROJECT 3A762760A806
MILITARY PREVENTIVE MEDICINE

Task 00
Military Preventive Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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a. PRIMARY	62760A	3A762760A806	00	034
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11. TITLE (Precede with security classification code)

(U)Epidemiologic Studies of Military Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 005900 Environmental Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
72 07	CONT	DA			C. In-House

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19a. RESPONSIBLE DOD ORGANIZATION

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NAME: Kirchdoerfer, Richard G., MAJ, MC

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede each with security classification code)

(U)Epidemiology; (U)Tuberculosis; (U)Congenital Malformations; (U)Drug Abuse

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23.(U) To identify, define, and study known and potential causes of disability in military populations using relevant, existing epidemiologic techniques and developing appropriate new methodology. To apply this information to the prevention and control of disability in military populations.

24.(U) Contemporary epidemiologic methods are applied to causes of disability in military populations. Multidisciplinary collaborative approaches are utilized and new methods developed as required.

25.(U) 74 07-75 06 Serologic testing by a glycolipid antigen hemagglutination system of material from human and primate tuberculosis studies was terminated. Followup of personnel identified during a possible outbreak hepatitis at AFEES, Des Moines, and evaluation of persisting biochemical liver abnormalities were completed. Analysis of demographic and medical data obtained in the testicular cancer study was completed. A study of the incidence and trends of congenital malformations in military populations was begun. A prospective study of the military performance of drug users identified during BCT is reported elsewhere (DA Project A833-Work Unit 103). For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 74-30 Jun 75.

*Applicable to contractor upon originator's approval.

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Project 3A762760A806 MILITARY PREVENTIVE MEDICINE

Task 00 Military Preventive Medicine

Work Unit 034 Epidemiologic studies of military diseases

Investigators:
Principal: LTC Herbert E. Segal, MC
Associate: Major Joel C. Gaydos, MC; Dr. Alan S. Morrison; SSG Michael C. Callahan; L. Charlene Evans

1. Serologic Tests for Human Tuberculosis

A two year serum collection for use in evaluating the soluble antigen fluorescent antibody (SAFA) test for tuberculosis diagnosis was completed at Fitzsimons Army Medical Center. Serum specimens were collected from all tuberculosis admissions, both newly diagnosed and old, from patients with diseases of the chest other than tuberculosis, from tuberculin skin test converters, and from normal persons skin tested as a result of case finding efforts. 2,384 clinical histories and serum specimens were collected and resulting information coded and keypunched.

All sera have been studied by the SAFA test and results coded and keypunched. Analysis of the sensitivity and specificity of the test antigens and of the associations between test results and clinical variables has been deferred. Separate evaluation of a passive hemagglutination test employing serologically active glycolipids in collaboration with University of Maryland investigators was terminated.

2. Laboratory Test Values Relating to a Copying Process

While undergoing a routine physical examination on 1 September 1972 a 37 year old asymptomatic officer was noted to have dark urine which was positive for bilirubin. Four days later a 20 year old enlisted man reported anorexia and dark urine which he had noticed initially about 28 August. Between 6 and 28 September, urine specimens were collected from 62 and blood specimens were collected from 64 of the remaining 65 AFEES employees. Tests were done for qualitative urine bilirubin (UB), serum glutamic-oxalactic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and serum alkaline phosphatase (SAP). According to limits established by the laboratories performing the tests, seven men, ranging in age from 20 to 39 years, had one or more abnormal results. None of the seven was symptomatic. Of the total of nine men with abnormalities SAP was elevated in all, SGOT in four, SGPT in five, and bilirubinuria was present in seven. Only three had SGPT levels over 100 units.

During October, 1972, elevated serum bilirubin was detected in seven, as was relative eosinophilia. Of those having relative eosinophilia, total eosinophil counts (TOT EOS) were available for five. Four of these had counts greater than 300 eosinophils/cu.mm. All had normal values for blood urea nitrogen and serum creatinine, and negative spot tests for infectious mononucleosis. None was positive for hepatitis B antigen by counter-electrophoresis, and urine specimens from all nine were negative for narcotic drugs by thin layer chromotography.

Four of the nine men were hospitalized for the purpose of diagnosis. Included were the two men with the most striking test abnormalities. Needle biopsies of the liver were performed on all four. The specimens from two men showed mild necroinflammatory disease consistent with subsiding viral hepatitis. In addition, fat vacuoles were observed in one specimen. The specimens from the other two men showed mild hepatocellular unrest and mild fatty change.

The nine were questioned on place of residence, social events, acquaintances, travel, meals, alcohol consumption, medical and dental care, and specific duties at work. The only common exposure was working in a poorly ventilated room where records were kept and documents were copied (files/reproduction room). All had worked in this room on a full or part time basis in August 1972. Of the remaining 53 employees interviewed, 36 (68%) had been in the files/reproduction room during the same time period.

No accidents involving chemicals had been reported. However, one of the nine men, the second individual to come to medical attention, had started working in the files/reproduction room in July and had difficulty mixing a diazo developer powder with water. He reported that the powder dispersed in the air and caused choking and nausea. Only two affected men reported mixing the developing powder after 1 July. Six of the 53 remaining employees interviewed reported mixing the powder during the same time period.

During a working day atmospheric samples were obtained from various sites in the room and analyzed. Levels of petroleum hydrocarbons and methylene chloride were extremely low, and no other organic contaminants were found.

Three copying machines were used in the files/reproduction room. The constituents of the substances used in two of the machines, an offset direct image reproducing machine and an electrostatic copier, were determined by chemical analysis. These contained no benzene, toluene, xylene, chlorinated hydrocarbons, alcohols, or ketones. The laboratory supporting this

investigation did not have the capability to analyze the diazo developer powder used in the third machine.

One brand of diazo developer which had been in use was expended just prior to this investigation, and the batch number could not be identified. The same brand of developer powder as that which had been expended, but possibly a different batch, was studied toxicologically. Parenteral administration in dogs, rabbits, rats, monkeys, and guinea pigs did not produce detectable chemical hematological, or histopathological changes consistent with hepatic damage. The results of the guinea pig sensitization test showed the powder to be a potential skin sensitizing agent.

These initial studies suggested that some of the abnormal results might have been related to exposure to the developer powder used in the diazo copying machine. Since this machine is used widely in the military further epidemiologic investigation was undertaken. At four additional midwestern AFEESs which used the same diazo copying process employees were asked whether, during the month preceding the interview, they had mixed the diazo developer powder, had used the diazo copying machine, had worked in the room where the diazo machine was used or the developer was mixed, or had used other copying processes. Blood samples were obtained and tested for total serum bilirubin (TSB), SAP, SGPT, and percentage of eosinophils on differential count (EOS). Urine samples were examined for bilirubin using the Ictotest^{R} method.

Interviews were completed for 267 (91%) of 294 employees. Of the 267 with completed interviews, blood chemistry results were obtained for 219 (82%), EOS for 236 (88%), and UB results for 246 (92%).

All urine tests were negative. Too few individuals were found to have elevated SGPT (>35 Sigma-Frankel Units) or TSB (>1.2 mg/100 ml)---five and six, respectively---to draw any conclusions concerning the relationship of these blood chemistry values to the measures of exposure which were obtained. Elevated EOS was 2.2 times as frequent in those who mixed the diazo developer powder as in the remaining employees. However, this association was not statistically significant (X_C^2=2.5, p>.10). Other differences between exposed and non-exposed persons were quite small.

In the fall of 1973 follow-up was initiated on all nine men who had abnormal results during the initial investigation. At that time one was known to have chronic liver disease but the specific diagnosis was uncertain. In December 1973, a needle biopsy specimen of his liver was interpreted as showing changes consistent with sclerosing cholangitis. On the basis of the biopsy report and other diagnostic studies, including endoscopic retrograde cholangiopancreatography, his condition was considered to be primary sclerosing cholangitis.

Table 1 - Percentages of personnel with elevated SAP and EOS according to four measures of exposure to the diazo copying process (survey of four AFEES stations).

Measure of Exposure		Percentage with Elevated SAP (>35 Internat'l Units)	N	Percentage with Elevated EOS (>3 percent)	N
Use other copying machine but not diazo machine	Yes	23.0	74	13.5	74
	No	28.0	82	13.8	94
Work in diazo copying or mixing room	Yes	26.9	167	15.6	180
	No	26.9	52	14.3	56
Use diazo copying machine	Yes	31.2	64	18.8	69
	No	25.2	155	13.8	167
Mix diazo developer powder	Yes	31.6	19	30.0	20
	No	26.5	200	13.9	216

The other eight men agreed to provide blood and urine specimens. Because of marked abnormalities in liver function tests one of the eight was hospitalized. A bromsulphalein test revealed 10% at forty-five minutes. His liver biopsy showed mild fatty metamorphosis. Medical observation of these men will continue, as a responsibility of the Health and Environment Division, Office of The Surgeon General.

3. Cryptorchidism, Hernia, and Cancer of the Testes

All patients included in this study had been hospitalized for the first time with primary testicular cancer while in the U.S. Army between 1950 and 1970. An initial roster of 1,034 names was assembled from lists provided by the Armed Forces Institute of Pathology and the tumor registries of the seven Army teaching hospitals. The military records of each patient were requested once through an office of the Army Surgeon General. Records were obtained from facilities located at Fort Benjamin Harrison, Indiana, Alexandria, Virginia, and St. Louis, Missouri. Information not available in the records of patients discharged from active service was requested from the Veterans Administration when it was possible to identify the specific facility in which the necessary records were located.

During the abstracting of records 98 patients were excluded either because it was found that there was no diagnosis of a primary testicular malignancy, or because this diagnosis was not first made during active Army service in the period 1950-1970. Records were obtained for 702 eli-

gibile patients. Four patients were excluded because year of discharge or age at diagnosis was unknown. Indication of histologic confirmation of a primary testicular malignancy was found in the records of 95.8 percent of the remaining eligible patients.

The sampling frame for controls was a list of one-tenth of one percent of persons on active service during the study period. This list was assembled by selection on the terminal digits of the Army Service Number or Social Security Account Number, and was furnished by the Follow-Up Agency, Division of Medical Sciences, National Academy of Science-National Research Council. A roster of 1,325 names was constructed from all the listed names of men on active duty from 1960 through 1970 at an age of 30 or greater, and, by further terminal digit selection, one tenth of the remaining names in the sampling frame. Four individuals whose names had been included incorrectly in the initial roster were excluded from the control series; the service records of 624 eligible controls were retrieved. An additional 102 patients and 22 controls were excluded from the present analysis because information on genital tract defects was not available.

If subjects found to be ineligible for the study are excluded from the denominator, the rate of record retrieval was 75.0 percent for patients but only 47.2 percent for controls. The discrepancy was due principally to a major fire at the St. Louis record facility which destroyed a large number of records of servicemen discharged before 1960. Requests for the records of most patients had been acted upon before this fire, but retrieval of control records was in progress. However, it seems unlikely that loss of records in this fire could have led to serious bias.

This study was designed to use information on "exposure" characteristics which was recorded before the development of malignancy. Status with respect to a specific characteristic was considered to be that which existed at or before entry on active duty as indicated in the earliest available military personnel, medical history or physical examination form which contained the necessary information. Data from physical examination were accepted only if this took place before diagnosis of testicular cancer.

Subjects were considered to have an undescended testis if this was noted at physical examination or if this condition had been treated surgically. A hernia was considered to have been present only if it had been surgically treated before age 15. No subject began active service with an existing hernia.

Risk ratios were estimated by the odds ratio. Estimates were similar with and without adjustment for age, year of discharge and ethnic group. Therefore crude risk ratio estimates are presented here. Ninety-five percent confidence intervals (C.I.) and two-tailed p-values were computed by a modified exact-procedure.

Undescended testis was noted in 17 patients and in 2 controls (Table 1). The risk ratio estimate was 8.8 (2.3-56.3, 95% C.I.). In 12 patients this condition was present at physical examination. In the remaining five patients and the two control subjects, the condition had been treated surgically. The side of occurrence of undescended testis and of testicular tumor were closely related. Information on both the side of tumor and the side of undescended testis was available for 14 patients with the unilateral defect. In 12 the tumor occurred in the testis which had been undescended. In seven patients this occurred on the lef and in five, on the right (p=.02).

Table 1. Numbers of patients and controls, and risk ratio, according to history of undescended testis.

Undescended testis	Patients	Controls	Risk ratio
Yes	17	2	8.8*
No	579	600	1.0
Total	596	602	

*2.3-56.3, 95% C.I.

Undescended testis also was associated with histologic type. Of tumors developing in patients with undescended testis 65 percent (11/17) were seminomas. Thirty-eight percent (212/552) of the remaining tumors for which information was available were seminomas (p=.04).

Four patients who had had a hernia also had an undescended testis. After exclusion of all subjects with undescended testis it was estimated that testicular malignancy was 2.9 (1.3-7.0) times more likely to occur in a person who had reported an operation for hernia (Table 2).

Table 2. Numbers of patients and controls, and risk ratio, according to history of hernia operation before age 15.

Hernia operation	Patients	Controls	Risk ratio
Yes	22	8	2.9*
No	557	592	1.0
Total	579	600	

*1.3-7.0, 95% C.I.

Unlike undescended testis, the side of the hernia was unrelated to the side of the tumor which followed, and a hernia was not associated with tumors of a specific histologic type. There were 10 patients for whom in-

formation was available on both the side of a unilateral hernia and the side of the tumor. Of six left-sided tumors, three followed a left-sided hernia and three followed a right-sided hernia. Of four right-sided tumors, two followed each type of hernia. Histologic type was not known for one patient who had had a hernia. Thrity-three percent (7/21) of the patients for whom the histologic type was known had seminomas.

4. Studies in Yaviza Village, Republic of Panama

Census and other demographic data were obtained for the population (1,466 persons) of the eastern Panamanian village of Yaviza. A random sample comprising the occupants of 56% of the dwellings in the town was selected for venapuncture. Four hundred and sixty-six people (31.8% of the total population) provided venous blood specimens. Hemoglobin, hematocrit, hemoglobin electrophoresis, glucose-6-phosphate dehydrogenase, and Hepatitis B antigen and antibody studies were completed and results reported (WRAIR Annual Progress Report 1 July 1973-30 June 1974, pp 824-5). Further serologic testing, analysis of data, and correlation with demographic variables has been deferred.

5. Personnel Identified as Drug Users During Basic Combat Training-Performance During First Military Tour

A prospective study is in progress in collaboration with Division of Neuropsychiatry investigators; results and discussion of this study are reported under DA Project: A762758A823, Work Unit 030, Military psychiatry (NP).

PROJECT 3A162110A821
COMBAT SURGERY

Task 00
Combat Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with security Classification Code)

(U) Gastrointestinal Responses to Shock and Trauma

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008800 Life Support 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 09	CONT	DA	C. In-House

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c. TYPE: d. AMOUNT:
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18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	3	30
FISCAL YEAR CURRENT 76	4	206

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Gurll, MAJ N. J. DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede each with security classification code)

(U) Splanchnic Hemodynamics; (U) Gastric Blood Flow; (U) Shock; (U) Vasoconstrictor Therapy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To elucidate the gastrointestinal response to shock and trauma. To evaluate the effects of epinephrine and norepinephrine on gastric blood flow. To evaluate the pharmacological effects of drugs currently used to control gastrointestinal hemorrhage. To study the effect of hemorrhagic shock on the development of stress ulceration. These studies relate to the gastrointestinal pathology that frequently occurs in combat casualties.

24 (U) To study the gastrointestinal hemodynamic response to experimental hemorrhagic shock in the primate and dogs. To study the functions of the gastric mucosal barrier during hemorrhagic shock. To evaluate the effects of adrenergic amines and vasopressin on the gastric circulation.

25 (U) 74 07 - 75 06. The primate gastric vasculature seems to be controlled predominantly by alpha adrenergic receptors in contrast to that in the dog. In the primate gastric circulation epinephrine, norepinephrine, and vasopressin produce sustained decreases in total gastric blood flow with no redistribution of flow or increased AV shunting. In contrast to previous statements concerning the use of these catecholamines for the control of upper gastrointestinal hemorrhage we now feel that epinephrine and norepinephrine may provide an alternative to vasopressin for the control of hemorrhagic gastritis. 2. Whereas total body oxygen consumption may not decrease during systemic hypotension as the result of hemorrhagic shock, gastric oxygen consumption decreases significantly. The results of this study indicated that this ischemia in the presence of normal amounts of acid back diffusion is a significant contributor to development of stress ulcerations following hemorrhagic shock.

For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 74-30 Jun 75.

* A registry or progress and evidence approval.

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Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Gastrointestinal responses to shock and trauma

Investigators
Principal: LTC David G. Reynolds, MSC
Associate: MAJ Nelson J. Gurll, MC; MAJ Michael J. Zinner, MC; and John C. Kerr, B.A.

I. Adrenergic Mechanisms in the Primate Gastric Circulation

A. Background and Statement of the Problem. The direct, selective catheterization of major arterial branches of the splanchnic viscera and the prolonged infusion of various vasoconstrictor agents have gained popularity for the control of upper gastrointestinal hemorrhage. Vasopressin and epinephrine have become the most popularly used vasoconstrictor agents in this respect.[1,2,3] Previous reports from this laboratory demonstrated autoregulatory escape and active vasodilation when epinephrine was infused into the canine gastric circulation. The phenomenon demonstrated in the dog would caution against the use of such agents if they acted in this manner in man. In other studies from this laboratory, species differences between dogs and primates have demonstrated that autoregulatory escape may not exist in the primate splanchnic circulation. Therefore, a study was conducted to evaluate the effects of adrenergic amines on gastric blood flow in baboons.

B. Experimental Approach. Following splenectomy, the baboon's celiac artery was isolated and blood flow measured electromagnetically. The hepatic artery was cannulated for intra-arterial injections and infusions of the adrenergic amines, epinephrine, norepinephrine, and isoproterenol. Arterial and portal pressures were monitored and vascular resistance calculated. The effects of these catecholamines were measured before and after alpha or beta adrenergic blockade.

C. Results and Discussion.

1. Epinephrine. The intra-arterial injection of epinephrine in doses from 10^{-3} to 10^{0} μg (base)/kg produced a dose dependent vasoconstrictor response. There was no evidence of the biphasic (constrictor and dilator) response seen in the canine model. When this drug was infused intra-arterially into the celiac artery for 10 minutes at a dose (.05 μg/kg-min) there was a sustained decrease in total gastric blood flow without evidence of autoregulatory escape. Alpha adrenergic blockade with phenoxybenzamine attenuated the constrictor response and beta adrenergic blockade with propranolol did not influence the responses at all.

2. Norepinephrine injections over the same dose range produced similar vasoconstrictor responses. However, these responses were of a lesser magnitude than those seen with the epinephrine at comparable doses. When norepinephrine was infused at the same dose, there also was a sustained decrease in gastric blood flow with no evidence of autoregulatory escape. Alpha adrenergic blockade attenuated the constrictor response while beta adrenergic blockade did not influence the responses.

3. Isoproterenol. Isoproterenol caused vasodilation whether injected or infused into the gastric circulation. The magnitude of these responses was significantly less than those seen in canine gastric circulations. Alpha adrenergic blockade did not affect the magnitude of these responses but beta adrenergic blockade significantly attenuated them.

These data suggest that the gastric vasculature in the primate is significantly different than in the dog. Whereas in the canine gastric circulation there appears to be abundant beta adrenergic receptors, the primate gastric circulation seems to have predominantly alpha adrenergic receptors. In the primate gastric circulation epinephrine and norepinephrine produced sustained decreases in total gastric blood flow. In contrast to our previous statements concerning the use of catecholamines for the control of upper gastrointestinal hemorrhage, we now feel that epinephrine and norepinephrine may provide an alternative to vasopressin for the control of hemorrhagic gastritis.

II. Regional Distribution and Arteriovenous (AV) Shunting in the Gastric Circulation

A. Background and Statement of the Problem. The role of AV anastomosis and redistribution of regional blood flow in the stomach has been indicated as an etiologic factor in the development of stress ulceration.[4] The opening of AV shunts in the submucosa of the stomach as a result of circulating catecholamines may be the reason for gastric mucosal ischemia during endotoxic shock. Vasopressin infusion is thought to be useful to controlling the bleeding from hemorrhagic gastritis because it is thought to open AV shunts in the stomach. A study was conducted on the effect of long-term intra-arterial infusions of epinephrine and vasopressins on blood flow, regional distribution, and AV shunting in the gastric circulation of baboons.

B. Experimental Approach. Total gastric blood flow was measured electromagnetically by placing a flow transducer on the celiac artery of animals that had been splenectomized and had common hepatic artery ligation. The hepatic artery was used for the intra-arterial delivery of epinephrine in doses of .05 μg/kg-min and vasopressin .005 units/kg-min for 60 minutes. Radioactive microspheres (15 $\pm$ 5 μ) with 3 tags were injected intra-arterially during a control period and 50 minutes after the initiation of each infusion to determine regional distribution of blood flow and AV shunting in the stomach. Arterial and portal venous pressures were monitored and vascular resistance calculated.

C. Results and Discussion. At these doses the intra-arterial infusions of epinephrine and vasopressin caused a decrease in total gastric blood flow of approximately 70 to 80%. Throughout the entire 60 minute period of the infusion there was no evidence of autoregulatory escape with either agent. Approximately 75% of the total gastric blood flow goes to the gastric mucosa and approximately 2% of the injected microspheres appear in the liver through AV anastomoses. There was neither a significant increase in the number of microspheres appearing in the liver nor any redistribution of flow with either vasoconstrictor agent. Arteriovenous anastomoses play a negligible role in gastric blood flow and are not influenced by epinephrine or vasopressin.

III. Stress Ulceration - Etiology

A. Background and Statement of the Problem. Acute superficial erosions of the gastric mucosa in the post-traumatic patient continues to be a serious source of life threatening hemorrhage. Although the etiology of this lesion remains unclear, recent attention has been directed at the role of the gastric mucosal barrier (GMB) in a pathogenesis of stress ulcers. Disruption of the normal gastric mucosal barrier results in increased permeability with greater back diffusion of hydrogen and increased luminal accumulation of sodium. It has been reported in critically ill patients that disruption of the GMB plays a role in the pathogenesis of stress ulceration.[5] We have investigated the effects of severe hemorrhagic hypotension on the integrity of the gastric mucosal barrier in dogs.

B. Experimental Approach. Dogs with internally drained Heidenhain pouches were prepared that had their blood supply solely on the splenic artery. The net fluxes of hydrogen, sodium, water, and potassium were determined in these pouches by instillation and recovery of an acid test solution containing a nonabsorbable marker. This was done during a control period, during the 3 hours of hemorrhagic hypotension, and the hour following reinfusion of shed blood. Total pouch blood flow was measured electromagnetically and the mucosal blood flow was measured by an aminopyrine clearance technique. Measurement of the pouch oxygen consumption was determined by knowing flow and measuring arterial venous oxygen difference across the pouch. The transmucosal electrical potential difference was also measured.

C. Results and Discussion

1. Systemic Hemodynamic Changes. Mean arterial pressure and cardiac output fell significantly during the 3 hour shock period and then increased following the reinfusion of shed blood. Total body oxygen consumption, however, did not significantly decrease during the shock period because of increased total body oxygen extraction. Even though there was no evidence of decreased oxygen consumption, there was a significant increase in the level of circulating lactate implying some areas of regional ischemia.

2. Gastric Pouch Hemodynamic Changes. There was a significant decrease in total pouch gastric blood flow as well as a significant decrease in mucosal blood flow. Although total pouch blood flow remained decreased through the 3 hours of hypotension, there was a slight increase in the mucosal blood flow during the shock period implying a possible redistribution of blood flow toward the mucosa during shock. Whereas there was no significant decrease in total body oxygen consumption, there was a significant decrease in pouch oxygen consumption during the shock period.

3. Potential Difference and Ionic Permeability. There was no increase in the ionic permeability to hydrogen or sodium during the shock period or following reinfusion. There was a steady rise in the amount of potassium appearing in the pouch lumen during the period of hemorrhagic hypotension and following reinfusion. There was a decrease in the net positive chloride flux during the hemorrhagic period. In concert with this fall in the chloride flux there was a fall in the transmucosal potential difference during the hemorrhagic period and following reinfusion.

4. Gross and Microscopic Examination. Nine of the 10 animals that were subjected to hemorrhagic shock with an acid test solution developed acute, superficial gastric erosions confirmed by histologic examination. The majority of dogs in this group had evidence of gross bleeding sometime during the shock period or recovery period. In a parallel group of dogs that underwent the same degree of hemorrhagic hypotension but had no acid present in their gastric pouch lumen, there were no gastric erosions or ulcerations.

Even with a severe anoxic and ischemic insult to the gastric mucosa there appears to be no increase in the ionic permeability and no breakdown of the gastric mucosal barrier in the canine model. However, acid in the lumen of the stomach in the presence of severe ischemia is sufficient to cause acute superficial erosions of the gastric mucosa. This probably results from an inability of the ischemic and anoxic mucosal cells to buffer the normal amount of acid that is back diffusing.

IV. Cardiovascular Effects of Urokinase

A. Background and Statement of the Problem. Urokinase is a plasminogen activator which has been used successfully to treat arterial and venous thrombosis. Large intravenous doses of urokinase have produced systemic hypotension in some patients, and direct intrapulmonary arterial injection has lowered right heart pressures in patients with pulmonary embolism. These clinical observations suggest that urokinase has vasomotor effects which have not been previously described. A variety of thromboembolic conditions such as acute mesenteric insufficiency have associated vasospasm which may be amenable to urokinase infusion.

B. Experimental Approach. Electromagnetic flowmeters were used to measure arterial blood flow in the femoral and mesenteric circulations of the dog. Urokinase isolated from both human urine and tissue culture was injected and infused intra-arterially and effects on arterial blood pressure, arterial blood flow, and venous pressure were recorded. The possible role of other well known vasodilator mechanisms (histamine, β-adrenergic receptors, kinin formation from fibrinolysis) was assessed using specific blocking agents.

Obstructing thrombi were produced in the femoral and mesenteric circulations of the dog. The effects of urokinase infusion relative to vasodilation, thrombolysis (arteriogram), and fibrinolysis (fibrin plate and euglobulin lysis time) were determined.

C. Results and Discussion. Human urine urokinase (3 different preparations) caused a dose-dependent increase in arterial blood flow in both the femoral and mesenteric circulations. This increase in flow was not attenuated by antihistamine treatment or beta adrenergic blockade. Inhibition of plasminogen activation by ε-aminocaproic acid (EACA) attenuated the vasodilation somewhat at a dose that completely inhibited fibrinolysis as measured by euglobulin lysis time and fibrin plate assay. However, this attenuation by EACA was nonspecific vasodilation due to isoproterenol was also attenuated. Tissue culture urokinase did not produce vasodilation. Trasylol (an inhibitor of kallikrein as well as activated Hageman factor) also failed to inhibit the vasodilation. Therefore, urokinase appears to be a direct vasodilator of the canine femoral and mesenteric circulations.

Local intra-arterial infusion of urokinase at a dose that produced only small increases in blood flow in a control experiment was successful in lysing ipsilateral obstructing femoral thrombi but not contralateral obstructing femoral thrombi. The contralateral thrombi were exposed to the systemic fibrinolysis of the urokinase but arterial flow was not re-established. Clots in the superior mesenteric artery were easily dissolved with urokinase at doses below those producing a vasodilation, and there was no difference between tissue culture urokinase and human urine urokinase in this thrombolysis.

V. Portal Venous and Hepatic Arterial Blood Flow Relationship

A. Background and Statement of the Problem. Experiments were conducted to test the premise that an inverse relationship exists between portal venous and hepatic arterial blood flows. The validity of this thesis is important to the management of surgical conditions that influence the flow in either circulation.

B. Experimental Approach. Hepatic arterial blood flow was measured electromagnetically in a group of anesthetized dogs. The effects of graded constriction of the portal vein, to reduce portal venous blood flow, were determined on hepatic arterial flow, portal venous pressure, and systemic arterial pressure.

C. Results and Discussion. Graded constriction of the portal vein caused a rise in distal portal pressure and significant declines in both hepatic arterial blood flow and aortic pressure. The data suggest no reciprocal relationship between flow in these two circulations.

VI. The Proceedings of The Symposium on the Splanchnic Circulation in Shock, sponsored by this Institute three years ago, have been completed and published through the Government Printing Office.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Gastrointestinal responses to shock and trauma

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6532	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A821	00	124
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Provide with Security Classification Code):

(U) Pathophysiology of Systemic Responses to Shock and Trauma

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:

012600 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA | | -	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	2	50
FISCAL YEAR CURRENT 76	2	82

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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20. PERFORMING ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME: Giordano, MAJ J.M.
NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide EACH with Security Classification Code): (U) Abdominal Abscesses; (U) Vein Grafts; (U) Irradiated Homografts; (U) Abscess Scanning

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Provide text of each with Security Classification Code.)

23 (U) To evaluate changes in physiologic mechanisms that may occur subsequent to shock or trauma. To evaluate the suitability of various materials for use as venous prostheses. These studies bear directly on improving vascular reconstructive surgery and understanding pathology resulting from combat wounds.

24 (U) The use of radioactive gallium and indium to identify abdominal abscesses was evaluated by scintillation scanning. The infectivity of various graft materials was evaluated in the abdominal aortas of dogs. Frozen, irradiated homogenous vein was tested for suitability for grafting into the femoral venous circulation.

25 (U) 74 07 - 75 06. Both radioactive gallium and indium proved to be effective in identifying experimental abscesses in the abdominal cavity after 5-7 days. However, gallium proved to have the widest clinical potential by yielding a better resolution of the scintigram and clearer identification. There was no significant difference in infectivity of dacron grafts, autogenous vein grafts, or bovine heterografts placed in the aortas of dogs challenged with intravascular microorganisms. Fresh frozen, irradiated homogenous vein grafts proved as suitable for vein grafting as autogenous vein suggesting their potential use in a clinical setting.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 124 Pathophysiology of systemic responses to shock and trauma

Investigators.
Principal: LTC Robert W. Hobson, II, MC
Associate: MAJ Joseph M. Giordano, MC

I. Adrenergic Mechanisms in the Cerebral Circulation

A. Statement of the Problem. A study was conducted to evaluate and contrast the adrenergic mechanisms in the cerebral and cephalic circulations of baboons.

B. Experimental Approach. Carotid arterial blood flow was measured electromagnetically with both internal and external branches open and then with the external branch obstructed. The effects of epinephrine (E), norepinephrine (NE), phenylephrine (P), and isoproterenol (ISO) were monitored by injecting and infusing the amines directly into the carotid artery.

C. Results and Discussion. Injections limited to the external carotid or cephalic circulation resulted in predictable dose dependent vasoconstrictive effects with E, NE, and P and vasodilator responses to ISO. However, no significant changes were seen with injections of the amines into the internal carotid or intracranial circulation unless associated with significant alterations in systemic pressure at the highest dosages. These data suggest the presence of reactive adrenergic receptor sites in the cephalic circulation of the baboon; however, these sites appear less reactive or possibly not pharmacologically related to the vasculature in the intracranial circulation of the baboon.

II. Scintigraphic Identification of Abscesses

A. Statement of the Problem. Identification of abscesses by isotopic scanning techniques has been useful to confirm clinical impressions of deep abscesses and assist in formulating treatment. A study was conducted to compare the effectiveness of radioactive gallium and indium in localizing abdominal abscesses.

B. Experimental Approach. Abscesses were created in the anterior abdominal wall of rabbits by fecal contamination and scanned following intravenous injection of ^{67}Gallium (Ga) citrate and ^{111}Indium (In) chloride.

C. Results and Discussion. The photoscans were clearest with established abscesses, 5-7 days old, 24 hours after intravenous injection of the isotopes. Although ^{67}Ga has had widest clinical application,

^{111}In appears to be an equally acceptable radiopharmaceutical for scintigraphic identification of abscesses. Further clinical evaluation is indicated.

III. Vincristine Induced Intestinal Pathology

A. Statement of the Problem. Vincristine sulfate, a vinca alkaloid, has been used successfully in the treatment of several malignant neoplasms. In a clinical report (Am Surg 39: 129, 1973), intestinal complications possibly caused by vincristine toxicity were described.

B. Experimental Approach. To evaluate the gastrointestinal toxicity of this chemotherapeutic, guinea pigs were treated with intraperitoneal and intravenous doses of vincristine sulfate. Changes in small bowel histopathology and intramural myenteric plexuses were evaluated.

C. Results and Discussion. Vincristine appears to have a two-fold effect on the small bowel; it interferes with normal mucosal renewal processes, producing mucosal atrophy, and it alters the structural appearance as well as catecholamine content of the intramural nerve plexuses. These factors may explain gastrointestinal side-effects of the drug.

IV. Infectability of Vascular Grafts

A. Statement of the Problem. Infection in vascular reconstructions is a serious complication frequently resulting in loss of limb or life. Direct or bacteremic contamination of the graft accounts for these infections.

B. Experimental Approach. To evaluate the influence of particular graft materials in infections of bacteremic origin, the canine infrarenal aorta was reconstructed with dacron (10 animals), autogenous vein (10 animals) and bovine heterograft (9 animals). Following reconstruction, the animals received 10^7 colony forming units of Staphylococcus aureus intravenously over a 2 hour period. Three weeks later, grafts were removed aseptically and cultured.

C. Results and Discussion. Four of ten dacron, two of ten autogenous veins, and one of nine bovine heterografts were positive for the bacteria infused. No significant difference in rate of infection is noted suggesting that the graft material itself is not a major factor in susceptibility of vascular reconstructions to infection of bacteremic origin in dogs.

V. Determination of Intestinal Viability

A. Statement of the Problem. Easily performed operative tests are not available to evaluate viability of small bowel after ischemia. The Doppler ultrasound device is capable of detecting flow in the small vessels on the serosal surface of the bowel. The feasibility of using the Doppler flowmeter to determine viability was evaluated.

B. Experimental Approach. In isolated loops of canine small bowel made ischemic by temporary mesenteric vascular occlusion Doppler signals were absent in intestinal segments subjected to arterial obstruction.

C. Results and Discussion. Following release of the vascular occlusion, presence or absence of Doppler sounds correlated with ultimate intestinal viability. Use of the Doppler ultrasound device is suggested to assist in determining intestinal viability prior to intestinal resection after trauma or mesenteric vascular accidents.

VI. The Development of a Suitable Graft for Use in the Venous System

A. Statement of the Problem. Previous work in this laboratory has documented the effectiveness of homografts as venous substitutes in canine femoral venous reconstruction. However, to make such a graft clinically useful an appropriate method of preservation must be found. Frozen, irradiated, homogenous vein was evaluated for suitability as vein grafts.

B. Experimental Approach. Venous homografts were frozen and subjected to 2 million rads of irradiation. Bilateral femoral cutdowns were performed in 20 mongrel dogs. A fresh autogenous graft was anastomosed into one femoral vein. On the opposite side a frozen irradiated homograft was anastomosed. Venograms were taken immediately after surgery and at 1, 3, and 6 weeks and 6 month intervals.

C. Results and Discussion. The venograms showed that there was no difference in patency between the autografts and the frozen irradiated homografts. We conclude from this study that frozen irradiated homografts may be a suitable substitute to autogenous veins for defects in the venous system. During the course of the investigation we found that at 6 months the frozen irradiated homografts recanalized at the same rate as the autogenous vein grafts. Autogenous vein grafts have been known to recanalize over a period of time. However, it has not been previously reported that frozen irradiated homografts or any other biological grafts can recanalize in the venous system.

VII. The Effects of Microaggregates on Pulmonary Vascular Resistance

A. Background and Statement of the Problem. It has been previously reported that microaggregates form in stored blood. A number of reports both experimentally and clinically have suggested that these microaggregates are the etiologic cause of the post-traumatic pulmonary insufficiency syndrome.

B. Experimental Approach. In a study recently completed we infused microaggregates into the left pulmonary artery following a shock period. If microaggregates had any effect on pulmonary vascular resistance, there should be a redistribution of blood from the left lung to the right lung.

Pulmonary blood flow and distribution were determined by central injections of microspheres.

C. Results and Discussion. The data show that despite massive amounts of microaggregates infused into the left PA, there was no redistribution of blood flow. We conclude that microaggregates do not affect pulmonary vascular resistance and may not be an etiologic agent in post-traumatic pulmonary insufficiency. We will repeat this study in a non-shocked preparation and in the baboon.

VIII. Renal Cortical Contractility

A. Statement of the Problem. Interstitial fibroblasts in the cortex of kidneys subjected to complete ureteral obstruction undergo transformation into cells resembling smooth muscle. Studies were conducted to assess the contractile capabilities of these cells.

B. Experimental Approach. Strips of cortical tissue were prepared from kidneys that had been subjected to 8 and 32 days of ureteral obstruction as well as from the contralateral, control kidneys. The strips were mounted in tissue chambers containing a balanced electrolyte solution and subjected to pharmacologic stimulation to yield isometric contraction.

C. Results and Discussion. Norepinephrine, angiotensin, and serotonin caused dose related contractions of the tissues. Incubation of the tissues with phenoxybenzamine attenuated the alpha adrenergic responses but not those resulting from angiotensin or serotonin. These transformed cells thus appear to be responsive through multiple mechanisms of excitation.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 124 Pathophysiology of systemic responses to shock and trauma

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6527	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A821	00	125
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 11AF			

11. TITLE (Procede with Security Classification Code)

(U) Control Mechanisms of Regional Circulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	2	62
FISCAL YEAR CURRENT 76	2	107

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Woods, MAJ M.

NAME: DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Oxygen Consumption; (U) Tissue Respiration; (U) Vascular Perfusion

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23 (U) To define the mechanism of regulating regional circulation, the influence of oxygen delivery and oxyhemoglobin affinity on tissue respiration. These studies apply directly to respiratory support of critically soldiers.

24 (U) Physiologic techniques have been employed to document the response of intact tissues to varieties of stress. Tissue respiration, lactate metabolism and viability were evaluated.

25 (U) 74 07 - 75 06. Control mechanisms of regional circulation. Completed studies in an isolated perfused canine hindlimb indicate that tissue oxygen uptake is related to the pH of the perfusing blood. Alkalosis is a profound stimulus to lactate production in an isolated canine limb. A rightward shift of the oxyhemoglobin dissociation curve (towards lower oxyhemoglobin affinity) permits increased oxygen off-loading at the tissue level and an increased oxygen uptake.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.

* Available to contractors upon requestor's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 125 Control mechanisms of regional circulation

Investigators.
Principal: MAJ Alden H. Harken, MC
Associate: MAJ Monty Woods, MC

I. Factors Influencing Tissue Respiration

A. Background and Statement of the Problem. Oxygen utilization by tissues is influenced by the chemical composition of the perfusing blood and cardiovascular reflexes. Variation in the chemical composition of the blood exerts influences both centrally and peripherally. Experiments were performed in isolated canine hindlimbs which were perfused under highly controlled conditions. The effects of variation in pH, O_2 and CO_2 content, lactate, and endotoxin on respiration were determined.

B. Experimental Approach. A membrane lung perfusion system was devised with which a vascularly isolated but viable extremity could be perfused with blood of controlled composition. The chemical composition of the perfusing blood was changed by altering pH, PO_2, PCO_2, using stored rather than fresh blood, and adding endotoxin and steroid. The effects of these alterations on tissue respiration were measured.

C. Results and Discussion. Oxygen consumption (V_{O_2}) was related to the blood's pH by the equation $V_{O_2} = 100.1\ pH - 1643$ ($r = 0.86$); thus a change in acidity of the perfusing blood by 0.1 pH unit causes a 10% change in limb V_{O_2}. When blood pH was above 7.3, lactic acid was produced and when pH was below 7.3, it was consumed. The relationship between the A-V lactate difference and blood pH is expressed by Lactate = 22.5 pH - 162 ($r = 0.75$). Lactic acid production does not reflect tissue oxygenation during clinical alkalosis. In seventeen dogs the addition of 5 mg of endotoxin to the perfusate reduced V_{O_2} significantly. The eventual addition of 60 mg of methyl-prednisolone returned V_{O_2} toward control. Variation of the hemoglobin-oxygen affinity by perfusing the legs with 2-4 week old blood revealed reduced V_{O_2} when the P_{50} of the perfusate was lowered.

These studies demonstrate several relationships between the chemical composition of blood and tissue respiration that are relevant to anesthesia, surgery, or blood banking. In addition evidence is presented in support of steroids being of benefit in shock therapy.

II. In Vitro Studies of Liver Respiration

A. Background and Statement of the Problem. The relationship between extracellular fluid oxygen tension and hepatocyte oxygen uptake was evaluated in 70 rabbits. Normal extracellular fluid (ECF) oxygen tension is 30 torr. It was expected that hepatocyte oxygen uptake would decrease at ECF oxygen tensions below 30 torr - presumably due to inadequate diffusion of oxygen into cells. Liver cell oxygen uptake at ECF oxygen tensions above 30 torr was not expected to change.

B. Experimental Approach. Oxygen consumption of slices of rabbit liver was measured in chambers fitted with oxygen electrodes. The effects of variation of pO_2 in the electrolyte solution and addition of endotoxin on tissue respiration was determined.

C. Results and Discussion. Oxygen uptake (V_{O_2}) was maximal at an extracellular fluid pO_2 of 30 torr. At a pO_2 of 10 torr V_{O_2} was significantly reduced. However, if pO_2 were increased to 90 torr, V_{O_2} was also reduced. These observations indicate that hepatocyte respiration is optimal at low pO_2s within a narrow range.

The action of endotoxin was evaluated on both slices and homogenates of liver. In both models, endotoxin induced a significant reduction of V_{O_2}. It would, therefore, appear that the hepatocyte cell membrane does not protect the cell from the detrimental effects of endotoxin.

III. The Clinical Use of a Pulmonary Artery Thermistor Catheter

A. Statement of the Problem. Much enthusiasm has been generated concerning the clinical value of a pulmonary artery thermistor catheter for the measurement of pulmonary artery wedge (left atrial) pressure and cardiac output.

B. Experimental Approach. The use of a pulmonary artery thermistor catheter for pressure measurement and thermodilution cardiac output determination was evaluated in eleven dogs. A pulmonary catheter was placed in the wedge position. A left atrial catheter was simultaneously placed via a superior pulmonary vein.

C. Results and Discussion. Pulmonary artery wedge pressure was a reliable index of left atrial pressure at end expiratory pressures less than 10 cm H_2O. Fluctuations in pulmonary artery temperature occurred at a frequency equal to the respiratory rate and an amplitude of 0.010° C to 0.086° C. Changes in amplitude were associated with changes in ventilatory waveform, respiratory rate and level of anesthesia. Intermittent and continuous positive pressure ventilation generally dampened and reversed the pulmonary artery temperature pattern exhibited during spontaneous breathing. This suggested that when end expiration is used to time indicator injection, cardiac output will be underestimated during

spontaneous breathing and overestimated during continuous or intermittent positive pressure ventilation. When indicator was injected at the same point in the ventilatory cycle, successive values of cardiac output deviated from one another by 0.0 - 6.7%. Deviations as large as 14% resulted if sequential injections were out of phase by half a respiratory cycle. Deviations in measured cardiac output can be minimized by injecting indicator at the same point in the respiratory cycle if it is not feasible to measure cardiac output during apnea. The clinical utility of a pulmonary artery thermistor catheter can be optimized through appreciation of its specific strengths and limitations.

IV. Systemic Oxygen Delivery, Oxygen Uptake, and Surgical Stress

A. Statement of the Problem. Recent advances in respiratory physiology and technology have permitted and promoted endotracheobronchial maneuvering on a scale not previously considered safe. The ventilatory and hemodynamic sequelae of these diagnostic and therapeutic interventions are often not fully appreciated by the operating surgeon. Straight and fiberoptic bronchoscopes at best only partially obstruct large bronchi with resultant decrease in ventilation: perfusion ratio. Conversely, continuous positive pressure ventilation may decrease pulmonary blood flow with resultant increase in ventilation: perfusion ratio. The purpose of this study was to evaluate the relationship between systemic oxygen delivery and tissue oxygen uptake during surgical stress.

B. Experimental Approach. Ten, 25 kg, mongrel dogs were anesthetized with pentobarbital (25 mg/kg). The dogs were intubated and ventilated (15 ml/kg) at a rate of 16 breaths per minute. A femoral artery catheter was placed. Cardiac output was measured by thermal dilution. All parameters (cardiac output, arterial and venous blood gases, pulmonary artery pressure and oxygen consumption) were measured as soon as all catheters were in place (period #1). Baseline values were again measured 15 minutes following the initial determination (period #2). All parameters were subsequently measured under the following conditions:

Period #3. 10 cm H_2O positive end expiratory pressure
Period #4. Right thoracotomy with equilibration of intrapleural and atmospheric pressure
Period #5. Right main stem bronchial occlusion with right chest up
Period #6. Right main stem bronchial occlusion with right chest down
Period #7. Right main stem bronchus released following several deep breaths, right chest up
Period [illegible]. 10 cm H_2O positive end expiratory pressure (pleural pressure at zero)
Period [illegible] 10 cm H_2O positive end expiratory pressure and 100% oxygen (FiO_2 = 1.0)
Period #10. Thoracotomy closed, intermittent positive pressure ventilation

Following the initial thoracotomy, a monitoring catheter (PE 240) was placed into the left atrium of each animal.

C. Results and Discussion. Systemic oxygen delivery (SOD) and oxygen uptake (V_{O_2}) decreased initially in parallel then SOD decreased more than V_{O_2}. Following closure of the thoracotomy, SOD was down by 60% and V_{O_2} was 80% of control levels. SOD and V_{O_2} did not relate to each other in a parallel fashion. Following moderate surgical stress, V_{O_2} at the end of the procedure was 80% of control levels.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 125 Control mechanisms of regional circulation

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PROJECT 3A762760A822
MILITARY INTERNAL MEDICINE

Task 01
Military Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OA 6469	75 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	01	120
b. CONTRIBUTING				
c. XXXXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Metabolic Response to Disease and Injury

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 003500 Clinical Medicine 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	75 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	9	225
	CURRENT 75	9	167

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

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NAME: Schaaf, MD, M. DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Metabolic; (U) Stress; (U) Endocrine; (U) Hormone

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Investigation into basic mechanisms of diseases of military importance and the metabolic response of patients during stress of disease and injury to provide rational approach to therapy.

24. (U) Metabolic balance studies with precise collection of biologic samples from patients under rigidly controlled diet, drugs, and activity. Development of techniques to measure alterations in homeostasis produced by disease or drugs. Provide clinical support and teaching for the Walter Reed Army Medical Center.

25. (U) 74 07 - 75 06. Hyperglucagonemia is said to play a role in the abnormal carbohydrate metabolism and negative nitrogen balance observed in infectious and other stresses. Insulin, growth hormone, glucagon and urinary C-peptide were measured in four individuals who were maintained in a constant caloric intake after receiving a standard dose of malaria parasites. All became ill but there was no consistent change in insulin-glucagon ratios or insulin secretion as reflected by urinary C-peptide. Sixty-one patients with galactorrhea were studied to determine if alterations of prolactin physiology might identify those with pituitary tumors. Tests of prolactin included thyrotropin releasing hormone (TRH) stimulation, chlorpromazine stimulation, L-dopa suppression, and water loading. L-dopa and water loading were not useful in the differential diagnosis. A high basal prolactin which failed to respond to either TRH or chlorpromazine was characteristic of pituitary tumors and idiopathic galactorrhea associated with amenorrhea. This work unit is being discontinued due to the transfer of the Metabolic Department from the Walter Reed Army Institute of Research to Walter Reed Army Medical Center. The work will continue in association with the Clinical Research Service. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 74-30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 120 Metabolic response to disease and injury

Investigators:

Principal: COL Jerry M. Harll, MC

Associates: Marcus Schaaf, M.D.; LTC Leonard Wartofsky, MC; MAJ Richard C. Dimond, MC; Joseph Bruton, Ph.D.

Description

This work unit is concerned with investigations into basic mechanisms of diseases of military importance and the metabolic responses occuring during stress of disease and injury. To provide rational approaches to diagnosis and therapy, metabolic balance studies are utilized which require precise collections of biologic samples from patients during rigid control of diet, drugs, and activity. In addition, support is afforded Walter Reed Army Medical Center in training house staff, four endocrine fellows, diagnosis and treatment of endocrine patients, and technical laboratory support to other departments. The unit maintains the capability of mounting field studies.

Progress

1. Polypeptide Hormone Metabolism.

Stressful illnesses such as severe infection, trauma, burns and fetal distress are said to be characterized by a negative nitrogen balance, an inappropriately low concentration of insulin and a high level of glucagon relative to glucose availability (R.H. Unger, Alpha and Beta Cell Interrelationships in Health and Disease, Metabolism, 23:581-592, 1974). Serum insulin, growth hormone, glucagon, glucose, and urinary C-peptide were measured in four human volunteers who received a standard dose of malaria parasites while being maintained on a constant caloric intake. Although all developed malaria, there was no consistent change in insulin-glucagon ratios or insulin secretion as reflected by the urinary C-peptide.

Sixty-one patients with galactorrhea were studied to determine which tests might differentiate normal from abnormal prolactin (PRL) secretion and separate patients with pituitary tumors from those with galactorrhea of other etiologies. Six types of galactorrhea were studied: (I) fourteen patients with pituitary tumors; (II) ten patients with galactorrhea associated with oral contraceptives; (III) ten patients with idiopathic galactorrhea and amenorrhea;

(IV) twenty patients with idiopathic galactorrhea and normal menses; (V) three patients with myxedema; and (VI) four patients with empty sella syndrome. Basal PRL: the highest values (8-4200 ng/ml) were found in patients with (I): only three of fourteen had normal PRL (<30); six of ten patients with (III) also had high PRL values (3.4-224). None of the patients with (IV), (V), or (VI) had elevated basal PRL. Tests of PRL secretion included thyrotropin releasing hormone (TRH) stimulation (normal >350% rise); chlorpromazine stimulation test (CST) (normal >200% rise); L-dopa suppression (normal fall to <60%). The number of normal responses in each type of galactorrhea were:

	TRH	CST	L-DOPA
I	1/10	0/6	5/6
II	3/3	3/6	9/9
III	4/10	2/8	6/7
IV	7/10	5/11	13/13
V	3/3	1/2	1/1
VI	2/4	1/4	3/3

The effect of water loading on PRL secretion was evaluated in nineteen patients; PRL did not suppress in any type of galactorrhea. It is concluded that L-dopa and water loading are not useful in the differential diagnosis of galactorrhea. Patients with idiopathic galactorrhea with normal menses, myxedema, or empty sella syndrome have normal basal PRL and respond to either TRH or CST. A high basal PRL and failure to respond to either TRH or CST are associated with pituitary tumors and idiopathic galactorrhea with amenorrhea.

Radioiodination of human growth hormone (hGH) with chloramine T yields several iodinated components that differ widely in their apparent molecular weights (MW_{app}). We have identified at least four such components by gel chromatography on Sephadex G-100, and have examined each of their activities in homologous radioimmunoassays (RIA, antiserum GP 2-5-19) and radioreceptor assays (RRA, cultured lymphocytes, IM9). Two immunochemical grade preparations of hGH (National Pituitary Agency, #1523D and #1394) were studied. Chloramine T iodinations (n = 4) were performed with ^{125}I to theoretically yield 1 mol ^{125}I/mol hGH. Each major iodinated component was examined individually as a radioligand by RIA and by RRA.

In both iodinated hGH preparations, component IV eluted with MW_{app} ~20,000 (monomeric ^{125}I-hGH) and displayed 50-60% binding in RIA (antibody dilution 1/2,000,000) and 8-14% specific binding in RRA. Component III eluted with MW_{app} ~40,000, was ~90% as active as

monomeric ^{125}I-hGH in RIA, but only ~20% as active in RRA. Component II eluted with MW_{app} ~60,000, and, relative to monomeric ^{125}I-hGH, displayed ~20% activity in RIA and <1% in RRA. Component I eluted in the void volume, had ~50-70% relative activity in RIA but <1% in RRA. In addition, there appeared to be another iodinated component I_B, with activities similar to component I, and eluting between components I and II with MW_{app} ~80,000. Each component was rechromatographed and eluted as before. The two unlabeled hGH preparations were examined by gel chromatography and RIA, and each contained four immunoactive components corresponding to iodinated components I, I_B, III, and IV.

These studies suggest the following: (1) relative to monomeric ^{125}I-hGH, there is marked disparity between immuno- and receptor activities among other components of iodinated hGH; (2) ^{125}I-hGH components I, IB, and III may result from iodination of hGH components intrinsic to the unlabeled preparation and may not be "damaged" products; (3) these components of iodinated hGH may serve as useful "markers" in structure-function studies of the multiple forms of pituitary and circulating hGH demonstrated previously in man.

2. Thyroid Metabolism.

Lithium and iodide have been demonstrated to have similar antithyroid effects, and both agents appear to inhibit thyroid hormone secretion. Patients who are rendered euthyroid after therapy for diffuse toxic goiter have been shown by L. Braverman and S. Ingbar, (New England Journal of Medicine, 281:816, 1969) to be extremely sensitive to the effects of iodide with the development of hypothyroidism after 3-6 weeks of therapy with this ion. We have studied a group of such patients to determine if they would be similarly sensitive to lithium. Seven patients (3 men, 4 women) who had been euthyroid for an average of 11 months following radioiodine or antithyroid treatment of diffuse toxic goiter were studied (mean T4 8.0 µg/100 ml, range 6.0-10.5 µg/100 ml; mean T3 138 ng/100 ml, range 73-189 ng/100 ml). The study was divided into control, experimental, and post-treatment periods which lasted 2, 8 and 2 weeks, respectively. Lithium was administered during the experimental period and TRH tests (500 µg i.v.) were performed in 5 patients after discontinuation of lithium. Six of 7 patients had a mean decrease in serum T4 of 3.5 µg/100 ml ($p<.02$), and a mean decrease in serum T3 of 57 ng/100 ml ($p<.05$). The mean nadir in T4 and T3 concentrations occurred on days 29 and 32 of lithium administration, respectively, and both T3 and T4 levels returned to their original values during the post-treatment period. Of the patients who had decreased T4 concentrations during lithium administration, TSH increased from 11.8 to >40 µU/ml in one patient (T4 decreased from 9.0 to 5.1 µg/100 ml), whereas the remaining 5 patients had TSH levels which remained 2.5 µU/ml. TRH tests were performed in 3 of these 5 patients; 2 had no TSH response and 1 had a moderate increase in serum TSH from <1 to 5 µU/ml. These data suggest: (1) patients rendered euthyroid following treatment of

diffuse toxic goiter are as sensitive to lithium as they may be to iodide; (2) such patients may therefore be particularly susceptible to the development of lithium induced hypothyroidism; (3) the mechanism of lithium induced hypothyroidism may be the same as that of iodide since increased thyroidal iodine content is a consequence of lithium therapy (Journal of Clinical Investigation 49:1357, 1970).

To gain a better understanding of the metabolic pathways of Triiodothyronine (T3) and Thyroxine (T4) in the fetus we modeled the known kinetics of iodide (I^-), T3 and T4 between mother, fetus and amniotic fluid in sheep using the SAAM computer program. The model for each of the above subsystems required exchange compartments within mother and fetus, exchanges between maternal and fetal circulations, and between the fetus and the amniotic fluid. The I^- model was developed from data using $^{131}I^-$ and $^{125}I^-$ tracers given to mother and fetus or amniotic fluid respectively under KI blocked or unblocked conditions. In general, the models for I^- in mother and fetus are comparable to similar models developed for man and dog, except for a somewhat larger I^- distribution volume in the mother, probably due to an exchange with rumen. The T4 model was developed from data on ^{131}I and ^{125}I T4 given to KI blocked maternal and fetal sheep. The model predicted similar concentrations for fetal and maternal serum and for amniotic fluid. The T3 model was developed from data on ^{131}I and ^{125}I T3 given to $KClO_3$ blocked maternal and fetal sheep. No fetal plasma T3 utilization and little T3 production, either via synthesis or T4 to T3 conversion could be accomodated in the model. The observed low fetal:maternal concentration gradient for T3 was supported by the model, which also predicted a high amniotic fluid: fetal gradient. The predicted T3 and T4 amniotic fluid levels are supported by measurements of I^-(3.3 ug%) and T4 (2.4 ug%) in fluid samples from 12 normal human term pregnancies, and of T3(40 ng%) in 3 sheep samples. Exchange between the fetus and the amniotic fluid appears to take place via urinary excretion and intestinal and/or biliary secretion into meconium, with reabsorption from swallowed amniotic fluid. Thus a comprehensive model integrating the kinetics of I^-, T3 and T4 in mother, fetus and amniotic fluid is presented which fits published kinetics and observed concentrations and which correctly predicts concentration gradients for the amniotic fluid.

3. Cyclic Nucleotide Laboratory.

Recent studies have suggested that cyclic AMP(CAMP) accumulation in rat seminiferous tubules may be regulated by several factors including FSH, phosphodiesterase, and prostaglandins. Although the Sertoli cell has been shown to be one site of intratubular CAMP accumulation (A.R. Means, Life Science, 15:371, 1974) there are no published studies defining the distribution of this nucleotide throughout the seminiferous epithelium. Using an indirect immunofluorescent staining technique we have attempted to localize CAMP in testes from rats 5 to 50 days of age. Preliminary brief fixation of fresh frozen testis sections with either acetone or cold formalin consistently maximized the intensity of

otherwise identical fluorescent staining patterns observed in unfixed specimens. Brilliant fluorescence was seen in the interstitial areas of all developmental stages while intratubular CAMP staining patterns were of lower intensity and changed with age. At 5 days CAMP appeared localized to perinuclear cytoplasm of both early Sertoli cells and spermatogonia. At 10, 15, and 20 days of age most tubules demonstrated positive staining around the developing spermatocyte nuclei with persistent fluorescence in Sertoli and spermatogonial elements. At 25 days a similar pattern persisted but CAMP staining was not seen in or around the newly appearing spermatids. From 30 to 50 days of age when developing spermatids accounted for the majority of tubule cells, positive cytoplasmic fluorescence was limited to the most peripheral areas occupied by Sertoli cell bodies, spermatogonia, and early spermatocytes. These observations confirm the presence of CAMP in the Sertoli cell. The additional finding of demonstrable CAMP surrounding the early germ cell nuclei suggests that this nucleotide may influence the metabolism of these cells. Absence of positive staining in spermatids suggests either that CAMP does not actively participate in the regulation of these more mature germ cells or that the levels of stainable nucleotide are below the detection limits of the system employed. Investigations are now being conducted to study the possible hormonal dependence of these staining patterns.

In rats the excretion of urinary cyclic GMP but not cyclic AMP was lowered by adrenalectomy and restored to normal by hydrocortisone suggesting a relationship between urinary cyclic GMP excretion and adrenal steriods (J.G. Hardman, et al, Journal of Biological Chemistry, 244:6354, 1969). In a retrospective study of 5 patients with Cushing's syndrome, we have measured urinary cyclic GMP and cyclic AMP in pre-treatment 24 hour urine specimens using radioimmunoassays. The cyclic nucleotide data is expressed both as total micromoles/day and as the ratio of micromoles/gm creatinine (means $\pm$ SE).

	Number of Specimens	Cyclic GMP Total	Cyclic GMP Ratio	Cyclic AMP Total	Cyclic AMP Ratio
Adrenal Carcinoma					
female	6	4.19±0.31*	3.40± .20*	3.49±38	2.82±0.23
Adrenal Hyperplasia					
female	1	2.70 *	2.90 *	3.58	2.76
male	4	2.01±0.11*	1.11±0.05*	2.02±0.33	1.11±0.16
Ectopic ACTH Syndrome (EAS)					
female	3	2.93±0.62*	4.60±0.61*	6.72±1.29*	10.55±1.11*
male	4	5.05±0.69*	3.56±0.61*	3.16±0.40	2.16±0.12
Controls					
female	(N=10)	0.71±0.08	0.68±0.12	2.81±0.41	2.43±0.31
male	(N=10)	0.57±0.09	0.33±0.05	2.55±0.39	1.35±0.18

Asterisk (*) indicates value 3 SD's from the mean of sex-matched controls.

Urinary cyclic GMP was increased in all patients whereas cyclic AMP was increased only in ectopic ACTH syndrome. Excretion of both urinary cyclic GMP and cyclic AMP was unchanged in adrenal carcinoma after 4 days of ineffective o,p'DDD therapy and in adrenal hyperplasia after four days of dexamethasone (2 ng/day x2 and 8 mg/day x2). In the EAS patient with elevated urinary cyclic GMP and cyclic AMP both nucleotides decreased after 4 days of aminoglutethimide. In the EAS patient with elevated urinary cyclic GMP and normal cyclic AMP only the increased nucleotide seemed to decrease after 4 days of metyrapone. These data do not identify the feature(s) of Cushing's syndrome causing the increased excretion of urinary cyclic GMP but do suggest a relationship to the action of adrenal steroids in man.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 0L Military Internal Medicine

Work Unit 120 Metabolic response to disease and injury

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6445	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADE	8A. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	01	121
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of Enteric Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
59 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	3	231
FISCAL YEAR CURRENT 76	3	365

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Diarrhea; (U) Dysentery; (U) Bacillary; (U) Salmonellosis; (U) Immunity; (U) Immunization

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To study the pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised. Diarrhea is a significant problem in military personnel operating overseas.

24. (U) The genetic control of O-antigen specificity of enteric pathogens is being studied since such cell envelope components are of importance in disease and its prevention through vaccination.

25. (U) 74 07-75 06 The genetic control of S. flexneri group antigen 6 has been shown to be under the control of a phage converting system which causes chemical and serological alterations to pre-existing shigella group 3,4 antigens. Shigella minicells are being exploited as a model for the study of pathogens of dysentery and as an oral vaccine against shigellosis. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 121 Pathogenesis of enteric diseases

Investigators.
Principal: Samuel B. Formal, Ph.D.
Associate: Peter Gemski, Jr., Ph.D.

Description.

The pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised.

By employing an integrated immunologic, cytologic and genetic approach (see previous annual reports) studies in this department are concentrating on further elucidation of (I) virulence factors and mechanisms involved in intestinal penetration and toxin elaboration by pathogens, key mechanisms by which enteric diseases are provoked; (II) the genetic control of O antigen specificity of enteric pathogens since such cell envelope components are of importance both in disease and its prevention through vaccination; (III) the application of genetic techniques for development of live oral vaccines against shigellosis.

1. A temperate phage, designated Sf6, has been isolated from Shigella flexneri 3a. Characterization of Sf6 revealed that it possesses the capacity for converting the S. flexneri 3,4 group antigen complex to group factor 6. Serological studies and chemical analysis of lipopolysaccharide from converted strains suggest that group factor 6 is a reflection of an acetylation of the pre-existing 3,4 antigen complex. Evidence is provided that the 3,4 group antigen complex functions, at least in part, as a cell surface receptor site for Sf6 adsorption.

2. By employing chemical mutagenesis, we have isolated mini-cell producing mutants of virulent S. flexneri2a, S. dysenteriae 1, E. coli and S. typhimurium. Characterization of these strains has shown that they retain their virulence properties (invasiveness and/or toxigenicity). Currently, efforts are geared toward preparing "pure"

suspensions of mini-cells via sucrose gradient fractionation and investigating such mini-cells for various properties associated with virulence and as potential oral vaccines.

Progress.

1. Phage conversion of S. flexneri group antigens. Our continuing investigations on the genetic control of S. flexneri somatic antigens have recently concerned the genetic determinant of group antigen 6. Our findings have revealed that group factor 6 can be under the genetic control of a temperate phage. Some of the properties of this newly isolated phage, its role in converting group factor 3,4 to group factor 6, and the chemical alterations in S. flexneri lipopolysaccharide (LPS) resulting from such antigenic conversion have now been characterized.

a. Isolation and host range of phage Sf6. In the course of characterizing a number of S. flexneri 3 strains, it was observed that S. flexneri 3a strain 3-19 spontaneously released a temperate phage. This phage, designated Sf6 was recovered by plating culture filtrates of strain 3-19 on S. flexneri Y hosts and was found to control antigenic conversion to group factor 6. After being cloned on a S. flexneri Y strain, a high-titer phage lysate of Sf6 was prepared by the confluent lysis method for screening studies to determine the host range of Sf6. Overnight broth cultures of the known serotypes of S. flexneri, various rough mutants of S. flexneri and a limited number of S. sonnei, S. dysenteriae, E. coli, and Salmonella strains were spread on Trypticase soy agar plates and spotted with drops of a Sf6 lysate ($\sim 10^{10}$ plaque-forming units per ml). After overnight incubation at 37 C and an examination for phage sensitivity, it was evident that the host range of Sf6 is limited. Only the S. flexneri Y strains were lysed by Sf6. When S. flexneri Y strains are employed as hosts in soft agar overlayers, Sf6 produces distinct, turbid plaques (1.5 to 2.0 mm in diameter) with haloes typical of many temperature phages.

b. S. flexneri group antigen 3,4 as an essential part of the surface receptor for phage Sf6. The results of the host range study suggest that S. flexneri group antigen 3,4 functions in the adsorption of phage Sf6. S. flexneri Y strains, the only serotype lysed by Sf6, represent a variant class of S. flexneri that produces only group 3,4 somatic antigen. S. flexneri Y strains are defective in their capacity to synthesize an additional type-specific determinant.

To establish unequivocally the that the group antigen 3,4 complex functioned, at least in part, as the receptor site for Sf6 adsorption, we studied the behavior of Sf6 on E. coli K-12 hybrids which express the S. flexneri group 3,4 antigen complex. Previous investigations have demonstrated that the group 3,4 antigen gene(s) is closely linked to the histidine operon of the S. flexneri chromosome and that it is possible to transfer this locus to E. coli recipients by conjugation. Thus, E. coli hybrid strain ABI133-H96 was prepared by mating S. flexneri 2a Hfr 256 with E. coli K-12 ABI133 and selecting for the inheritance of the his-group 3,4 antigen chromosomal segment. Unlike the original parent E. coli ABI133 hybrid derivative ABI133-H96 produces the 3,4 group antigen complex of S. flexneri. This strain was found to be sensitive to lysis by Sf6, plating it with an efficiency approximately that of the natural host, S. flexneri Y (e.o.p. 0.5 to 1.0). The E. coli parent ABI133 was resistant to Sf6. The results of phage adsorption tests performed with E. coli hybrid ABI133-H96 and parent ABI133 revealed the following. Unlike the parent E. coli ABI133, hybrid strain ABI133-H96 efficiently adsorbs phage Sf6. The sensitivity of this hybrid to Sf6 is thus considered to be a direct consequence of its inheritance and expression of the 3,4 group antigen complex.

c. Group antigen conversion by phage Sf6. The results of serological studies revealed that phage Sf6 functions in S. flexneri group 6 antigen conversion. S. flexneri Y strains FHI0 and F3 and E. coli hybrid ABI133-H96, which agglutinate in group 3,4 antiserum but not in group 6 antiserum, were lysogenized with Sf6 and tested for their agglutination properties. Lysogenization was achieved by depositing drops (0.01 ml) of a Sf6 lysate (10^9 plaque-forming units/ml) on lawns of host cells prepared on Trypticase soy agar and incubating overnight at 37 C. Isolates, prepared from the secondary growth in the area of lysis, were cloned twice and then scored for their lysogenic properties. Clones that expressed immunity to lyses by Sf6 and released Sf6 plaque-forming units were considered to be Sf6 lysogens. Unlike the parental strains, such lysogens were found to agglutinate strongly in group factor 6 serum in addition to retaining a weak reactivity in the group 3,4 antiserum. Slide agglutination tests on over 200 independent Sf6 lysogens established an absolute correlation between Sf6 lysogeny and agglutination in group factor 6 serum. The group 6-converting property of Sf6 was confirmed by agglutinin absorption tests (Table I). Comparative tube agglutination tests with various antisera were performed with S. flexneri Y strain FHI0, E. coli hybrid ABI133-H96, and Sf6 lysogens of

them. Unlike the parental strains, the Sf6 lysogens agglutinated at significant levels in group 6-specific levels in group-6 specific serum. In addition agglutination in group 3,4 antiserum was observed with these lysogens, indicating the presence of group antigens 3,4. The expression of both group complex 3,4 and group factor 6 by Sf6 lysogens was confirmed by preparing antisera against E. coli hybrid ABI133-H96 (Sf6). This antiserum contained agglutinins against group antigens 3,4 (Table I). Furthermore, after absorption with the non-lysogenic parent ABI133-H96 to remove the 3,4 agglutinins, the antisera behaved as a typical group 6-specific serum. A wild-type S. flexneri Ib strain was also included in these tests. This strain, 2381-0, which produces group factor 6 and group antigen 4, agglutinated as expected in all antisera.

d. Analyses of LPS. Lipopolysaccharides were isolated from S. flexneri Y strain F3 and a representative lysogenic phage converted derivative, F3 (Sf6), and analyzed to determine the chemical basis of antigenic conversion to group factor 6. The results of LPS analysis of strain F3 and F3 (Sf6) are summarized in Table 2. It can be seen that both strains yield approximately the same amount of LPS per 100 g of cells (wet weight). In addition, monosaccharide analyses support the conclusion that both strains produce a complete LPS core. The heptose/hexose (glucose plus galactose) ratios for strains F3 and F3 (Sf6) were found to be 1.0:2.2 and 1.0:2.4 respectively. These values are in close agreement with the expected ratio of 1.0:2.0, based on the complete core structure of S. flexneri LPS.

Comparison of rhamnose/O-acetyl ratios, on the other hand, yielded a significant difference between the LPS of strain F3 and its phage-converted derivative, F3 (Sf6). The rhamnose/O-acetyl ratios of 1.0:0.1 for F3 and 1.0:0.7 for F3 (Sf6) indicate elevated levels of O-acetyl groups in the phage-converted strain. In addition the average length of O side chains of the phage converted strain F3 (Sf6) appears to be shorter than that of F3 (Table 2).

2. Development of oral living Shigella vaccines. An important area of our research on the pathogenesis of enteric disease centers on the development of oral vaccines against shigellosis. Earlier studies from this department yielded avirulent hybrids and mutants of S. flexneri for possible use as vaccine strains for providing protection to challenge against virulent S. flexneri. On the basis of human volunteer experimentation performed by the university of Maryland Vaccine testing group, some of these strains were shown to be relatively safe and significantly effective (see previous annual reports). Since it is

impossible to obviate the theoretical possibility that these strains could revert to a virulent state, the practical use of these strains is severely limited.

To eliminate such complications with safety of living oral vaccines, an alternative approach for genetically constructing vaccines was attempted (see last year's annual report). In principle, our approach was to prepare by intergeneric hybridization, avirulent E. coli strains which express the serological characteristics of the genus shigella. Since the E. coli parent strain for such hybrids is naturally avirulent, the problem of reversion to virulence is, a priori, obviated. As summarized in last year's annual report, limited human volunteer studies with this new breed of vaccine candidates have been disappointing.

As a consequence, we have initiated investigations during this past year on the possible use of "mini-cells" as an oral vaccine. Mutants of E. coli K-12 have been described recently which express an aberrant cell division cycle that results in the production of anucleate spherical cells at the polar ends of rod-shaped bacteria. These entities, termed mini-cells, have been shown to be incapable of cell division but still possess functional cell wall, membrane, ribosomal and metabolic systems. Since the uniqueness of the mini-cell system has not been exploited as a model for the study of mechanisms of pathogenesis and as potential oral vaccines our efforts have been geared to prepare and characterize mini-cell producing mutants of enteric species.

a. Isolation of mini-cell producing mutants. By employing chemical mutagens, we have isolated mini-cell producing mutants of S. flexneri 2a, S. dysenteriae 1, toxigenic E. coli and S. typhimurium. Preliminary characterization of these strains have shown that they retain the invasive and/or toxigenic properties of their virulent parents.

At the present time, emphasis is being placed on investigating the mini-cell-producing mutant of S. flexneri, termed MC-1. This strain has retained its properties of invasiveness and efficiently produces large yields of mini-cells by cultivation in either minimal or complete medium. Pure mini-cell suspensions (less than one bacillus per 10^6 mini-cells) have been prepared by sucrose gradient fractionation at concentration levels approaching 10^{12} mini-cells per ml. Preliminary attempts to employ such mini-cell suspensions as an oral vaccine against shigellosis have not been successful. Six monkeys receiving a single oral dose of about 10^{12} pure mini-cells exhibited no resistance to challenge by virulent S. flexneri 2a and failed to show an elevation in

serum antibody titer against S. flexneri. It is premature on the basis of this limited trial to assess the potential usefulness of the "mini-cells" system as a vaccine. Further studies hopefully will define this.

Summary and Conclusions.

1. Our results support the conclusion that lysogenization with phage Sf6 results in appearance of group antigen 6. Immunological tests indicate that the group 6 factor controlled by Sf6 is typical of that produced by a native S. flexneri Ib. Furthermore, it is apparent that lysogenization of hosts with Sf6 does not result in a complete conversion to group antigen 6. Such strains, in addition to expressing factor 6, retained reactivity in group 3,4 antisera.

Our comparative analyses of LPS from a S. flexneri Y strain and its Sf6 lysogenic derivative revealed an elevated level of O-acetyl residues in the phage-converted strain.

On the basis of these observations it appears that phage Sf6 functions by converting pre-existent group 3,4 O-repeat units to group 6 specificity. The significant increase in the level of O-acetyl groups in the LPS of the group 6-converted strain F3 (Sf6), suggests that this phage determines a specific acetylase which results in the acetylation of rhamnose residues in the 3,4 O-repeat unit and the subsequent expression of the group 6 antigen.

2. Studies on the potential use of "mini-cells" as a vaccine and as a model for the pathogenesis of enteric infections have been initiated. Mini-cell producing mutants of virulent S. flexneri 2a, S. dysenteriae 1, toxigenic E. coli and S. typhimurium have been isolated and are being characterized.

Table 1. Agglutination reactions of strains converted to group antigen 6 by phage Sf6

Strain	Group-specific 3,4[a]	Group-specific 6[a]	Anti-AB1133-H96	Anti-AB1133-H96 (sf6)	Anti-AB1133-H96 (Sf6) absorbed with AB1133-H96
S. flexneri Y FH10	+ (640)[b]	- (<20)	+ (2,560)	+ (640)	- (<20)
S. flexneri Y lysogen FH10 (Sf6)	+ (640)	+ (2,560)	+ (640)	+ (2,560)	+ (1,280)
E. coli hybrid AB1133-H96	+ (640)	- (<20)	+ (5,120)	+ (1,280)	- (<20)
E. coli hybrid lysogen AB1133-H96(Sf6)	+ (640)	+ (2,560)	+ (2,560)	+ (2,560)	+ (1,280)
S. flexneri 1b 2381-0	+ (320)	+ (640)	+ (320)	+ (640)	+ (640)

[a] Prepared according to absorption protocols of Edwards and Ewing.

[b] Tube agglutination titer.

Table 2. Analysis of LPS[a]

Organism	LPS (g)/ 100 g cells	Total[b] carbohydrate	Glucosamine	Heptose	Hexose[c]	Rhamnose	O-acetyl[d]	N[e]
F3	1.33	32.6	89.0	21.9	48.9	99.3	10.9	3.2
F3 (Sf6)	1.21	15.1	54.2	20.6	49.4	25.2	18.3	0.8

[a] Values are reported in micromoles/100 mg of LPS.
[b] Expressed in micromoles of glucose equivalents.
[c] Total hexose = glucose and galactose.
[d] Assayed by the hydroxylamine method.
[e] N is the average number of O-repeat units per side chain. It can be calculated by multiplying the rhamnose/heptose ratio by 2/3 according to the revised Shigella O-repeat unit structure.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 121 Pathogenesis of enteric diseases

Literature Cited.

Publications.

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3. Levine, M.M., Rice, P.A., Gangarosa, E.J., Morris, G.K., Snyder, M.J., Formal, S.B., Wells, J.G., Gemski, P. Jr., and J. Hammond. An outbreak of Sonne shigellosis in a population receiving oral attenuated shigella vaccines. Am. J. Epidemiol. 99:30-36, 1974.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6446	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A83C	01	123
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Histopathologic Manifestations of Military Diseases and Injuries

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	4	220
	CURRENT 76	5	265

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immune responses; (U) Enteroviruses; (U) Intestine; (U) Electron Microscopy; (U) Infection

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code.)

23 (U) To define histopathologic manifestations of injuries and diseases which have current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and immunologic responses with infections and injuries. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries of military personnel.
24 (U) Various morphologic techniques including histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy are employed. Various immunologic techniques have also been utilized.
25 (U) 74 07 - 75 06 Experimental Entamoeba histolytica infections have further clarified the early responses of the gut mucosa subsequent to epithelial penetration by trophozoites; excessive epithelial cell loss secondary to lysis of granulocytes caused by invading amebae results in microulceration and vascular changes characterized by increased vascular permeability and occlusive thrombosis by platelets are important features. EM study by freeze-etching has demonstrated regularly spaced spherical pores on nuclear membrane and evidence of active secretion mechanisms. EM studies of experimental viral infections by murine adenovirus and canine coronavirus have determined the site of viral replication; the adenovirus grows exclusively in the nucleus of three types of epithelial cells and the coronavirus in intestinal columnar cells. Further studies of delayed hypersensitivity have shown that extravascular fibrin accumulation results from increased vascular permeability caused by cell-mediated immune mechanism.
For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Diseases and Injuries

Investigators.
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Description:

To define histopathologic manifestations of injuries experimentally produced and diseases which present current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and immune responses due to infections and injuries. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries in military personnel.

Approach to the Problem

A multi-disciplinary approach including conventional histology, histo- and cytochemistry, autoradiography, radio-tracer methods, various immunologic techniques, immunofluorescent microscopy, transmission and scanning electron microscopy is employed.

Progress

In the past, this work unit was primarily concerned with studies of histopathologic manifestations of acute diarrheal diseases of infectious origin. In the past three years, we have expanded this work unit and included studies of diseases of the digestive tract of non-infectious origin and experimental infections and injuries in the kidneys. In addition, this work unit has been conducting various interdepartmental collaborative projects on immunopathology at WRAIR and WRGI.

1. STUDIES OF HOST-INDIGENEOUS MICROBE RELATIONSHIP IN THE DIGESTIVE TRACT

Medical microbiology has been concerned primarily with the potentially pathogenic members of indigeneous microflora. Symbiotic species

are of at least equal importance because they maintain essential anatomical and physiological function with the host (Dubos 1967). The lumen of the digestive tract is now acknowledged as the site of a dynamic ecological system composed of extremely large populations of different microbes maintained in balanced proportions. Studies have indicated that indigeneous microbes in the gastrointestinal flora of mammals predominantly populate within certain anatomical and histological divisions of the digestive tract (Dubos et al. 1962). Some of these microbes are preferentially localized in a close proximity to the surface of gastrointestinal mucosa, while others are predominantly found in the glandular lumen of the crypt (Savage, Dubos & Schaedler 1968). A study indicated that the large concentration of epithelial-associated indigeneous microbes resist access of pathogenic bacteria to the intestinal epithelial surface (Savage 1972). Since the adherence of indigeneous bacteria is required for colonization of epithelial cells of the buccal mucosa of man, it has been suggested that the bacterial adherence may be inhibited by the presence of abundant secretory immunoglobulin (Williams & Gibbons 1972, 1974).

Little is known about "gastrointestinal epithelial cell-associated indigeneous microbes" in mammals including monkey and man (Nelson & Mata 1970). For this reason, we have studied spiral-shaped microbes infested at the surface of the colonic epithelium of the rhesus monkey and man and also gastric spirilla in the stomach of rhesus monkeys. (See 73-74 Annual Report, Dept. of Experimental Pathology, WRAIR.)

We have been studying another example of epithelial cell-associated microbes in the stomach of the mouse, a common laboratory animal.

A. Studies of Adherence of Lactobacilli and Yeasts to the Murine Gastric Mucosa

In the stomach of the mouse, lactobacilli localized on the surface of the squamous epithelial portion (Figs. 1 and 2), while yeasts colonize on the glandular portion. These two microbial layers could coexist in the stomach of the same animals and were mutually exclusive. We analyzed this unique microbe-host relationship in conventionally housed mice of Swiss-Webster strain by conventional light microscopy (LM) and cytochemical electron microscopy (EM). For the demonstration of acid mucopolysaccharide, ruthenium red (RR) was used according to Luft (1965 and 1968).

Results

EM showed that the luminal plasmalemma of the surface squamous epithelial cell was invaginated and consisted of electron opaque layer in the center sandwiched by the thick inner membrane and thin outer membrane in the squamous portion fixed in glutaraldehyde-osmium sequence omitting ruthenium red. Lactobacilli were covered by the fine fuzz of the capsule. Parts of the bacilli were contacted with the outer leaflet of the luminal cell membrane by the fine capsular fuzz. The capsular fuzz was better visualized by the ruthenium red containing fixative. In thin sections without counter-staining by lead or uranyl acetate, the reaction product was seen in keratohyaline granules and also along the discharged granules on the surface cells. The reaction was positive all around organisms. Lactobacilli contacted the surface of discharged keratohyaline granules or outer leaflet of the surface with thick capsular fuzz. In interfaces between bacteria and host cells, bacteria were adhered to the outer leaflet of the host cell membrane with the capsular fuzz, while in the host the reaction was mostly absent except for focal positive reaction on the outer leaflet of the luminal plasma membrane (Fig. 3). The capsular fuzz measured up to 150 nanometers in length. On the other hand, the closest distance between the bacterial cell wall and the outer leaflet of the host plasma membrane was approximately 130 nanometers, which allowed bacteria to adhere to the luminal plasma membrane with bacterial muco-substance alone.

In the glandular portion, the tissue, fixed in conventional glutonaldehyde-os sequence without RR, showed no visible attachment between yeasts and epithelial cells. In tissues processed through a ruthenium red containing fixative, strongly positive reaction was present on the extraneous coat of the luminal membrane, the capsule of yeasts and also mucus strands in the lumen. The capsular fuzz of this yeast shows a contact with the extraneous coat. Note positive reaction on the desquamated microvilli in the lumen (Fig. 4). The ruthenium red reaction could be further intensified when thin sections were stained by lead citrate; the capsular fuzz and extraneous coating of the luminal membrane were much more strongly stained. These were well stained mucus strands. The host cytocomponents such as mitochondria and cytoplasmic granules and also those of the yeast were demonstrated by the counter stain with lead citrate.

Fig. 1 - A squamous epithelial portion of murine stomach. Multiple lactobacilli are in the gastric lumen (GL) and some are adhered to the surface of the stratified epithelium (SP). LP: Lamina propria. 1 μ thick section stained by methylene blue azure II. X 400

Fig. 2 - Higher light micrograph showing attachment of lactobacilli (arrow) to the surface cell of the squamous epithelium, murine stomach. Gastric lumen: GL. X 950

Fig. 3 - Luminal lactobacilli attach to surface of keratinized cell of squamous epithelium, murine stomach. The bacteria adhere to the outer leaflet of the luminal cell membrane with their fuzzy dense RR positive mucosubstances (arrow) produced by the bacteria. RR reaction is fairly positive on the host cell membrane. X 33,000

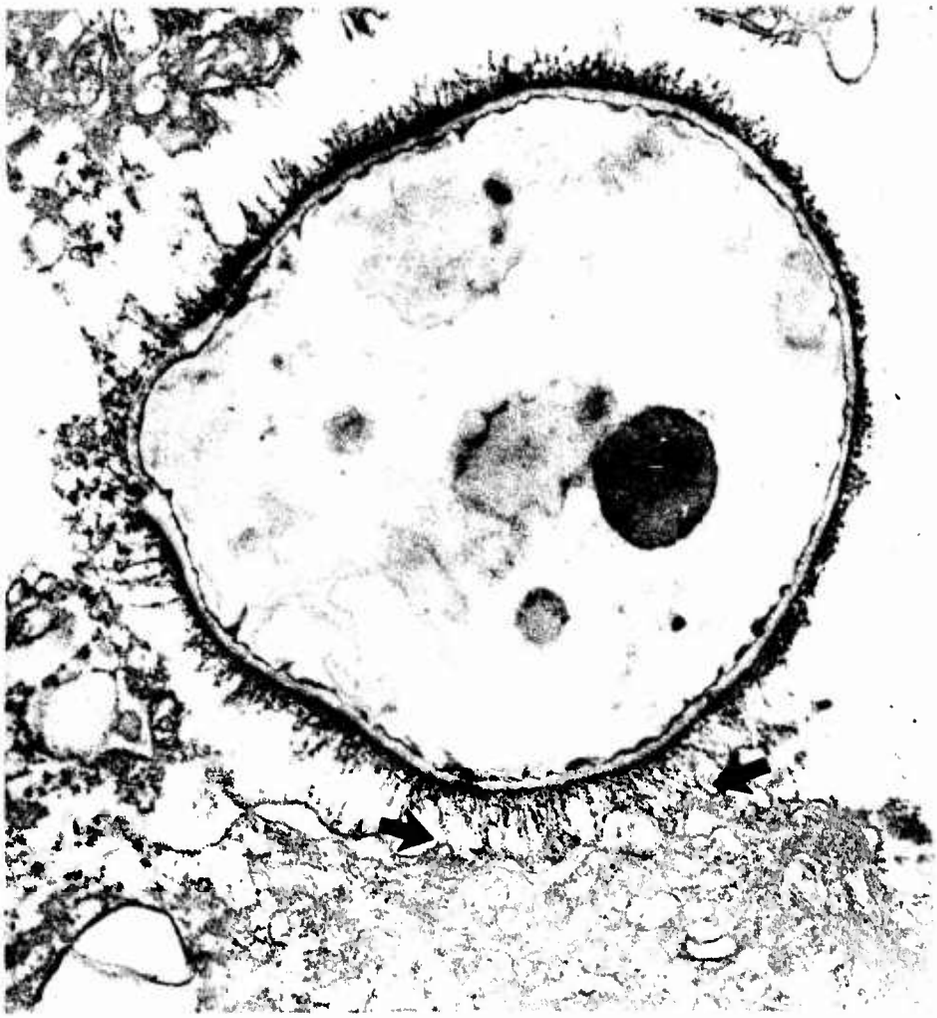

Fig. 4 - Attachment of (arrow) yeast to surface of the glandular epithelium of murine stomach. Ruthenium red reaction product is present on the luminal membrane of host cell and also on the cell wall of the organism. X 24,000

In some instances, yeasts were present away from the host cell and appeared to adhere to ruthenium red positive mucus strands. These mucus strands showed projection from the extraneous coating layer of surface epithelial cells. The capsular fuzz of yeasts averaged 120 nanometers in length.

Conclusion and Recommendation

Since Luft has introduced the method of ruthenium red, this chemical compound has been proven a reliable method of ultrastructural localization of acidic mucopolysaccharide in many tissues. In contrast, the application of ruthenium red on studies of microbes has been limited to the demonstration of the capsular fuzz of bacteria in vitro which included Chondrococcus columnaris, Diplococcus and Klebsiella pneumoniae.

In this study, we have utilized ruthenium red in ultrastructural localization of the acid mucopolysaccharide in a host-microbe relationship. In the murine stomach, lactobacilli adhered to the stratified squamous epithelium by their capsular fuzz while yeasts adhered to the glandular epithelium by connecting their own capsular fuzz with luminal mucus and also extraneous coat of the surface epithelial cell.

Further studies on other epithelial cell-associated indigeneous bacterial flora under different conditions by RR and other cytochemical compounds will provide additional new information on the host-microbe relationship in the gastrointestinal tract.

II. STUDIES OF HOST-PATHOGENIC MICROBE RELATIONSHIP IN THE DIGESTIVE TRACT

A. Studies on Penetration of the Colonic Epithelium by Entamoeba Histolytica

Background

In man, acute diarrhea caused by E. histolytica is attributable to colonic lesions associated with invading amebae. The most common colonic lesions in human patients are acute ulceration which initially developed in the cecum. Cecal ulcers often complicate the prognosis of patients because they develop frequently into perforation of the bowel wall followed by peritonitis and also because they are an initial source of extra-enteric amebic lesions. Yet the pathogenesis of acute amebic ulceration has never been satisfactorily clarified. Some believe that ulcers develop from necrosis of bowel tissue by "lytic enzyme" produced by invading amebae, while others postulate that secondary invasion of bacteria is responsible for ulcer formation in the colon. This discrepancy had been mainly related to as yet unclarified mechanism of initial penetration of the gut mucosa by amebae and the subsequent early changes of the mucosal tissue surrounding invading amebae. Some believe that the ameba penetrated the epithelium by mechanical means. Others postulate that necrosis of gut mucosa by cytolytic enzymes produced by the ameba is responsible for penetration and establishing tissue infections.

By electron microscopy, Griffin (1972) and Pittman et al. (1973) studied rectal biopsy specimens from human patients with E. histolytica infections. Although these studies clarified several aspects of amebic-colonic mucosa interactions, they did not demonstrate penetration of colonic epithelium by amebae and their effect on the epithelial cell. This may be due to the limitations inherent in rectal biopsy material and the fact that human cases clinically encountered represent a relatively advanced stage of the disease.

It has been found that young germfree guinea pigs, inoculated intracecally with cultured E. histolytica and the enteric flora from a patient with acute amebic colitis, develop lesions similar to those of human amebiasis and thus provide a good experimental model for studies on the pathogenesis of amebic disease. Using this model at an early stage of infection when trophozoites invade the cecal mucosa, we have demonstrated for the first time, ultrastructurally, how amebae penetrate from the gut lumen to the lamina propria through the cecal

epithelium and how cytoplasmic components of epithelial cells respond to penetrating amebae. (See Annual Report, 73-74, Dept. of Experimental Pathology, WRAIR.)

We have continued to study the responses of host cells in the gut mucosa during invasion by trophozoites. We have clarified the interactions between invading amebae and host cells, and the vascular changes involved early in the development of acute amebic colitis.

Experimental Infections and Morphologic Methods

NIH Hartley strain germfree guinea pigs were used as experimental hosts. The animals were obtained by Caesarian section, maintained in Reynier's series 500 stainless steel isolators on dietary regimen L-445 and monitored at weekly intervals by procedures described previously (9). All animals were inoculated at the age of 12-17 days and each received a 1.0 ml inoculum containing 200,000 E. histolytica. CDC J-190 strain amebae were injected directly into the cecum during laparotomy under sodium pentothal anesthesia. They were maintained in vitro in Locke's egg-rice flour medium with enteric flora from the patient, incubated at 37° C and transferred thrice weekly. Inocula were prepared by pooling the sediment from 48 hour cultures and quantitating with a hemocytometer. Control animals were treated just as the experimental group except they were inoculated with only the enteric flora without amebae.

Guinea pigs were killed at post-inoculation intervals of 7-12 days by ether anesthesia and autopsied in a conventional manner. The cecum was removed immediately and immersed in chilled physiological saline wherein the cecal wall was opened and the luminal contents carefully removed. Multiple sections were taken immediately from grossly normal mucosa of the cecum. Each section was divided into two pieces and processed for light (LM) and electron microscopy (EM).

Observations

LM examinations of sections of paraffin and Epon embedded tissue of infected guinea pigs showed trophozoites in the gut lumen and on or near the surface (interglandular) epithelium and some in the crypt (glandular) lumen (Figs. 5 and 6). Luminal amebae were often intermixed with cell debris and eosinophilic exudate. Occasional amebae were seen in direct contact with the surface or crypt epithelium.

Fig. 5 - Invasion of the cecal mucosa by trophozoites at interglandular site. Two amebae (1 and 2) are in the lamina propria and one (3) separates degenerating pale epithelial cells which are in a process of desquamating into the lumen (arrows). Two other amebae (4 and 5) are in the lumen. X 650

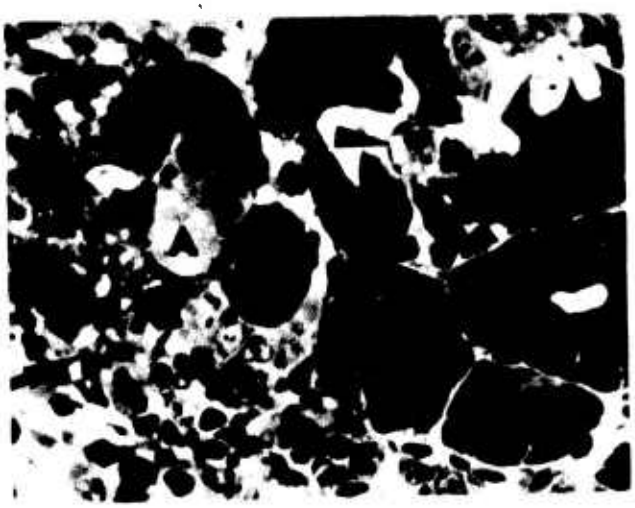

Fig. 6 - Invasion by trophozoites of the crypt gland epithelium. Two amebae (A) have penetrated through the crypt epithelium and are surrounded by cellular infiltrate (arrow). An ameba (arrowhead) is in the crypt lumen. In the lamina propria, many extravasated RBC are present. Capillary lumen (C) is filled by RBC.
X 460

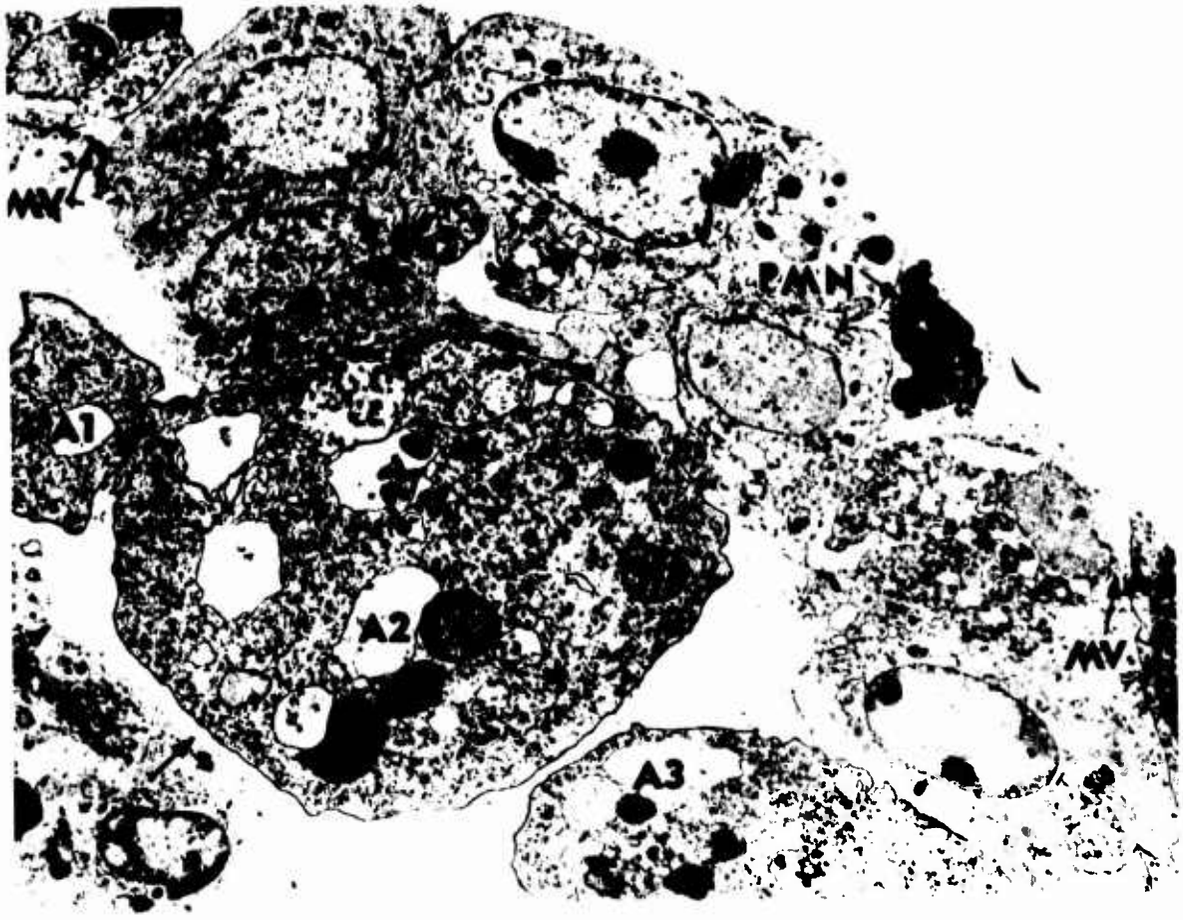

Fig. 7 - Amebae in a process of penetration into interglandular portion of the cecal mucosa. Amebae (A2 and 3) are between epithelial cells and basal lamina (arrows). A1 appears to be pseudopodium of A2. Luminal ameba (A4) is close to altered microvilli (MV) of degenerating epithelial cells. Polymorphonuclear leukocyte (PMN) shows degeneration. X 4,400

Usually a single but occasionally several amebae were seen apparently in a process of passing through the surface and crypt epithelium and reaching the lamina propria (Figs. 5 and 6). The epithelial cells in proximity of penetrating amebae were often cuboidal with shortened brush borders and pale cytoplasm, while those distal to the site of amebae penetration were tall columnar cells with regular brush borders and dark cytoplasm (Fig. 5). The site of penetration by trophozoites showed a focal discontinuity of the epithelium (Figs. 5 and 6). Occasional epithelial cells appeared to be desquamating from the site of amebae penetration. Goblet cells were decreased in number and often absent, particularly in areas of mucosal invasion by amebae. Crypt glands were often distorted, with their lumina dilated, and contained occasional organisms (Fig. 2). Some amebae were observed deep in the lamina propria (Figs. 11 and 12), whereas others were in the subepithelial region. The number of PMN leukocytes varied from area to area in the lamina propria, but were usually concentrated in areas of amebic invasion. The lumina of capillaries and venules were dilated and filled by erythrocytes, PMN leukocytes and aggregates of platelets (Figs. 11 and 12). Extravasated erythrocytes were occasionally present within the epithelial lining. Aggregates of bacteria were seen in the gut lumen but none were identified in the gut mucosa.

EM observations confirmed the LM findings and provided further details. During passage of amebae through the epithelial intercellular spaces, cytoplasmic alterations became progressively evident in epithelial cells proximal to penetrating amebae, while distal cells were little, if at all, affected. Epithelial changes included accumulation of lipid droplets and increased number of autophagic vesicles in translucent cytoplasm, shortening and disappearance of microvilli, dilatation of endoplasmic reticulum (ER), reduced numbers of ribosomes and swelling of mitochondria with emergence of large dense spherical granules between deranged cristae in a clear matrix (Figs. 8 and 9).

When amebae were within the epithelium, epithelial cells proximal to the amebae often appeared to be in a process of detaching from both the basal lamina and the lateral plasmalemma of adjacent cells (Fig. 8), and frequently were seen projecting into the lumen (Fig. 9). Partly or completely detached epithelial cells often formed clumps or sheets that shed into the lumen (Fig. 9). Sometimes, clumps of more than a dozen epithelial cells were shedding from an individual site of ameba penetration, thus leaving a wide gap in the epithelial lining, resulting in a focal defect of the epithelium or microerosion.

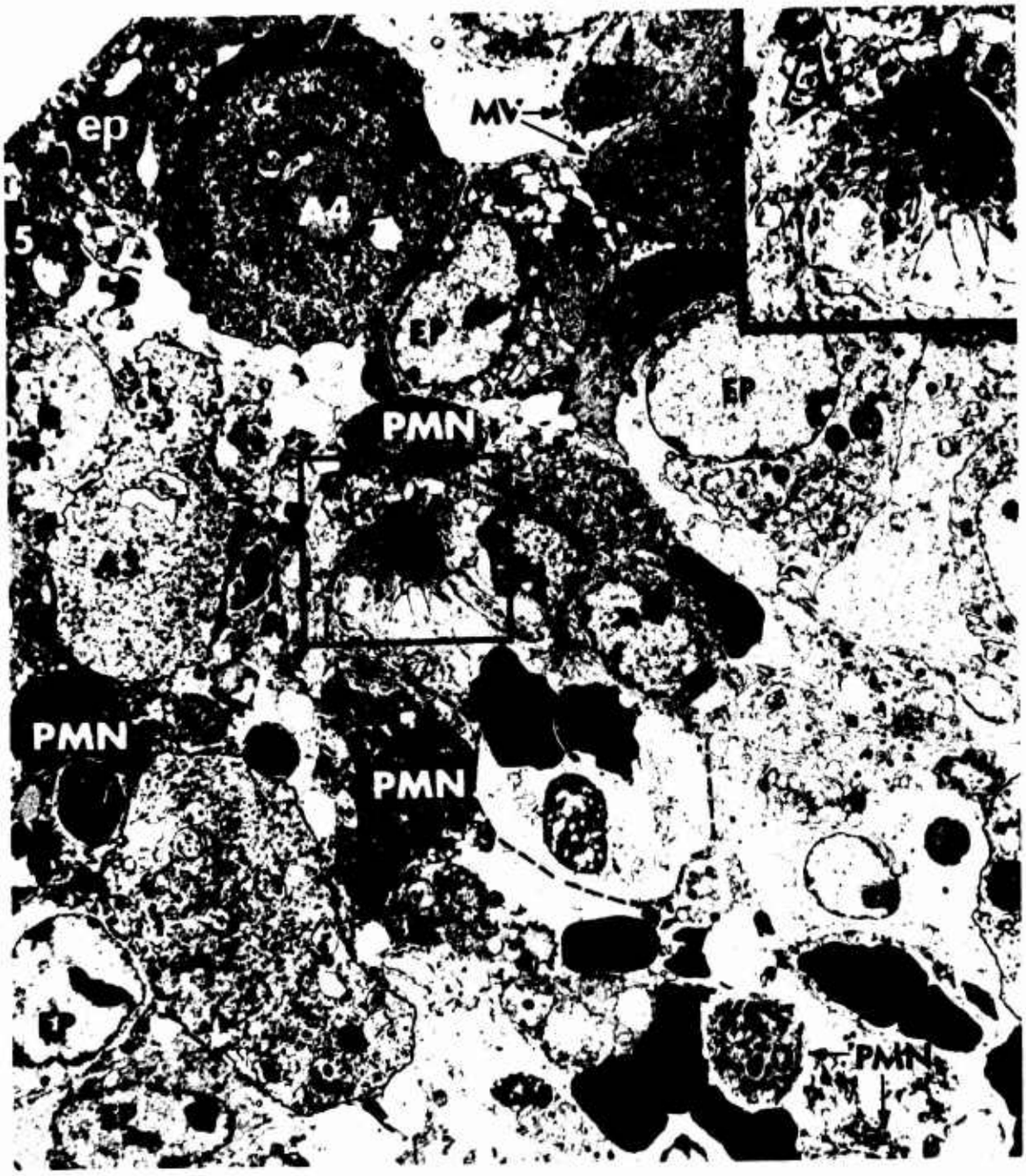

Fig. 8 - Invasion of interglandular portion of the cecal mucosa by multiple amebae. Epithelial cells (EP) show a variety of cytoplasmic changes. Shedding of cells (ep) produces a discontinuity of epithelium. An ameba (A2) has completed epithelial penetration. PMN in the proximity of amebae (white letters) show degeneration, while PMN in the distal amebae (black letters) appear intact. Subepithelial capillary (square) shows platelet aggregation and swollen cells. Basal lamina is outlined by dotted lines. Inset magnified from square shows aggregated thrombocytes (arrows).

Fig. 9 - Desquamation of epithelial cells into cecal lumen. Amebae (A) are in lumen and are in contact with degenerating epithelial cells. A clump of epithelial cells in process of shedding (SHEP) projects into lumen. Notice an intraepithelially migrating PMN. X 2,300. Circled inset shows mild cytoplasmic changes in a shedded epithelial cell, including accumulation of lipid droplets (LD). Square inset shows severe epithelial degeneration including altered ER and mitochondria with dark granules (arrows).

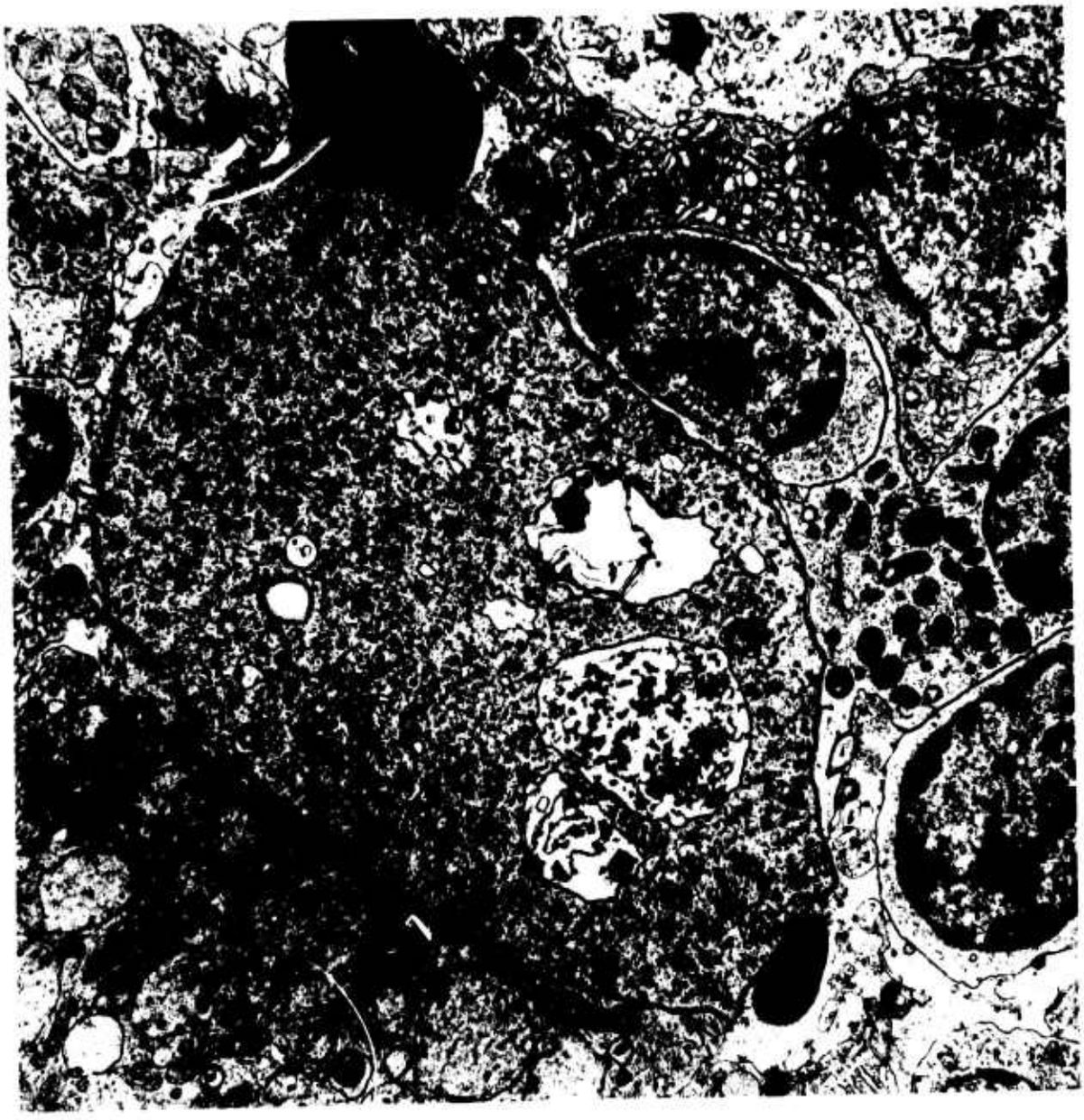

Fig. 10 - Ameba in the lamina propria. Ameba is surrounded by lymphocytes (LY), eosinophilic leukocyte (E), macrophage (MAC), polymorphonuclear leukocyte (PMN) and red blood cell (RBC), all of which are unaltered. X 8,500

Cytoplasmic alterations were usually extensive in extruded epithelial cells (Figs. 8 and 9), but occasional cells in the lumen showed only a minor degree of cytoplasmic change (Fig. 9). Desquamating or desquamated epithelial cells were often in contact with amebae (Figs. 7-9). In severely altered cells, microvilli had disappeared completely, and the cytoplasm showed a translucent matrix. Cytoplasmic blebs sometimes developed from these altered cells and were observed in the process of discharging cytoplasmic organelles into the gut lumen. During the final stage of epithelial alterations, a portion or remnants of altered cytoplasmic organelles of degenerating cells were frequently seen in contact with amebae or in a process of being phagocytosed by them.

Concomitant to the changes observed in the epithelium, degenerative alterations occurred in the PMN migrating into the epithelium at the sites of invasion. These PMN alterations have been described previously (Takeuchi & Phillips, 1975) and included depletion of cytoplasmic glycogen, homogenation and condensation of nucleoplasm and cytoplasm, pinching-off of the cytoplasm containing granules, and disruption and breakage of cell membrane with release of cellular contents into the extracellular space (Figs. 7 and 8).

In the lamina propria, amebae often exhibited pseudopodia, showed no signs of degeneration and appeared as normal as those in the lumen and epithelium. Some were in direct contact with mesenchymal cells (Fig. 10), while others were in the intercellular space without such contact. Cells of the lamina propria included fibroblasts, smooth muscle cells, macrophages, lynphocytes, plasma cells, eosinophils, RBC and PMN leukocytes. With exception of PMN, all other cells (Fig. 10), as well as collagen fibers and nonmyelinated nerve bundles, showed no significant changes when in contact with or in the proximity of amebae. As PMN contacted amebae or were topographically close to the organisms, they showed degenerative processes identical to those described above.

When amebae were confined to the epithelium, the structure of capillaries in the subepithelial region remained unchanged. However, emigrating PMN and RBC were often found in perivascular and intraepithelial locations. When amebae were present in the subepithelial region, the lumina of capillaries and venules were dilated and contained large numbers of erythrocytes, PMN, lymphocytes, thrombocytes and fibrin suspended in condensed plasma (Fig. 16). When amebae were found deeper in the lamina propria, both subepithelial capillaries

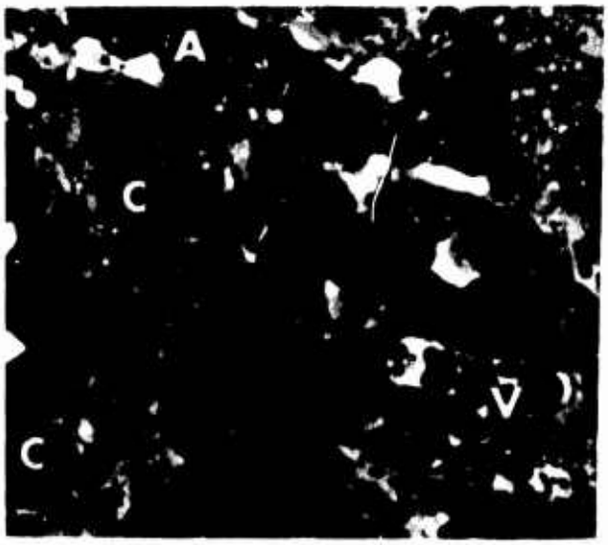

Fig. 11 - Invasion of lamina propria by amebae (A). Capillaries (C) are filled by RBC, and venule (V) contains platelets (arrows) and RBC. Note numbers of RBC in the extravascular space.
X 650

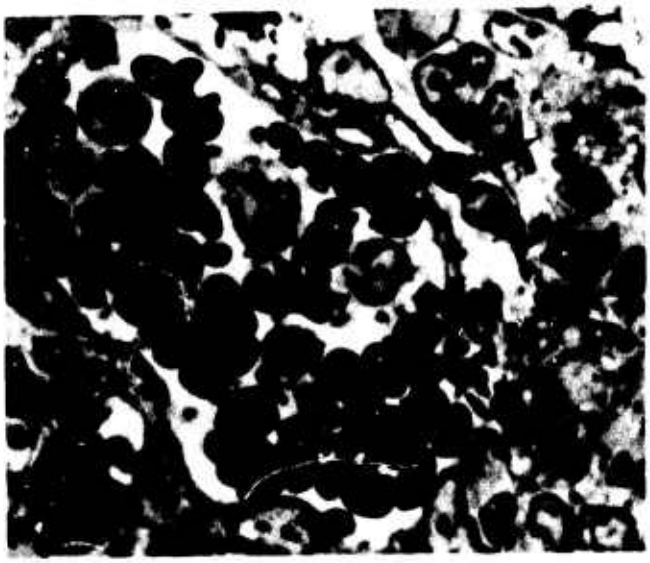

Fig. 12 - Invasion of lamina propria by amebae (arrows). Venular lumen contains many RBC and mononuclear cells.
X 840

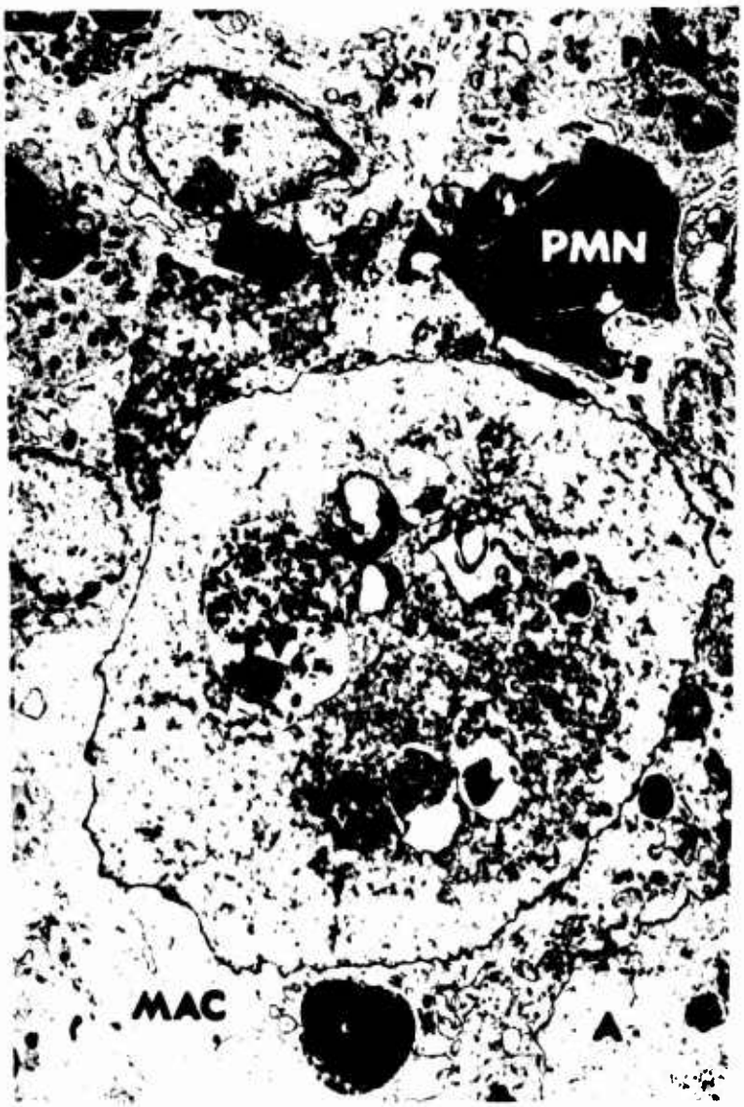

Fig. 13 - Ameba in the lamina propria. Ameba contains food vacuoles (FV). Most of amebic cytoplasm is replaced by irregular light areas due to the extraction of glycogen during alcohol dehydration. PMN close to or in contact with ameba (white letters) show released granules (arrow) and cytocomponents and homogenation of the nucleoplasm. PMN away from ameba (black letters) are unaltered. Portion of a second ameba (A) is seen at right lower corner. Macrophage (MAC) and fibroblast (F) are unaltered. X 7,200

and venules deep in the lamina propria frequently showed various degrees of endothelial damage. In both types of blood vessels, however, endothelial cells showing degenerative processes adjoined endothelial cells which remained unaltered (Fig. 15). Mild degenerative endothelial changes included cellular swelling, loss of cytoplasmic density, dilatation and swelling of ER and mitochondria (Fig. 15). The fenestrae of capillaries were usually unaltered, but the intercellular junction of capillaries and venules occasionally showed gap formation. PMN and RBC were sometimes identified in an apparent process of passing through the cell junctions of endothelial cells in both capillaries and venules. In severely altered vessels, the intercellular tight junctions were separated more frequently, and fenestrae occasionally developed gaps (Fig. 8). Their lumina often contained aggregations of degranulated platelets and fibrin. Such thrombotic processes occurred topographically close or attached to the separated cell junction and fenestrae (Fig. 8). The space between altered endothelium and the basal lamina was often widened. The pericytes of vessels, however, remained unchanged.

Isolated fragments and clumps of fibrin were seen not only in the vascular lumen (Fig. 15) but also were frequently observed in abundance in the extravascular space of the lamina propria (Fig. 14). Most fibrin in both intra and extravascular locations lacked the typical striation of 220 A. Indeed, very little such striation was seen. There was no evidence of extravasated thrombocytes in the lamina propria.

When amebae were topographically close to endothelial cells, multiple small vesicles of uniform size were observed between the basal lamina of the endothelium and the amebae; these appeared to have developed from the endothelial cell membrane (Fig. 16). In some instances, blebs of different sizes were identified in a process of pinching-off from the endothelial cytoplasm into the lumen (Fig. 16).

Discussion

The present series of studies on pathogenesis of acute amebic colitis demonstrates the potential of the developing field of gnotobiology in certain biomedical investigations. When virulent amebae from acute human amebiasis are inoculated intracecally into conventional guinea pigs, the animals may develop either acute or chronic disease or, as is often the case, remain completely uninfected. In contrast, if the same amebic inoculum containing patient's enteric

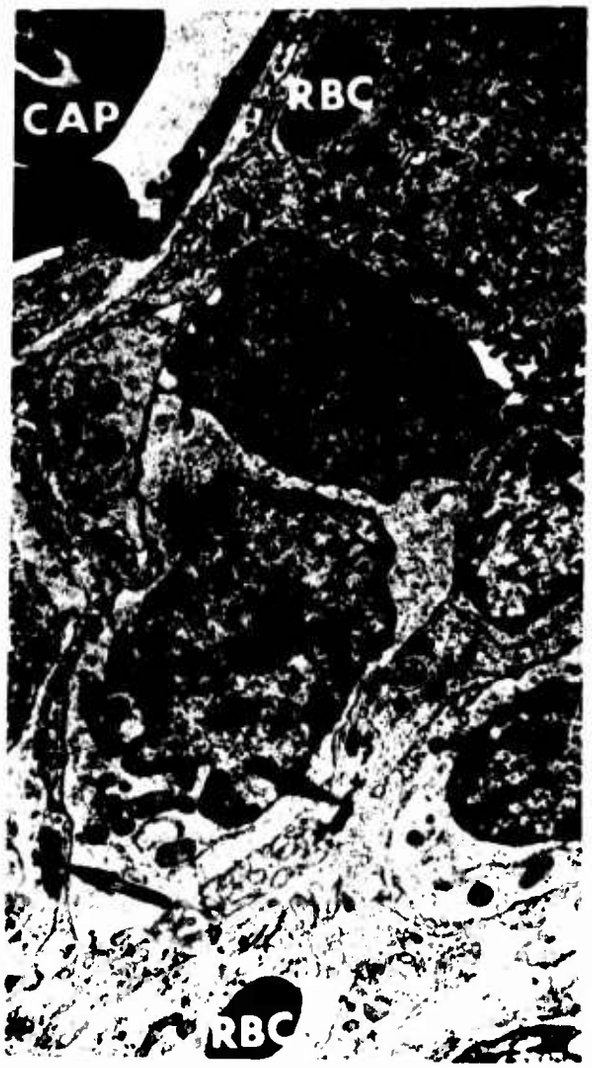

Fig. 14 - Clumps of fibrin are in the extravascular space of lamina propria (arrows). Note extravasated erythrocytes (RBC), capillary (CAP), PMN leukocyte (PMN), lymphocyte (LY).
X 10,500

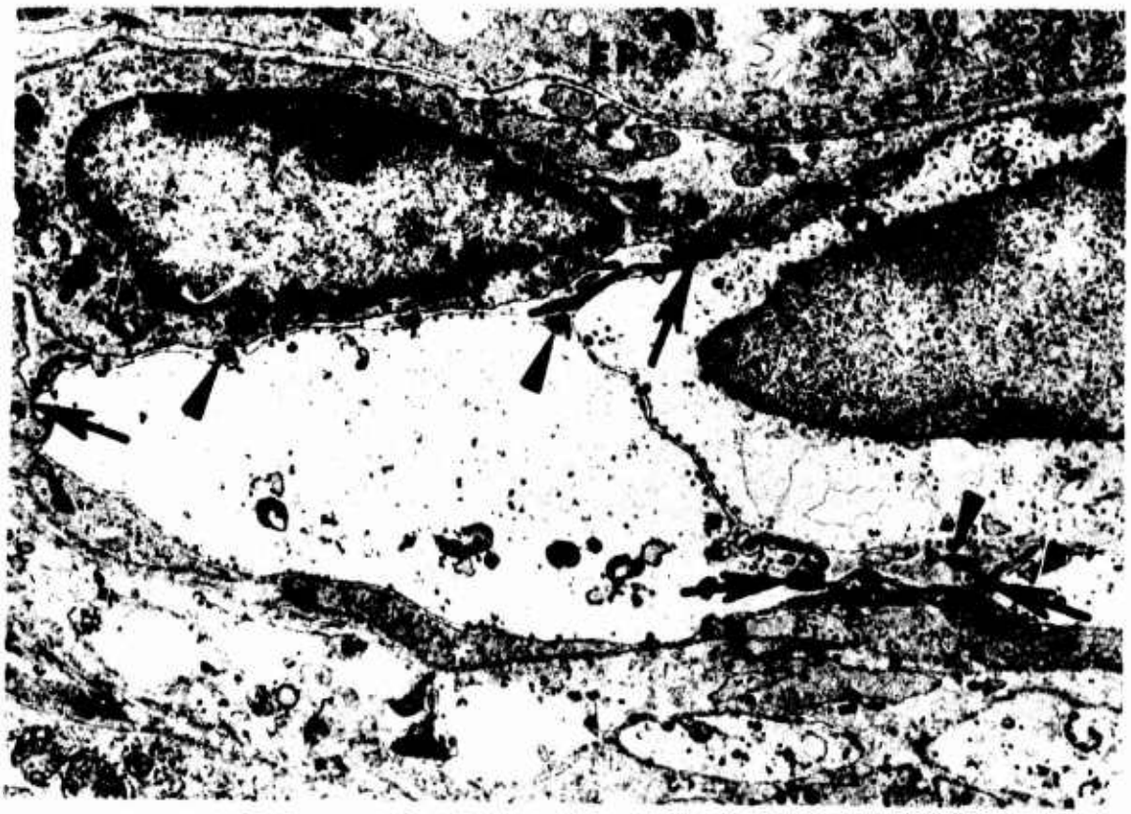

Fig. 15 - Capillary changes below crypt epithelium (EP). Markedly swollen endothelial cells with clear cytoplasmic matrix almost obliterate the lumen. Slit-like lumen contains fibrin-like structures (arrowheads). Pinocytotic vesicles are concentrated at the luminal edge of cytoplasm of altered endothelial cells. Note that the adjacent endothelial cell at left is well preserved. Intercellular tight junctions (arrows) and the basal lamina appear unchanged.
X 13,000

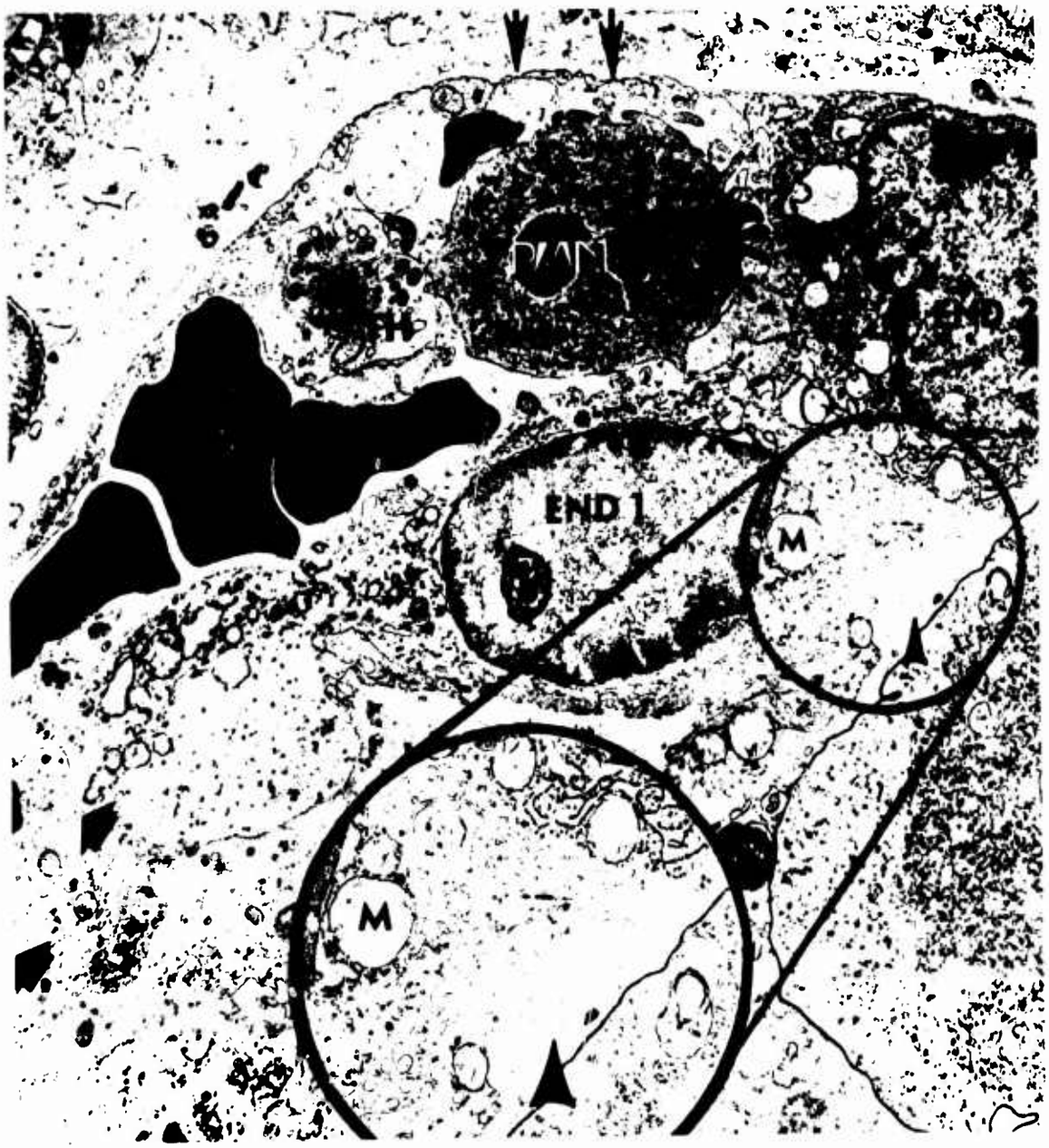

Fig. 16 - Ameba (A) is close to a capillary. Lumen contains RBC, PMN, and aggregates of thrombocytes (TH). Endothelial cell (END2) shows swollen ER and mitochondria in dense cytoplasm, while an adjacent endothelial cell (END1) shows lighter cytoplasm. In capillary lumen, cytoplasmic blebs (thick arrows) of various sizes appear to develop from luminal side of pale endothelial cell (END1). Fenestrae are intact (thin arrows). X 11,800. Inset circle shows light endothelial cytoplasm with swollen mitochondria (M). Cytoplasmic blebs (arrowhead) are also present in the extravascular space.

flora is administered to germfree guinea pigs, it produces a uniformly acute pathologic process which is similar to that in the human patient. Since it has been demonstrated that different bacteria may influence appreciably the many facets of enteric amebic disease (Phillips et al., 1968), this model offers obvious advantages over study of the ameba in a conventional animal harboring an enteric flora that is characteristically much different from that encountered in the human patient. Use of this experimental model and the specific sampling method described herein have facilitated observation, at the ultrastructural level, of detectable morphological events attending the invasion of the intestinal mucosa by E. histolytica.

Worthy of mention are the abnormal detachment and shedding of epithelial cells at the sites of amebae penetration. Under normal conditions, intestinal epithelial cells produced in the crypt migrate toward the surface of the mucosa and are then extruded singly into the lumen. The kinetics of this epithelial cell turnover are relatively constant at different levels of the gut in each individual animal species. However, during acute enterocolitis such as produced by Shigella (Takeuchi et al., 1968) and Salmonella (Takeuchi & Formal, 1967), excessive cell loss occurs not only at the normal extrusion site, but also elsewhere on the epithelial surface. Under these conditions, the kinetics of epithelial renewal are significantly altered (Abrams et al., 1965). Similar considerations are applicable to amebic infection; excessive cell loss occurred at multiple sites of epithelial amebic penetration and also between sites of penetration.

The present study has provided additional information on the penetration of intestinal mucosa by amebae. Following invasion of the lamina propria, additional amebae were often found at the gaps between epithelial cells (microerosion) and appeared to be passing preferentially through them, as if they were attracted to the microerosions (Figs. 5 and 8). Given the high requirements of serum for the successful growth and motility of axenic E. histolytica, it is conceivable that, in view of the morphologic evidence of increased vascular permeability, the luminal amebae might be chemically attracted to the microerosions where leaked plasma proteins were presumably present in abundance.

Besides clarifying with greater detail the interaction between epithelial cells and amebae, the present EM evidence has shed further light on the interaction between amebae and PMN. In the lamina propria, PMN in the proximity of invading amebae, but not necessarily in contact with them, showed a variety of changes, ranging from minor defects to cell death. These changes were essentially the same as those described previously at the site of epithelial penetration (Takeuchi & Phillips, 1975). Lysis of PMN upon contact with amebae has been reported to occur both *in vivo* and *in vitro*, but contradictory observations have also been reported. Weissman has pointed out that potent toxins derived from certain microbes are capable of lysing PMN. However, although proteolytic enzymes are present in many strains of amebae, including some free living species, toxic agents have not been demonstrated in *E. histolytica*. Studies utilizing various techniques, including enzyme cytochemistry and immunochemistry, are needed to clarify the interaction between amebae and PMN.

On the basis of ultrastructural observations, Eaton has proposed that surface lysosomes of amebae are important in causing lysis of host cells (1970). Although cytochemical studies on *E. histolytica* *in vitro* and *in vivo* (Lushbaugh, 1974) have demonstrated acid phosphatase activity on the external cell membrane, there has been no definite cytochemical evidence that externally released lysosomal enzymes from the cytoplasm of trophozoites are capable of causing lysis of host cells. Such activity seems highly unlikely in view of the fact that mesenchymal cells in direct contact with amebae remain structurally unaffected, whereas under the same conditions PMN showed lysis of cell membrane with release of contents.

Lysates of PMN, on the other hand, may be responsible for some of the other phenomena which occur during invasion of the lamina propria by *E. histolytica*. PMN lysates have been shown to contain hydrolytic enzymes such as lysosomal hydrolases and vascular permeability factors (Weissman, 1973), which undoubtedly affect the physicochemical functions of tissues with which they come in contact. We were much impressed with the concomitant occurrence of various vascular changes in the areas of PMN lysis.

The capillaries of the bowel mucosa characteristically contain multiple pores closed by a thin diaphragm (Palade, 1970). They are the so-called "fenestrated capillaries", which are found also in

renal glomeruli and certain endocrine glands. Hurley and McQueen, who studied the response of fenestrated vessels of the small bowel of rats to applications of mustard oil into an intestinal loop, observed that an intravascular carbon marker was found at the gap formed at the intercellular junction of endothelial cells, but not at the fenestrae (Hurley, 1971). They stated that the gap formation was reversible and provided a route for leakage of plasma protein. During invasion of the cecal mucosa by amebae, gaps were formed at cell junctions between endothelial cells in both capillaries and venules. In the latter instance, the gap formation was found not only at the intercellular junction but also at the diaphragms. Vascular changes in both capillaries and venules included not only formation of gaps, but also a variety of endothelial changes. These occurred both in areas directly invaded by the organism and away from them. Severe endothelial changes were accompanied by occlusive thrombosis by platelets and fibrin and swelling of the thrombotic vessel wall. Similar endothelial changes have been reported previously during invasion of the intestinal mucosa of guinea pigs and monkeys by Salmonellae and Shigellae. Thus, it is possible that, regardless of the type of enteric pathogen involved, similar mechanism(s) operate in the development of endothelial changes in the early stages of many acute enteric infections.

Many physical, chemi ective and immunologic stimuli elicit a delayed and prolonged response which involves both capillaries and venules (Cotra). In an EM study on vascular response of the skin of rats to Clostridium toxin, Cotran observed an intravascular carbon marker at the intercellular gap between endothelial cells and thrombosis associated with disrupted endothelial cells (Cotran, 1968). He proffered three possible explanations: that the endothelial injury might result from chemical mediators, or from the effect of prolonged stasis and ischemia on the endothelium, or possibly from the direct action of the Clostridium toxin. As shown in Fig. 12, the endothelium in contact with or in close proximity to amebae showed a pinching-off of the cytoplasm from the surfaces toward the amebae, suggesting a direct effect of the amebae upon the endothelium. Yet, such endothelial changes were rare. Hence, we believe that endothelial changes seen in this study might be caused in part by the direct action of the amebae, but mostly, perhaps, by other mechanisms, such as chemical or immunologic mediators derived from lysed PMN or ischemia.

Conclusion and Recommendation

Germfree guinea pigs were inoculated intracecally with Entamoeba histolytica and the enteric flora derived from a human patient with acute amebic colitis. Animals were sacrificed at post-inoculation intervals of 7-12 days. The invasion of cecal mucosa by the ameba was examined by light and electron microscopy. Early amebic penetration of the lamina propria then is accompanied by continued epithelial shedding, PMN degeneration, and changes in both capillaries and venules consisting of endothelial damage and occlusive thrombosis. Vascular changes appeared to be related to PMN degeneration caused by interaction of PMN with the invading amebae.

Further studies on histolysis of lamina propria and alterations of microcirculation in the colonic mucosa subsequent to the penetration of the lamina propria by amebae are highly recommended. These studies will provide new information in the pathogenesis of acute ulceration of the colon, the hallmark of Entamoeba histolytica infection in man.

B. Study of the Ultrastructure of Pathogenic Trophozoites of Entamoeba Histolytica

Recent application of the freeze-etching technique to the study of biological specimens has added important information on the intricacies of various cell membranes and cytoplasmic organelles not previously disclosed by other electron microscopic techniques.

In search of new information on the ultrastructure of E. histolytica, we have studied its trophozoite stage by the freeze-etching technique (FE) of electron microscopy. In this study, we have attempted to describe its ultrastructure as revealed by FE and to correlate it with observations made by the thin-sectioning technique (TS). Particularly, attention has been given to the complexity of the cell membrane and the fine structure of the nucleus.

Amebae:

Amebae used in this study were the CDC-J190 strain of Entamoeba histolytica (Healy and Gleason, 1966). Cultures of the trophozoites and the accompanying bacterial flora from the original patient were maintained in Locke's egg-rice flour medium at 37° C and transferred 3 times each week. The amebae were harvested from 48 hour cultures and processed for both thin-sectioning (TS) and freeze-etching (FE) techniques of electron microscopy.

Observations

General Appearance of Trophozoites:

In replicas of FE specimens, trophozoites were easily recognized; many of them were fractured through various planes (Fig. 17), while others were not fractured and could be seen as intact cells, irregular in shape and enveloped by a wavy, intricate, and convoluted cell membrane. In the environ, many granules and rod-shaped bacteria were interspersed between membranous and granular structures of different size and shape which were identified as fragments of cytolysed amebae. The cleaved surfaces of amebae exhibited a granular and lumpy cytoplasm in which nucleus, vacuoles and vesicles of various size and shape were readily discernible (Figs. 17 and 21). Many of the vacuoles contained bacteria, membranous and granular material. Structures which could be interpreted as mitochondria, endoplasmic reticulum and Golgi complex were not identified. This was confirmed by TS observations and was in agreement with other reports (Miller et al.

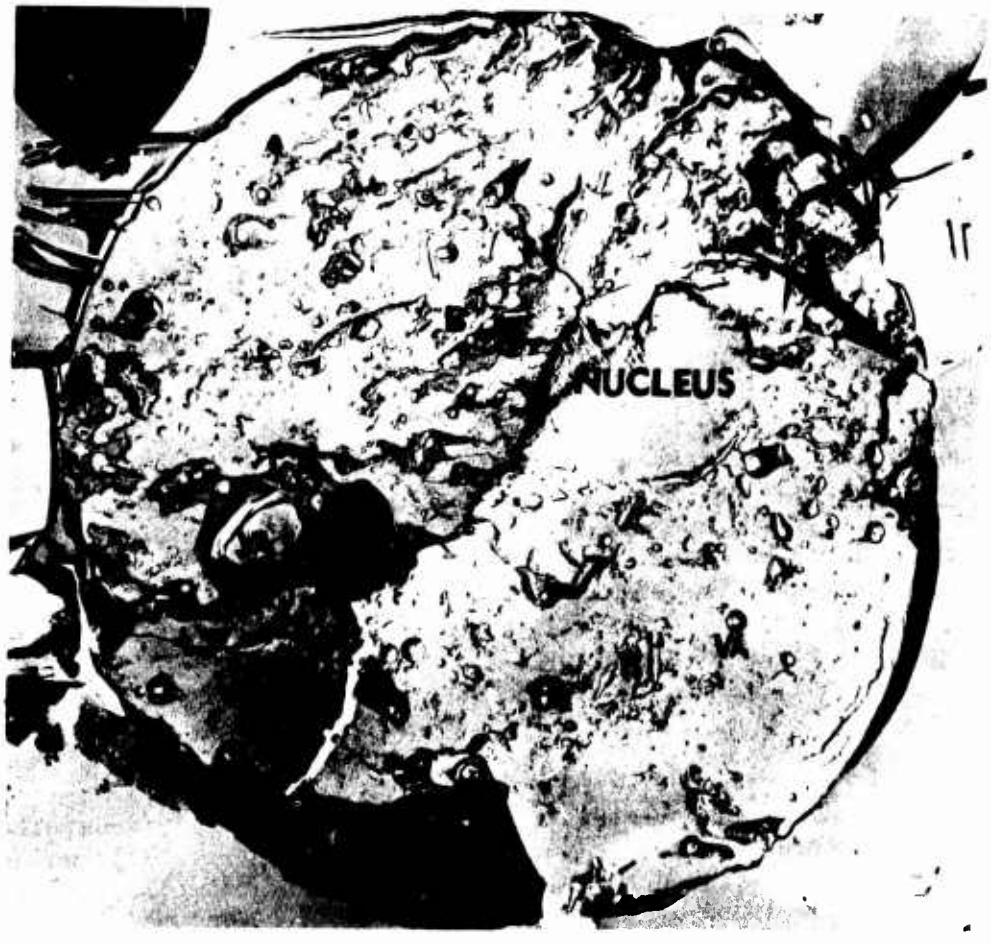

Fig. 17 - FE trophozoite of E. histolytica. The fractured surface of the cytoplasm shows many vesicles and vacuoles (VA) of different sizes. Phagocytosed bacteria (B) are numerous. Nuclear pores cannot be identified in the nucleus at this low magnification.
X 6,000

1961; El-Hashimi and Pittman 1970; Griffin and Juniper 1971; Lowe and Maegraith 1970; Ludvik and Shipstone 1970).

Cell Membrane:

In replicas of unfractured amebae, cell membranes were convoluted and displayed numerous infolds; some organisms had a wavy or creased membrane (Figs. 19 and 21), while others showed pseudopodia (Fig. 18). In favorably oriented specimens, pseudopodia were wrapped around bacteria. Sometimes fragments of membranes and ribosomal helices, presumably derived from cytolysed amebae, adhered to the outer surface of the cell membranes (Fig. 24). Spherical protrusions and depressions ranging from 0.5 μ to 1.0 μ in diameter were uniformly seen on extracellular surfaces of unfractured cell membranes. In favorable replicas, fractured amebae showed these depressions and protrusions, measuring up to 1.0 μ in width, on the inner and outer aspects of the cell membrane (Figs. 18, 19 and 22). In contrast, structures suggestive of surface projections and depressions were rarely recognizable in TS specimens.

Cytoplasmic Organelles:

In freeze-etched amebae, vacuoles and vesicles were present in abundance (Figs. 17 and 21). They were usually spherical or ovoid and enclosed by a single membrane. Their size varied greatly from 0.2 μ to 9 μ in width. Some vacuoles and vesicles were apposed and their membranes were fused together (Figs. 20 and 21). In certain instances, the enclosing membrane invaginated, forming "teardrop" shaped vesicles (Fig. 23). Some bacteria were tightly enclosed, while others were found loosely enveloped in vacuoles together with ribosomal helices, vesicles and fragments of membranous structures (Fig. 20).

In TS specimens, abundant polyribosomes and glycogen particles were seen throughout the cytoplasm and were identified also in replicas of FE amebae. Structures which were of similar dimensions as ribosomal helices observed in TS cytoplasm were occasionally seen on the unfractured cell membrane (Fig. 22). Several parallel membranes were found in the peripheral cytoplasm. They were separated by 0.2 μ to 0.5 μ intervals and varied in length from 0.5 μ to 1.0 μ, thus corresponding to the crystalline ribonuclear protein helices of Rosenbaum and Wittner (1970) and the crystalline aggregates of El-Hashimi and Pittman (1970). They were serpentine in shape and failed to show saclike ends like those of the Golgi complex.

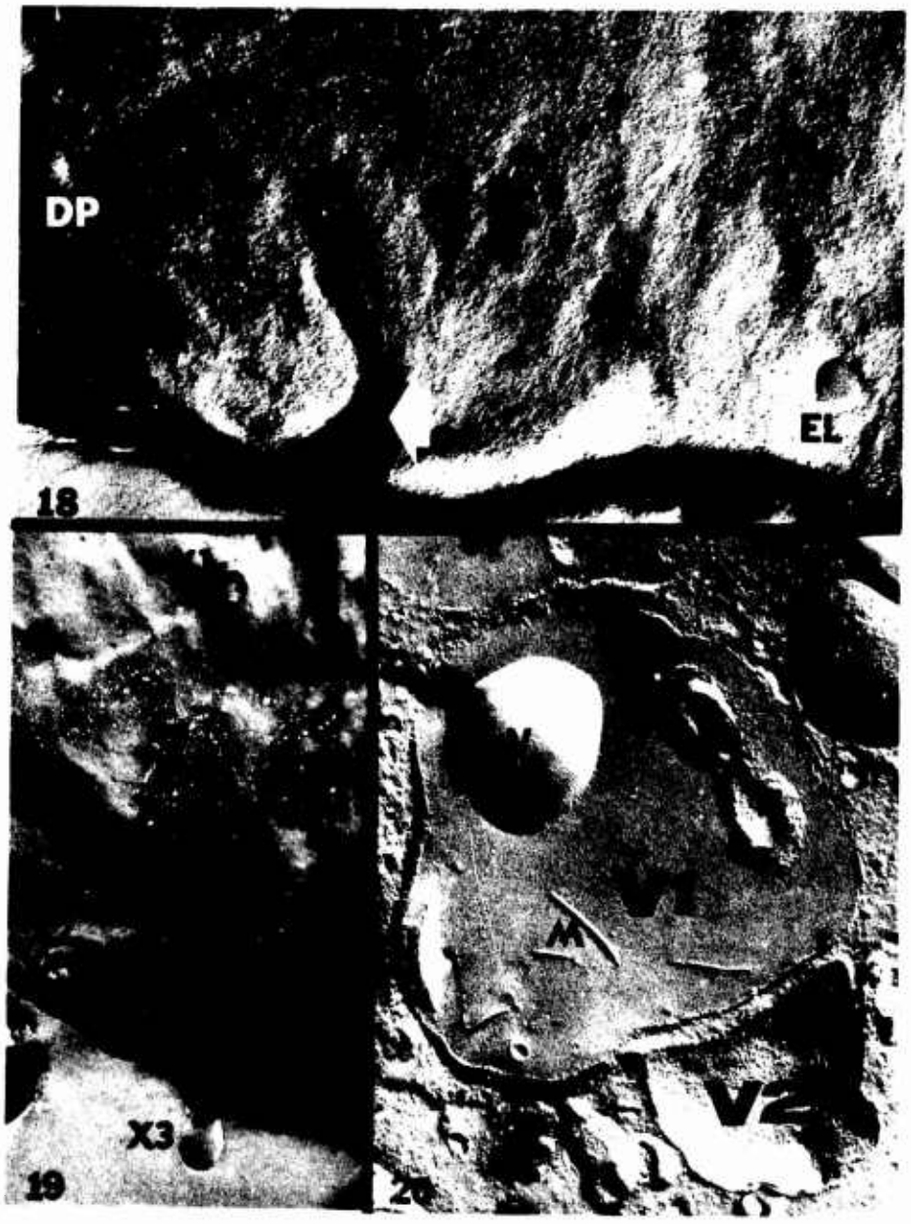

Fig. 18 - FE replica of intact cell membrane. The surface is creased (white arrow) and shows a convoluted cell membrane on which spherical elevations (EL) and depressions (DP) are seen. X 24,300

Fig. 19 - FE replica of intact external cell membrane. The surface is smooth, wavy, and contains spherical elevations (X1, X2, X3). Elevations X1 and X2 have been fractured so as to reveal their interiors. X3 is seen in profile. X 22,000

Fig. 20 - FE vacuoles. Vacuole (VA1) is enclosed by a membrane and contains bacteria (B), membrane fragments (M) and unspecified vesicles (V). Vacuole (VA2) is closely apposed to VA1. The membrane of VA2 is unfractured and appears to be fused to to membrane of VA1 at the site of contact. X 45,300

Fig. 21 - FE ameba. The cell membrane (CM) is smooth and convoluted. Homogenously lumpy cytoplasm shows numerous vacuoles (VA). Arrows indicate where a vacuole (VA1) is fused with another vacuole (VA2). X 10,000

Fig. 22 - FE replica of unfractured external cell membrane. Numerous spherical elevations and depressions are easily discernible. Ribosomal helices (small arrows) are dispersed on the surface. X 16,200

Fig. 23 - FE vacuole. Vacuolar membrane shows teardrop invagination (arrow). X 50,000

Nucleus:

By TS technique, the nucleus was enclosed by double membranes which appeared to be parallel and were separated by an electron translucent space 240 A thick (Fig. 26). Each membrane was a typical triple layered unit membrane, 120 A thick. The two membranes of the nuclear envelope appeared to be interrupted by numerous regularly spaced nuclear pores. No additional structures were identified at the pore openings or in their vicinity. Inside the nucleus was the nucleoplasm, which consisted of a heterogeneous matrix with central electron translucent heterochromatin and peripheral electron opaque euchromatin (Fig. 26). In the latter were occasionally seen membrane-enclosed bodies which contained electron dense material and ranged from 0.5 μ to 3 μ in diameter. These intranuclear bodies corresponded to the button-like bodies of Ludvik and Shipstone (1970), or the small vesicular inclusions of El-Hashimi and Pittman (1970).

By FE technique, the nuclear profiles were easily identified by their double parallel nuclear membranes and characteristic nuclear pores (Fig. 24). Freeze cleavages often occurred between the outer and inner nuclear membranes, as exemplified in Fig. 25. In such fractured specimens, the cytoplasmic surfaces of both outer and inner membranes showed regularly spaced circular nuclear pores approximately 640 A in diameter and were studded by numerous globules. Counts were made on the number of nuclear pores at a photographic magnification of approximately 30,000 times; the approximate figure for the density of the nuclear pores at the FE nuclear surface was about 35 pores per square micron. Distance between the pores was also approximately 640 A. By FE technique, the nucleoplasm appeared homogenously granular, as the euchromatin could not be distinguished from the heterochromatin. Neither was it possible to demonstrate intranuclear membrane-bound bodies.

Discussion

Trophozoites of E. histolytica, demonstrated by the FE technique, exhibited intricate infolds, convolutions and undulations of their membranes and a surface topography of the nuclear envelope and its pores which have not been shown by TS technique. Confirmed by FE were the absence of a Golgi apparatus, of a well formed endoplasmic reticulum, and of mitochondria, as previously reported by other investigators (Miller et al. 1961; El-Hashimi and Pittman 1970; Griffin and Juniper 1971; Lowe and Maegraith 1970; Ludvik and Shipstone 1970).

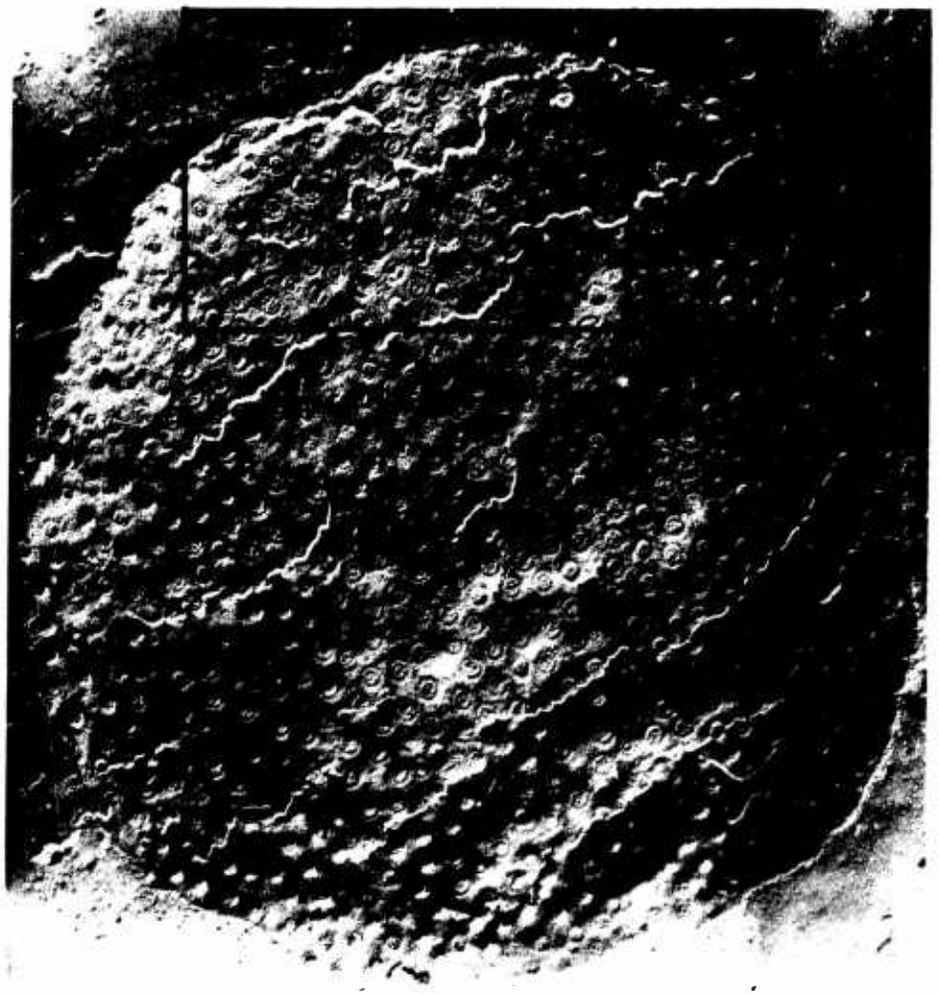

Fig. 24 - FE replica of fractured nucleus. The fracture plane is irregular and has occurred between the outer and inner membranes of the nuclear envelope. Both membranes show their cytoplasmic surface (A face). Fenestrating the nuclear membrane are circular pores (arrows) which are spaced at regular intervals. Numerous minute globules are present on the A face of both membranes. Square is magnified in Fig. 25.
X 13,000

Fig. 25 - Detail of FE nucleus, magnified from the square in Fig. 24. The fracture plane through the nuclear membranes shows outer (OM) and inner (IM) membranes. Some pores on the inner membrane have been fractured (parentheses), while others are unfractured and are covered by remnants of the outer membrane (arrowhead).
X 26,300

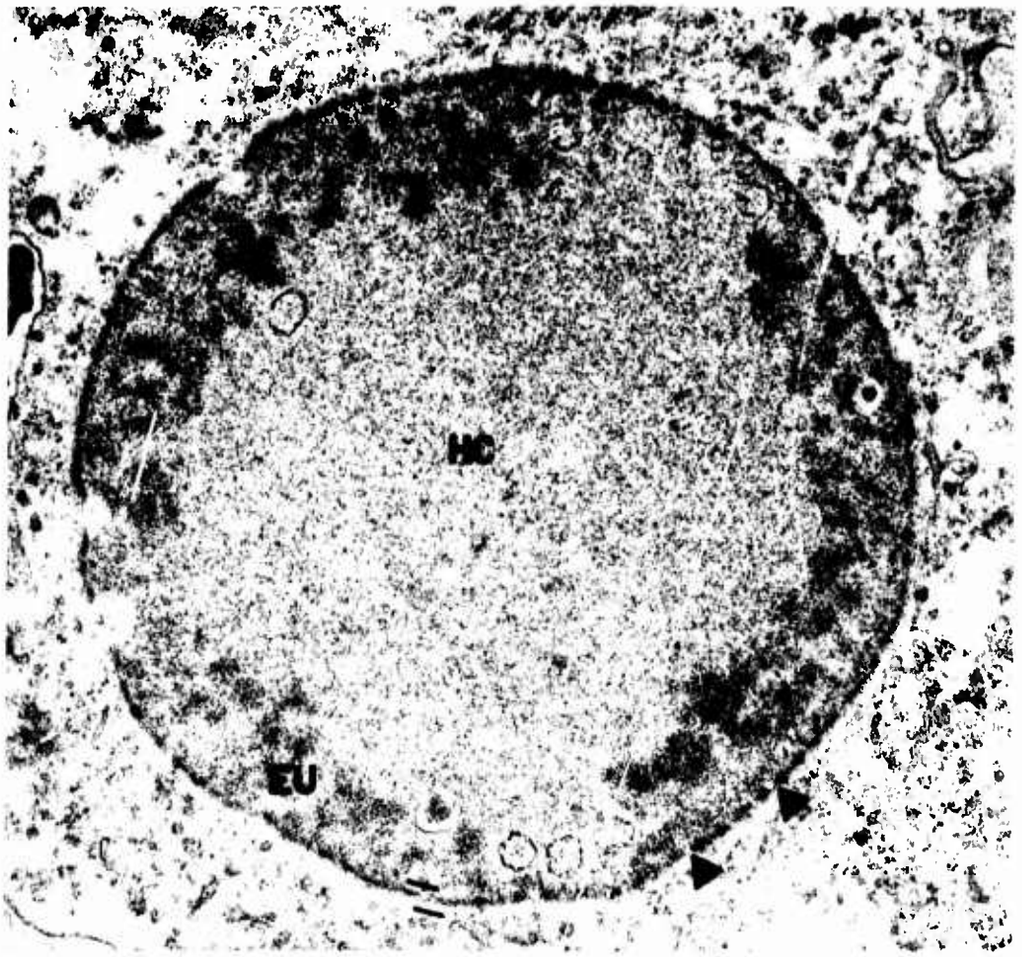

Fig. 26 - TS nucleus. The TS nucleus is enclosed by a porous (arrowhead) double unit membrane (brackets), separated by an electron translucent space approximately 240 A wide. Inside the nucleus is a heterogeneous nucleoplasm, as the heterochromatin (HC) can be distinguished from the euchromatin (EU). Membrane-bound inclusions (parentheses) are confined to the euchromatin.
X 22,000

Surface replicas of the intact cell membrane of trophozoites clearly showed small focal depressions and elevations. In FE studies of the mechanism of release of secretory granules of endocrine cells of mammals, Smith et al. (1973) and Amherdt et al. (1973) have clearly demonstrated similar profiles of spherical depressions of the cell membrane. They postulated that such depressions represented exocytotic release of secretory granules. Thus, it is possible that depressions in the cell membrane of amebae, as shown in Figs. 18 and 22, might represent openings of exocytotic vesicles into the environ. On the other hand, elevations (button bodies) on cell membranes, exemplified in Figs. 18, 19 and 22, were very similar to the filiform extensions of surface lysosomes described by Eaton et al. (1969). Based solely on morphological observations, Eaton et al. (1969) have speculated that these filiform extensions release lysosomal products when touched by particles or cells in the environ. Utilizing cytochemical techniques for demonstrating lysosomal enzymes, Lushbaugh and Miller (1974) further postulated that the filiform extensions were filled with lytic enzymes and ruptured upon contact with host cells after detaching from the cell membrane of E. histolytica. Upon examination of numerous electron micrographs by both the FE and TS techniques, it was established that there was a great uniformity in both diameter and elevation of the button bodies. From these observations and from the similarities of the button bodies with structures described by the already cited investigators, we believe that both depressions and elevations on the cell surface represent secretory mechanisms of cytoplasmic vesicles from E. histolytica into the extracellular space.

Although many authors have reported the presence of food vacuoles, phagosomes, and lysosomes in E. histolytica (Rosenbaum and Wittner, 1970; Ludvik and Shipstone, 1970; Griffin and Juniper, 1971), the relationships among these cytoplasmic vacuoles have yet to be demonstrated by TS technique. Intake of food stuffs was rarely observed in the present TS observations; food vacuoles were formed mostly by pseudopodial extensions of the cytoplasm, which loosely enclosed bacteria and starch material from the environ. Since FE preparations included various planes of cellular details, phagocytic processes and formation of food vacuoles were commonly observed in replicas. Food vacuoles were often in contact with or attached to vacuoles or vesicles. Some of them showed the disappearance of membranes at the point of contact. Zucker-Franklin and Hirsch (1964) and Henson (1972) have shown similar findings by TS in phagocytic cells and postulated that the fusion of vacuoles represents transfer of lysosomal contents into phagocytic vacuoles.

Most clearly defined in FE amebae was the topography of the nuclear membrane. Surfaces of the nuclear membrane were regularly fenestrated by circular pores. On observation of E. histolytica by TS, Deas et al. (1961) reported the nuclear membrane as being composed of "numerous discontinuities", which comprised 50% of the total nuclear surface, while Osada (1959) saw only "several minute holes" on the nucleus of methacrylate embedded amebae. In the present FE observations, counts of nuclear pores were possible and were estimated as 35 per square micron. Nuclear pores were arranged in regular arrays and the distance between pores was similar to the pores' diameter. Flickinger (1970) has elegantly demonstrated by TS that in Amoeba proteus nuclear pores contain a central gap and are situated at the center of a fibrous lamina which arises from the inner nuclear membrane and radiates into the nucleoplasm to form honeycomb layers. By neither FE nor TS were we able to demonstrate such structures on the nuclear membrane of trophozoites of E. histolytica. Since nuclear pores occupy such a large area in the nuclear envelope of E. histolytica, the transfer of substances should be readily accomplished between two major compartments of the cell. Yet, it has not been documented whether nuclear pores provide free communication between nucleoplasm and cytoplasm in E. histolytica.

Conclusion

Pathogenic Entamoeba histolytica trophozoites were studied by a new technique of electron microscopy, the freeze-etching (FE) method. The study has provided the following new information on the ultrastructure of this enteric pathogen. Spherical depressions and elevations varying from 0.5 μ to 1.0 μ in diameter were commonly present on the external cell membrane and appeared to represent an extracellular secretory mechanism of trophozoites. Vesicles and vacuoles ranged in diameter from 0.2 μ to 9.0 μ. Some vacuoles tightly enveloped bacteria, while others enclosed bacteria together with host cytocomponents including ribosomal helices. Occasional vesicles and vacuoles appeared to be fused to each other.

Replicas of FE nucleus were enclosed by double nuclear membranes which were fenestrated by numerous spherical pores measuring approximately 640 A in diameter and spaced at intervals of 640 A. Counts of nuclear pores were possible and indicated 35 pores per square micron on the nuclear envelope. Golgi apparatus, mitochondria and well formed endoplasmic reticulum were absent in FE replicas. This was in agreement with electron microscope observations on thin sections previously reported by other investigators.

III. STUDIES OF INTESTINAL INFECTIONS WITH ENTEROVIRUSES

Background

Abundant information is available concerning the physical, chemical, antigenic, infective and epidemiologic properties of enteroviruses. Most of these data have been obtained from studies of virus-cell interactions in tissue culture system rather than observations on enteric infections *in vivo*.

In natural virus infections the precise role of the gastrointestinal tract either as the portal of entry or as the initial site of virus replication has not been well established. It is generally considered, however, that it is an initial site of replication for many enteroviruses (Dowie, 1963; Stenhouse, 1970). For instance, Enders and his colleagues demonstrated that the Lansing strain of the poliomyelitis virus multiplied in suspension cultures in human embryo intestinal tissue more than 25 years ago (Enders et al. 1949). Yet, little is known about host cell-virus relationship in the gastrointestinal tract *in vivo*.

In collaboration with two groups of investigators, light and electron microscope studies on enteric infections by enteroviruses were undertaken. We used a murine adenovirus K87 and a canine coronavirus 1-17. Special attention has been given to site of viral replication in the gut mucosa.

A. Study of Experimental Enteric Infection of Mice with a Mouse Adenovirus K87

Dr. Hashimoto, Prof. of Microbiology, Keio University, Tokyo, Japan, has kindly collaborated on this project. He has infected mice with his adenovirus in his laboratory and supplied us with all necessary virological data and infected intestinal tissues.

Adenovirus strain K87 was originally isolated by Hashimoto from the feces of a healthy inbred mouse strain DK1 (Hashimoto et al. 1966). Strain K87 is identified as an adenovirus and grows well in the mouse kidney tissue cultures (Hashimoto, 1967). Both oral and parenteral administrations of K87 into the mice result exclusively in replication of the viral antigens in the bowel but produce no symptoms in the infected animals (Hashimoto, 1969). When DK1 mice

Fig. 27 - The mouse ileum at 14 days after oral infection with K87 adenovirus. Fluorescent structures at the epithelium of both villi and crypts. The viral titer is high in the stool and ileal tissue of this animal. Immunofluorescent micrograph X 480.

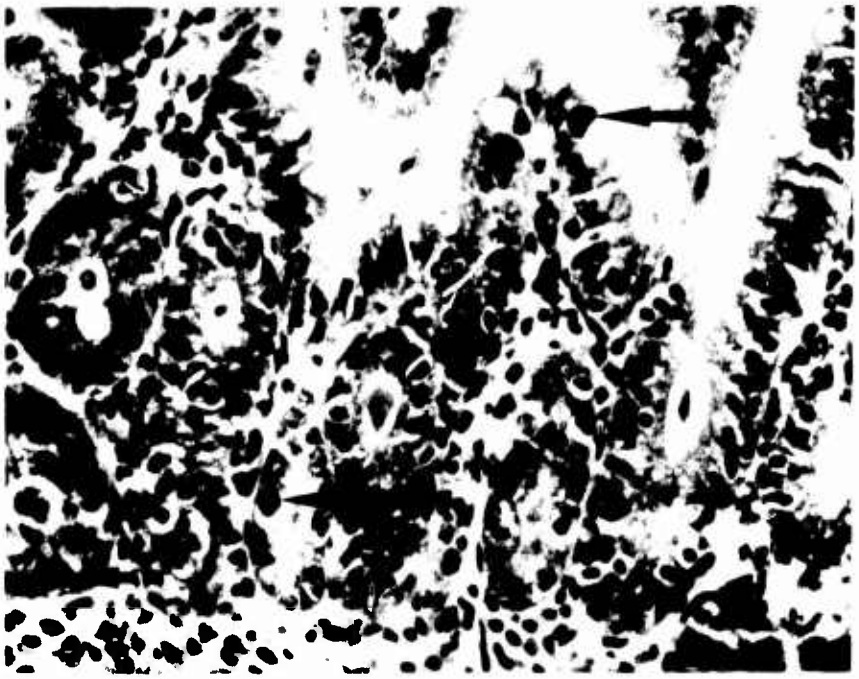

Fig. 28 - The ileal mucosa of a mouse at 14 days after infection with adenovirus K87. Spherical and bizarre shaped large inclusions were seen within the epithelium (arrows). X 560. Paraffin section stained by H & E.

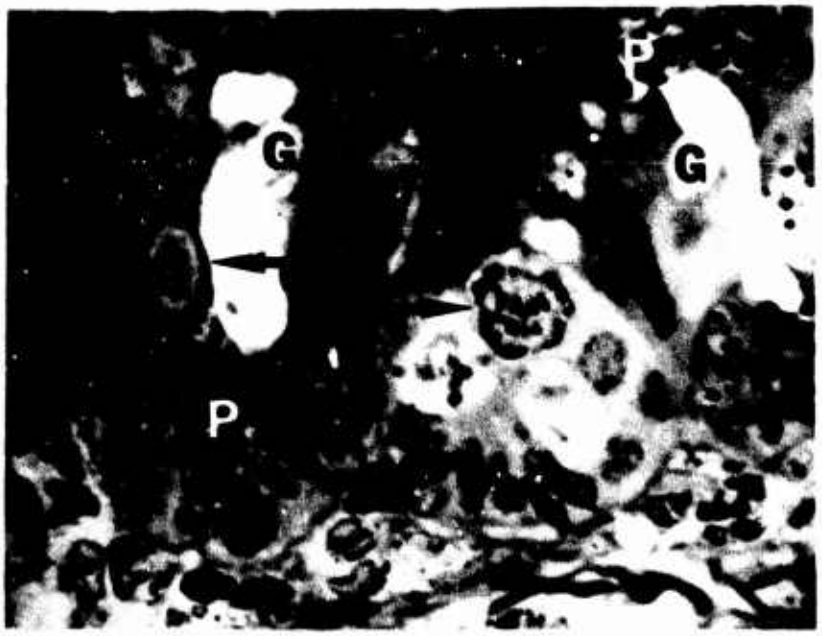

Fig. 29 - Crypt epithelium of the ileum with adenovirus infection. One μ thick section from Epon embedded tissue shows details of intraepithelial inclusions (arrows); their shape is oval at left and angular at right. Inclusions consist of dense globular masses at periphery and numerous less dense granules and globules in the lightly homogeneous nucleoplasm. Note prominent granules in Paneth cells (P). G: mucus granules of goblet cells. Methylene blue azure II stain. X 900.

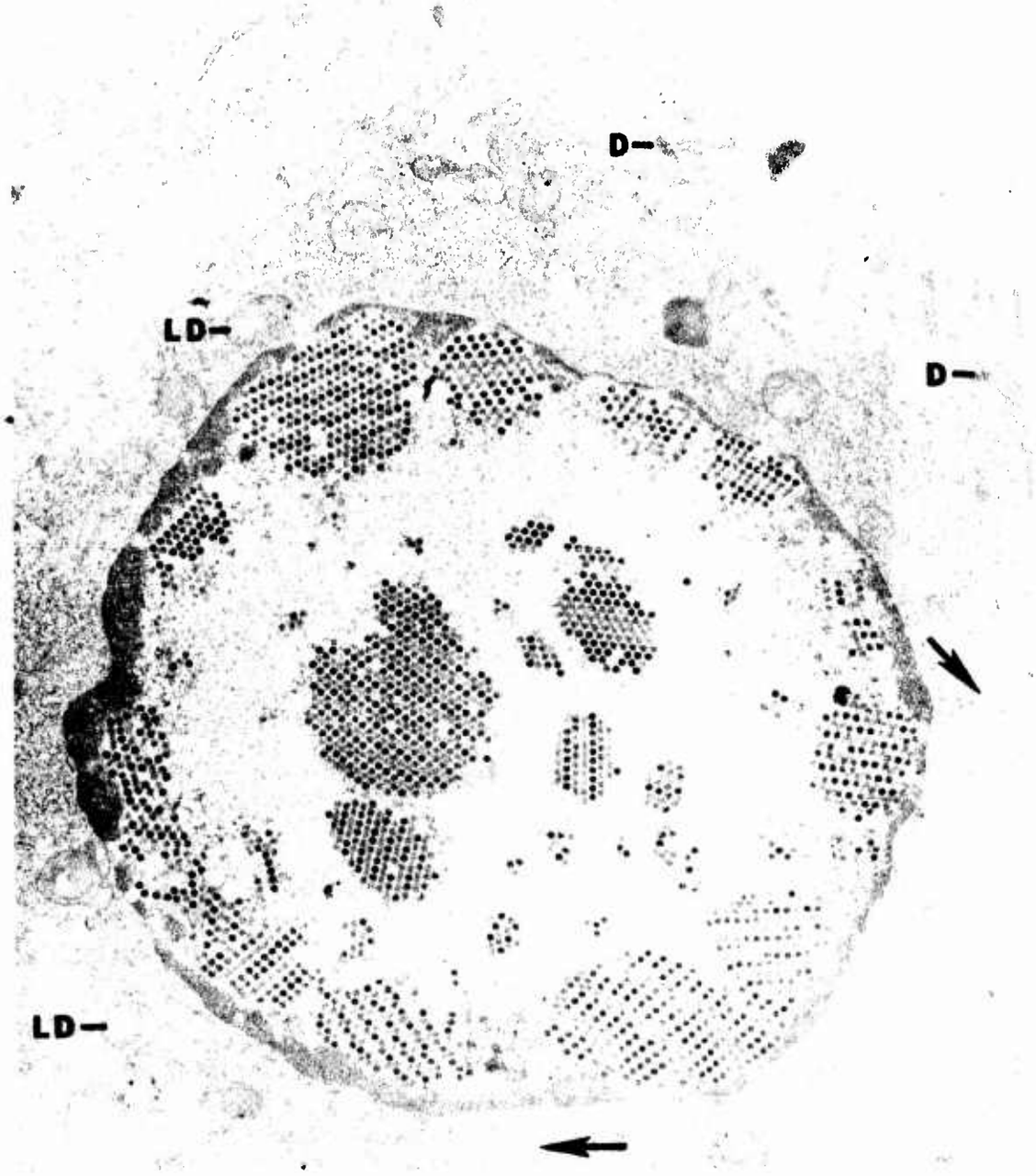

Fig. 30 - Intestinal columnar cell at mid-villus, ileal epithelium, mouse infected with adenovirus. The enlarged nucleus contains virus particles showing crystalline arrays. Mitochondria occasionally show spherical lipid droplets (LD). Split-like electron translucent spaces are present in the unaltered cytoplasmic matrix (arrows). Microvilli are normal. Intercellular desmosomes: D. X 62,000

are orally infected with suspension of K87 grown in the mouse kidney culture cells, viral antigens start to increase at 3 days and reach maximum between 7 and 14 days after infection (Hashimoto, 1970). At the height of the viral replication in the gut, ample numbers of nuclear inclusions emerge in the epithelial lining of the distal small intestine; this made an electron microscope observation on the adenovirus host cell interaction in vivo feasible.

At the peak of infection, fluorescent antibody technique shows that viral antigens are found in the epithelium of the crypt and villus of the small intestine (Fig. 27). Light microscopy on conventional paraffin sections stained by hematoxylin-eosin exhibits nuclear inclusions are a large bizarre shape which is stained uniformly basophilic (Fig. 28). In thick sections from Epon embedded tissue the nuclei of infected cells are large and globular, and have several dense masses (Fig. 29). Under EM, infected cells bear the characteristics of the epithelial cell. Their nuclei exhibit crystalline arrays of virus particles (Fig. 30). The virus averages 80 mμ in diameter and shares ultrastructural characteristics of other adenoviruses. In the cytoplasm the virus does not form crystalline arrays but lies in aggregates. Otherwise, infected cells show no cytopathic effect. The virus infection elicits neither inflammatory response nor architectural alteration in the gut mucosa.

Further studies are needed to characterize the cell site of the viral replication and clarify the nature of the cytopathic changes in host cells.

B. Studies of Enteric Infection of Neonatal Dogs with Canine Coronavirus 1-71

Virological, histological, histochemical and immunofluorescent aspects of this viral infection in vivo has been studied in detail. (See 73-74 Annual Report, Dept. of Vet. Microbiology, WRAIR.) Our preliminary findings indicate that viral particles are identified at ultrastructural level mostly in the cytoplasm of intestinal absorptive cells at the villus portion of the ileal epithelium (Fig. 31) and are totally absent in crypt epithelial cells. This localization is corresponded to those of immunofluorescent microscopy. In the cytoplasm, many mature virions are included in membrane-bound vesicles, while some are in the process of budding from smooth ER (Fig. 32).

Further studies are required to clarify the exact nature of epithelial cytoplasmic changes associated with coronavirus replication.

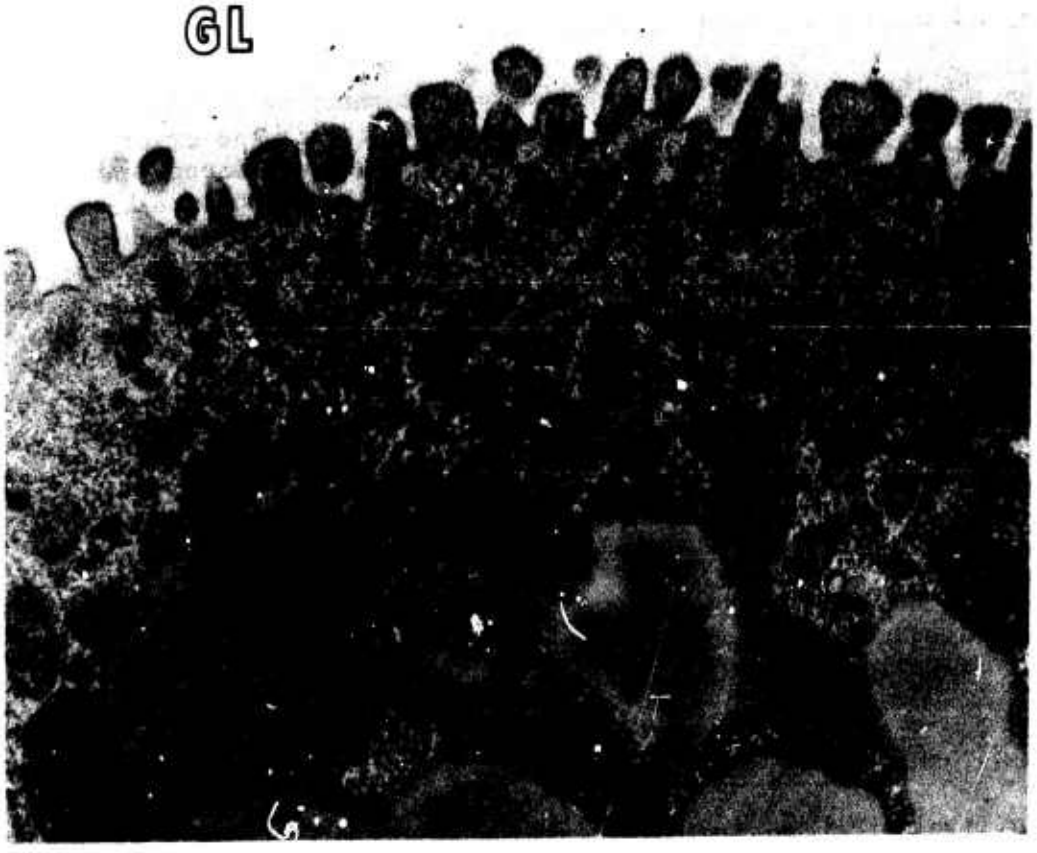

Fig. 31 - Intestinal columnar cell at apical epithelium of villus, ileum, neonatal dog orally infected with coronavirus. Viral particles are enclosed in membrane-bound vesicles (arrows). Microvilli are short, blunt and irregular. Note numerous lipid droplets (LD). M: mitochondria. GL: gut lumen.
X 31,000

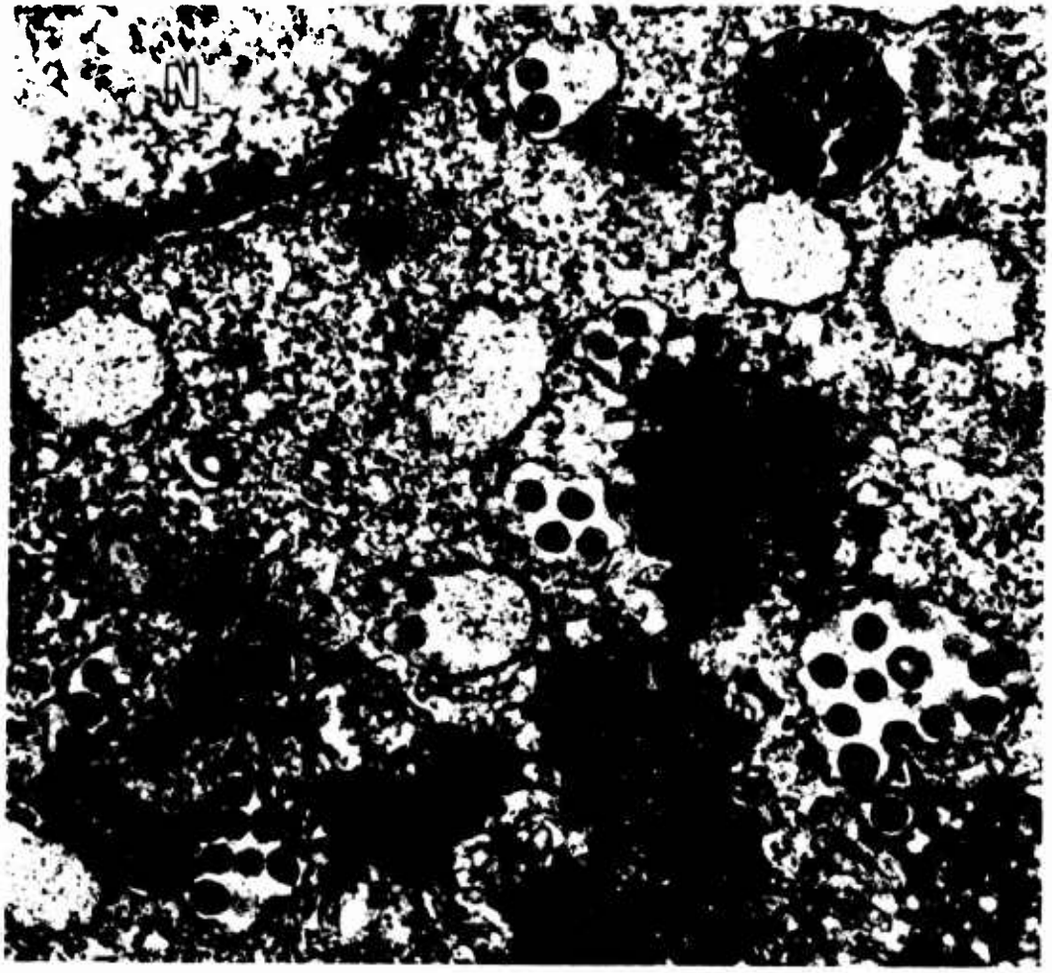

Fig. 32 - Perinuclear region, columnar cell, apex of villus, ileum, neonatal dog infected with coronavirus. Viral particles are present within membrane enclosed vesicles. Some particles are in process of budding from smooth endoplasmic reticulum (arrows). N: nucleus.
X 68,000

IV. STUDIES OF CELL-MEDIATED HYPERSENSITIVITY

A. Study of Kinetics of Fibrinogen/Fibrin Accumulation and Vascular Permeability Changes in Cell-Mediated Hypersensitivity

Background

Recent studies have demonstrated that fibrin accumulation is a prominent and consistent feature of delayed hypersensitivity (DH) skin reactions and allergic contact dermatitis in man.

To gain a clearer understanding of the relationship between the clotting system and DH we have performed further quantitative studies in the guinea pig. In this species two distinct forms of cell-mediated hypersensitivity have been recognized: 1) classic DH, a long-lasting state of hypersensitivity mediated by lymphocytes with relatively high avidity for antigen and characterized by extensive mononuclear cell infiltrates, and 2) cutaneous basophil hypersensitivity (CBH), a sometimes transient state of hypersensitivity mediated by lymphocytes of lower average avidity and characterized by extensive infiltrates of basophilic leukocytes. In neither reaction had significant fibrin been observed previously.

In the present investigation, radioactively labeled fibrinogen and albumin were employed as tracers to measure changes in vascular permeability, fibrinogen accumulation, and fibrin formation in CBH and DH skin test reactions.

Results

Classic delayed hypersensitivity (DH) reactions to Old Tuberculin and to the azobenzenearsonate hapten were characterized by a progressive increase in the fibrinogen (^{125}I-HF) content which exceeded that of the albumin tracer (^{131}I-HSA) and paralleled the development of induration and erythema. Accumulation of ^{125}I-HF could be related both to increased vascular permeability to ^{125}I-HF and, more specifically, to retarded efflux of extravascular ^{125}I-HF from tuberculin reaction sites. Warfarin inhibited ^{125}I-HF accumulation and the formation of urea-insoluble ^{125}I-HF (cross-linked fibrin) as well as induration in tuberculin reactions. Immunofluorescence studies revealed the site of Fib deposition to be extravascular, among the connective tissue fibers of the dermis, similar to that in DH reactions in man.

In contrast, little ^{125}I-HF accumulated in cell-mediated reactions rich in basophils - cutaneous basophil hypersensitivity (CBH) reactions to keyhole limpet hemocyanin, ovalbumin, and dinitrochlorobenzene - due in part to less vascular leakage of macromolecules and to decreased formation of urea-insoluble fibrin. By immunofluorescence Fib deposits were found in CBH reactions in a pattern similar to that in DH reactions, but the intensity of staining was appreciably less.

Conclusion

Based on this study, fibrin accumulation can distinguish DH from CBH reactions and is very likely responsible for the induration characteristic of DH reactions.

B. Studies of Macrophage Cell Surface Fibrinogen/Fibrin

Background

Peritoneal macrophages adhere to each other and to the peritoneal serosal cells following i.p. antigen challenge in guinea pigs primed for delayed hypersensitivity. This phenomenon, termed the macrophage disappearance reaction (MDR) by Nelson (1965), can be inhibited by warfarin or heparin and can be mimicked by i.p. administration of thrombin. Although such data suggest that macrophages might interact via fibrin formation, attempts to identify fibrin on macrophages by electron microscopy were unsuccessful. Because fibrin deposition has recently been shown to be a characteristic feature of classic cell-mediated hypersensitivity reactions in the guinea pig and in man, we have reexamined the possibility that mononuclear phagocytic cells might directly interact with Fib.

Results

The peritoneal cavity of guinea pigs proved to be a rich source of mononuclear cells (34-52%) with fibrinogen and/or fibrin (Fib) on their surface. Such Fib was readily detected on living cell suspensions by the binding of fluoresceinated anti-guinea pig fibrinogen and occurred either in a speckled distribution, similar to cytophilic IgG, or in a distinctive net-like pattern, the latter believed to represent fibrin formation on the cell surface. Fib binding required calcium, but not magnesium, and could occur in vitro during incubation in heparinized plasma containing fibrinogen concentrations comparable

to that in normal peritoneal fluid (0.58 mg/ml). Cell bound Fib was more susceptible to plasmin and trypsin digestion than cytophilic IgG. By morphologic and physiologic criteria, cells exhibiting surface Fib were chiefly, if not exclusively, macrophages. Granulocytes, erythrocytes and lymph node and thymus lymphocytes had no appreciable Fib. Cells with surface Fib were observed rarely among mononuclear cells prepared by Ficoll-Hypaque sedimentation of guinea pig and human blood (1.4 and 4.6% respectively). Alveolar macrophages, known to be distinctive functionally from peritoneal macrophages, also differed in their lack of surface (Fib 0.8%).

Polymerization of Fib on the surface of macrophages might participate in certain cell adherence phenomena, such as the adherence of free peritoneal macrophages during the antigen induced macrophage disappearance reaction (MDR). The MDR elicited in the presence of ^{125}I-fibrinogen was accompanied by a 2.4-fold increase in cell associated ^{125}I-Fib.

Conclusion

The unexpected and intimate relationship between macrophages and a major constituent of the clotting system provides a potential means for significant interactions between these important components of the biological defense system.

V. STUDIES OF PATHOLOGY OF RENAL DISEASES

A. Study of Renal Interstitial Fibrosis

A variety of chronic renal diseases reveal variable degrees of interstitial fibrosis. A statistical correlation has been shown between the degree of interstitial fibrosis and decreased renal function in human biopsies, yet the pathogenesis of the renal interstitial fibrosis remains unclarified. The present study, using an animal model which leads to diffuse interstitial fibrosis, was designed to study the kinetics of the initial cell proliferative response.

Autoradiographic determinations of the number and distribution of replicating cells within the renal interstitium were made at 24, 48, and 72 hours in rabbits following unilateral complete ureteral obstruction. Animals were given a series of five intravenous injections of ^{3}H-thymidine (0.5 μCi. per gm.) at 30-minute intervals. Kidneys obstructed at 24, 48, and 72 hours revealed a mean nuclear labeling count of 6.1, 7.8, and 21.1 labeled cells per high power field (HPF) within the cortical interstitium as compared with mean counts of 0.6, 1.4, and 0.7 in sham-operated controls. The interstitium of the outer medulla revealed mean counts of 10.1, 14.4, and 27.0 labeled cells per HPF during these same time intervals. The inner medulla revealed a feeble proliferative response, with mean counts of 2.1, 1.5, and 5.0 labeled cells per HPF. The contralateral kidney revealed a variable degree of low grade proliferative response.

These results indicate a continuous stimulation of cell replication in this injury extending over the first 72 hours. In addition, it appears that there are local differences in the response, with the cells within the interstitium of the outer medulla being most responsive.

VI. STUDIES OF DISEASES OF DIGESTIVE TRACT OF NON-INFECTIOUS ORIGIN

A. Gastric Secretory Changes of Stress Ulcers in Rats

Analyses of gastric juice withdrawn 3 hours after the pylorus was ligated and of plasma corticosterone and blood glucose after animals were exposed to rotational stress revealed that gastric secretion was highest in controls, intermediate in stressed rats that developed ulcers, and lowest in stressed rats that did not develop ulcers. Neither high nor low gastric secretion correlated with stress-ulcer formation. When initial mucosal ischemia and secretory inhibition, which occurred in all stressed rats, were considered, those that developed ulcers manifested gastric hypersecretion when compared with those that did not develop ulcers. Mean plasma corticosterone was highest in stressed rats that developed ulcers, next highest in stressed rats that did not develop ulcers, and lowest in control rats. Compared with normal rats, mean blood glucose was lowest in stressed rats and highest in controls. Hypoglycemic changes were more marked in stressed rats that developed ulcers than in stressed rats that did not develop ulcers.

B. Study on Comparative Histochemistry of Gastrointestinal Mucosubstances

In taking advantage of two relatively simple and reproducible staining techniques, this study was undertaken to describe and compare the distributions of epithelial mucosubstances in the gastrointestinal mucosa of the human and of a variety of mammalian species commonly used as laboratory subjects for the study of gastrointestinal disorders. The two staining sequences used are the alcian blue periodic acid-Schiff technique (Mowry 1963) and the high iron diamine alcian blue technique (Spicer, 1965, as modified by Sheahan et al., 1970). These techniques which differentiate neutral from sulfated as well as non-sulfated acid mucosubstances, underscored gross anatomical differences in the various parts of the gastrointestinal tract of different species (Fig. 33) and disclosed an unexpectedly wide variation in the distribution of such mucosubstances in different areas of the gastrointestinal tract of the same species and in the same anatomic areas of different species (Fig. 34). The significance of such variations is, as yet, unknown. Knowledge of the normal variation in the

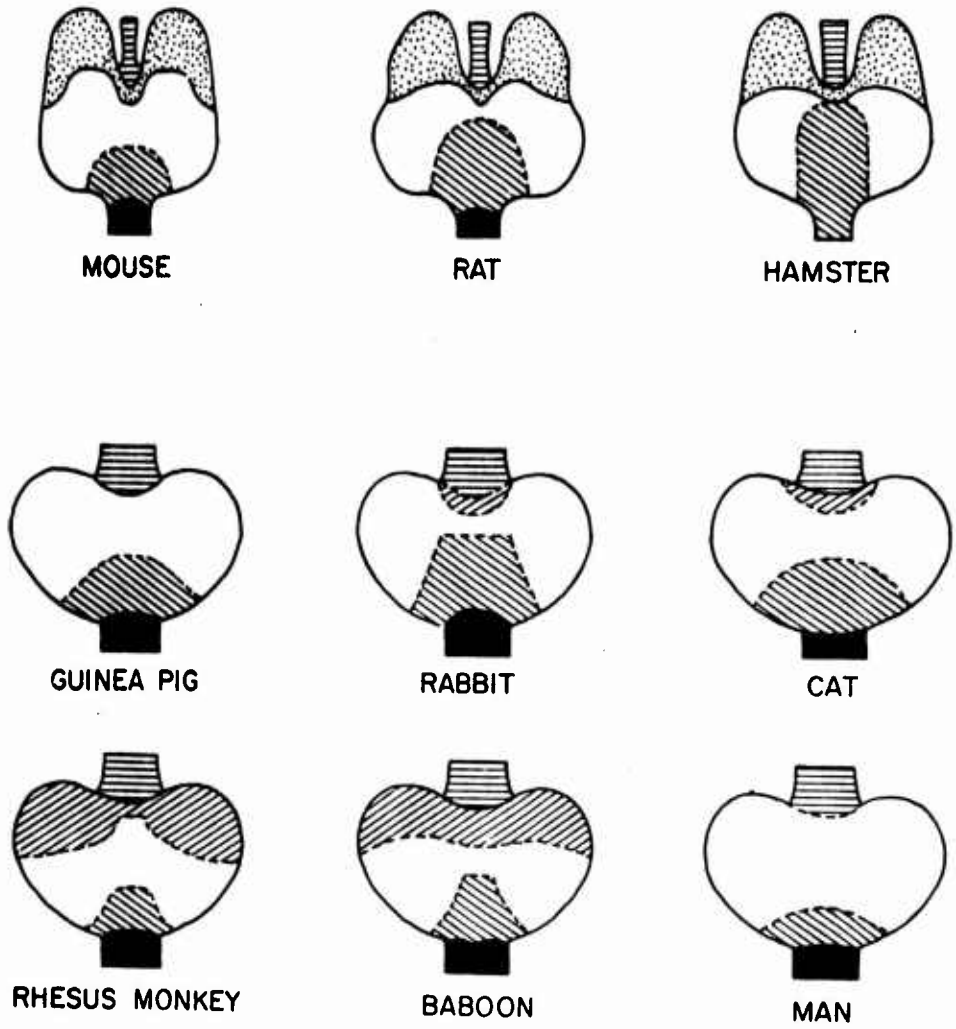

Fig. 33 - Schematic representation of the gross anatomy of gastric mucosa indicating species variations in the gross topography of cardia, corpus and antrum.

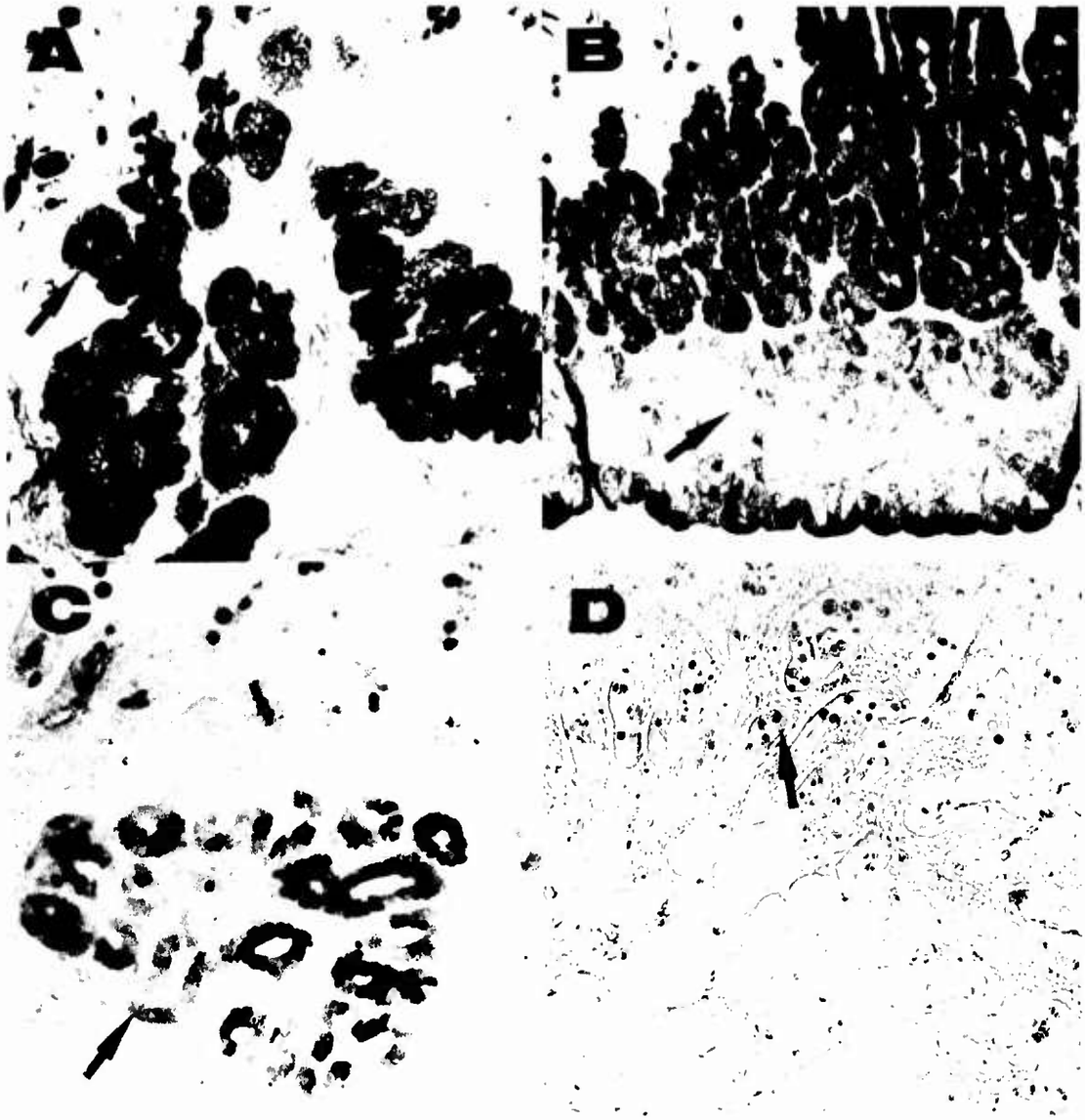

Fig. 34 - Brunner's glands stained with the high iron diamine alcian blue sequence. Neutral mucins are unstained, as in D, while the sialomucins, stained blue, appear grey in the photomicrographs (arrows), and the sulfomucins stain black. A: Rabbit; B: Guinea pig; C: Baboon; D: Man. A, C, D X 150; B X 400.

anatomic distribution of mucosubstances in the gastrointestinal tract is, however, an essential prerequisite to accurate interpretation of observed alterations which occur in the distribution of such moieties as a result of naturally occurring or experimentally induced diseased states.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Disease and Injuries

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6403	75 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	01	126
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Infectious Hepatitis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	1	50
FISCAL YEAR CURRENT 76	1	12

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Conrad, Dr. M. E.

NAME: Flannery, LTC E. P. DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Liver Disease; (U) Hepatitis; (U) Australia antigen

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Viral hepatitis has been a major cause of morbidity in military populations and a significant hazard in the military blood program. Investigations have been undertaken related to the etiology, epidemiology, and prevention of this group of disorders.

24. (U) A randomized double-blind study is in progress to ascertain if HBAb hyper-immune gamma globulin prevents transfusional hepatitis, and if transfused blood positive for hepatitis B surface antigen only by radioimmune assay (but not by other methods of testing) causes hepatitis.

25. (U) 74 07 - 75 06. A study is in progress among patients undergoing cardiac bypass surgery who receive multiple blood transfusions to determine if gamma globulin containing HBAb in high titer is useful in the prevention of hepatitis B. To date, 354 patients have volunteered for this study, which compares two types of gamma globulin and a placebo solution. Studies have been completed to characterize the significance of some of the other agents often mentioned as possible etiological causes of posttransfusion hepatitis. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon addressee's approval.

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Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 126 Infectious hepatitis

Investigators.
Principal: MAJ Robert G. Knodell, M.D., MC
Associate: Marcel E. Conrad, M.D., University of Alabama
LTC E. Patrick Flannery, M.D., MC, Letterman General Hospital
Allen L. Ginsberg, M.D., George Washington Medical School

Objectives: To determine if gamma globulin with high anti-HB_S titer provides protection against transfusion hepatitis and whether transfused blood positive for HB_SAg only by radioimmune assay (but not by other methods of testing) causes hepatitis.

Technical Approach: During past years there has been a considerable incidence of hepatitis among patients undergoing cardiac bypass surgery at WRAMC. Estimates of the incidence of hepatitis in this group based upon the detection of elevated transaminase determinations 3 months after surgery are 10-20% of patients. It is believed that this is caused by the requirement of using many pints of blood and blood products from multiple donors in these patients.

Since August 1972, all volunteers who are undergoing cardiac bypass surgery receive a 10 milliliter injection of either high titer anti-HB_S gamma globulin, conventional gamma globulin, or an albumin placebo solution. These injections are administered double blind under code. Blood is drawn from the volunteers before gamma globulin injection, weekly after surgery while the patient is hospitalized, and 3, 6, and 9 months after surgery. Determinations for HB_SAg, anti-HB_S, SGPT, and serum bilirubin are done on each blood specimen. In addition, a history is obtained from each patient at intervals after surgery. All blood used for transfusion is tested by radioimmune assay for HB_SAg and anti-HB_S.

It is estimated that a minimum of 300 patients will be required for completion of the above study. The biologic materials used in this study include a high titer anti-HB_S lot of gamma globulin prepared by the Massachusetts State Laboratories and currently used under NHLI contract in several national studies, a lot of gamma globulin used in 60,000 soliders in Korea, and a placebo solution used in 40,000 U.S. soldiers in Korea without known complications. All solutions are tested in accordance with U.S.P. regulations.

Progress and Results: This is a collaborative double blind study being conducted at Walter Reed Army Medical Center and at Letterman Army Medical Center. Under the direction of Dr. Allen Ginsberg, George Washington University of Medicine was scheduled to become a study center and begin assessing patients during FY 75. However, it was determined that the study would likely be terminated before GWUM could assess and follow a significant number of patients, and the subcontract with GWUM was terminated 31 December 1974.

Since the inception of the study, 354 patients undergoing cardiac bypass surgery at either WRAMC or LAMC have volunteered as participants in the study. Each patient has received under code either 10 ml of high titer anti-HB_S gamma globulin, conventional gamma globulin, or an albumin placebo solution. There have been no known adverse reactions to the administration of either the gamma globulin or placebo solutions. Transaminase elevations have been observed in approximately 20% of patients 3-6 months after surgery. Only 10 patients have had clinical icteric hepatitis. Only 12% of patients with either icteric hepatitis or elevated transaminase determinations have had HB_SAg-related hepatitis as measured by radioimmunoassay. Approximately 7% of the patients have been transfused with blood that was HB_SAg positive when tested by radioimmunoassay; all blood was HB_SAg negative when tested by counterimmunoelectrophoresis.

Surprisingly, in the majority of hepatitis cases, hepatitis B virus cannot be implicated, and different viral agents must be hypothesized. A 90% followup of 9 months is being achieved largely by mail and telephone. Surveillance of the study is being maintained by the National Heart and Lung Institute, NIH, through the Hepatitis Data and Safety Monitoring Committee. This committee has been reviewing uncoded data three times yearly to insure safety and that significant differences have not already been achieved between the three coded groups. Accessions to the study were halted 31 December 1974 when supplies of the high titer anti-HB_S were exhausted. Currently followup is scheduled to be completed by 30 September 1975, and final data analysis can begin. Projected completion date for this project is approximately 31 December 1975.

Conclusions: This collaborative study has been in progress since August 1972. Data collection should be complete by 30 September 1975 when final data analysis can begin. It is presently funded by a contract with NHLI. It will provide worthwhile information on transfusional hepatitis and the usefulness of gamma globulin in its prevention.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 126 Infectious hepatitis

Literature Cited.

Publications:

1. Knodell, R. G., Conrad, M. E., Dienstag, J. L., and Bell, C. J.: Etiological spectrum of posttransfusion hepatitis. Gastroenterology 68: 927a, 1975.

2. Knodell, R. G., Conrad, M. E., and Bell, C. J.: Posttransfusion hepatitis: A changing spectrum. Gastroenterology 67: 804, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 05 67	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GIDEP INVEST'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760AB22	01	128
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Include with Security Classification Code)

(U) Biochemical Methodology and Laboratory Automation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	PRECEDING/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	75	6	90
	76	6	189

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME: Bass, B. G. MS
NAME: Neeley, MAJ W. E. DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide KWOC and security classification Code) (U) Gas Chromatography; (U) Automation; (U) Clinical Chemistry; (U) Toxicology; (U) Drugs of Abuse

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Provide text of each with security classification Code.)

23. (U) The technical objective of this work unit is to develop and establish modern automated methods for qualitative and quantitative chemical analyses of military importance in medical laboratories and to adapt these systems for use in support of combat medical operations.

24. (U) Automated analytical instruments, on-line digital computers, electronic digital or analog processors and semi-automated manual techniques will be developed and adapted to identify and quantitate various compounds for clinical and research use. Emphasis will be placed on precise, accurate, and fast analytical systems, on simultaneous identification of a variety of compounds, on analytical standardization and on practical systems for laboratory and intensive care facilities use. Efforts will be focused on fast identification and quantitation of drugs of abuse and their principal metabolites, the utilization of simplified automated analytical systems for data collection and on standardization of techniques and equipment for automated laboratory systems. Operating systems will be miniaturized and ruggedized for field use.

25. (U) 74 07 - 75 06 Analytical chemistry methodology development and application was continued in this work unit. Five automated 4-channel, high speed, miniaturized, continuous flow analyzers have been constructed and installed in the WRAMC clinical laboratory for operational evaluation. Interface completed of electronic programmable desk top calculators to automated instruments. An ultra-micro high speed analyzer has been developed. Unit requires only 40microliters of sample. A fluorometric method for analysis of N-acetyl procainamide has been developed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

* Applies to continuation pages as appropriate.

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Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 128 Biochemical methodology and laboratory automation

Investigators.

Principal: LTC (P) Douglas J. Beach, MSC

Associate: MSG Vaughn G. Ayer; Billy G. Bass. M.S.; Betty J. Boone, Ph.D.; Nesbitt D. Brown, B.S.; John I. Davis, B.S.; LTC Gale E. Demaree, MSC; James E. Doolittle, A.A.; MAJ Thomas P. Gibson, MC; Leo Kazyak, B.S.; Robert T. Lofberg, Ph.D.; PFC Leighton C. Makanui; Edward J. Matusik, B.S.; Jean E. (Lewis) Matusik, B.S.; SP5 Michael E. McKay; MAJ William E. Neeley, MC; Dominic F. O'Donnell; Helen C. Sing, M.S.

The objectives of this work unit are to develop and exploit new laboratory methods for qualitative and quantitative analyses of biochemical substances in support of military medicine and to apply automation to analytical procedures to provide rapid, precise and accurate results. During the reporting period, efforts have been focussed on the following areas:

1. Development of a four-channel, high speed, miniaturized continuous flow analyzer.

2. Application of electronic programmable desk top calculators to on-line clinical laboratory analyses.

3. Design and construction of a new ultra-micro high speed analyzer for hospital chemistry laboratories.

4. Investigation of procainamide in man.

5. Analysis of N-acetylprocainamide by spectrophotofluorometry.

6. Automation of techniques in drug analyses.

7. Amino acid analysis.

1. Development of a four-channel, high-speed, miniaturized continuous flow analyzer.

A prototype four-channel microanalyzer has been constructed, tested and placed in routine daily operation in the clinical laboratory, Walter Reed Army Medical Center. This system is set up to analyze the electrolytes Na, K, Cl, CO_2 in patient specimens. The analyzer

has been operating reliably for three months at the rate of 120 samples/hour. The instrument it replaced operated at a rate of 60 samples/hour. The test system's reagent consumption is one tenth that of the older instrument.

The prototype analyzer is one of four such instruments planned for the clinical laboratory at WRAMC as an operational performance evaluation in collaboration with the Department of Pathology, WRAMC. The use of the microanalyzer has demonstrated a substantial reduction in reagent consumption and the potential savings of substantial equipment lease costs (e.g. SMA 6/60 at $35,000.00 p.a.). Laboratory personnel acceptance has been enthusiastic. A high-speed dual channel analyzer has placed in the clinical laboratory for testing and for developing new analytical procedures using enzymatic methods. The test model is glucose determination. The more specific enzyme methods are expected to provide more accurate results with less interference from other drugs. The higher costs for enzymes will be offset by the reduced reagent requirements for the miniaturized analyzer.

Arrangements have been completed for the construction of ten miniaturized analyzers by the U.S. Army Biomedical Research Unit, Fort Detrick, MD. These analyzers will be evaluated in various clinical laboratories to fully evaluate their potential savings in manpower and time and to assess their usefulness to the U.S. Army Medical Department.

2. Application of electronic programmable desk top calculators to on-line clinical laboratory analyses.

The feasibility of interfacing existing continuous flow systems with programmable calculators has been demonstrated. A multiplexor was designed and constructed to interface a Hewlett-Packard 9810A calculator with Technicon AA II multichannel analyzers. Since April 1974, the clinical chemistry laboratory, WRAMC has had in daily operation a dual channel AA II creatinine-BUN analyzer linked to the programmable calculator. The demonstrated advantages of this system are:

a. Printed display of instrument calibration results as soon as the standards are analyzed, thereby making available immediately the status of the system prior to specimen analysis.

b. Improved accuracy of results due to use of multiple point rather than single point calibration.

c. Technician time saved by eliminating tedious hand calculations of analog signal output.

d. Elimination of errors in manual calculations.

e. Test results available immediately upon analyzing specimens - no waiting for manual calculations.

The four channel electrolyte analyzer was interfaced with a programmable calculator through a four-port multiplexor. The more complex program required for this system demonstrated the effectiveness of the tape cassett memory extension available with the calculator. This cassette feature has made possible the implementation of a new result reporting system to be explained later. The high speed of the calculator and the analyzer has made possible the processing of STAT specimens along with routine work since the time involved from sampling to printout is only 2 3/4 minutes. The programmable calculator has been successfully interfaced with an Abbott Bichromatic Analyzer. A three-analyzer hookup through a multiplexor to a 9810A calculator with cassette memory is planned.

A data reporting system for a clinical laboratory has been designed and tested using the 9810A calculator tape cassette system. The data from the analytical instruments (electrolyte analyzer, Abbott Analyzer, etc.) are stored on the tape cassettes. These cassettes are manually transferred to a Model 9830A calculator (a much higher level calculator) where the data are collated with other patient information and a complete printout of specimen results provided. A method whereby interim results are printed on gummed labels for attaching to work sheets and reports is being tested. A high speed card reader for inserting manually generated data into the Hewlett-Packard 9830 has been tested with encouraging results.

3. Design and construction of a new ultra-micro high speed analyzer for hospital chemistry laboratories.

A unique continuous flow analyzer system consisting of very small scale parts and totally solid state electronic components has been designed and constructed. The analyzer consists of a digital electronics controlled sampling device requiring only 40 microliters of specimen, a variable speed micro-peristaltic pump (5 X 8 X 9 cm) with a roller head three centimeters in diameter, a miniaturized manifold, totally solid state colorimeter, high efficiency 1.8 microliter volume flow cell and an electronic bubble gate that allows the bubble stream to pass directly through the flow cell by electronically eliminating the bubble artifact.

NOTE: Considerable time and effort was spent by personnel involved in the work outlined above in exhibiting the results of their work at various locations throughout the country. The more prominent demonstrations are listed below.

a. Symposium on Current Concepts in Pathology, El Paso, Texas.

b. Johns Hopkins Medical School, Baltimore, MD.

c. University of Maryland Medical School, Baltimore, MD.

d. Association of Clinical Chemists, Las Vegas, NEV.

e. U.S. Air Force Laboratory Symposium, New Orleans, LA.

f. Association of Military Surgeons, San Diego, CA.

g. American Society of Clinical Pathologists, Washington, DC.

h. AACC - Neonatal Conference, Washington, DC.

4. Investigation of the acetylation of procainamide in man.

Fourteen subjects were given 500 mg of procainamide (PA) orally as the hydrochloride salt. For each patient, acetylation phenotype was determined by the method of Scott and Wright (1). Each urine voided for 96 hours after PA ingestion was analyzed for PA and N-acetylprocainamide (NAPA) using a gas chromatographic method.

The fourteen subjects eliminated 52 $\pm$ 4% of the dose as PA and 16 $\pm$ 2% as NAPA. Four fast acetylators eliminated 23 $\pm$ 3% of the dose as NAPA compared to 12 $\pm$ 1% by the slow acetylators ($p<0.05$). The amount of PA excreted by fast and slow acetylators was not significantly different, 50 $\pm$ 4% and 53 $\pm$ 4% respectively. There appears to be a positive correlation between the ability to acetylate isonicotinic acid hydrazide and the ability to acetylate PA.

5. Analysis of N-acetylprocainamide by spectrophotofluorometry.

A spectrophotofluorometric method for accurately estimating the concentration of N-acetylprocainamide in serum in the presence of varying concentrations of procainamide has been developed. This method was used in the procainamide acetylation investigation outlined in paragraph 4. The method is based on the measurement of quantum yields of PA and NAPA as a function of pH. There is a ten-fold increase in the quantum yield of NAPA at pH 1.0 compared to pH 11.0. Conversely, the quantum yield of PA is maximum at pH 11.0 and is significantly diminished at pH 1.0.

6. Automation of techniques in drug analyses.

A Real Time Executive Multiprogramming System is fully operational on the 2116C minicomputer. With this programming of gas chromatograph equipment interface drivers complete, up to four automated gas chromatographs have operated continuously for periods up to 32 hours

without operator intervention, and program development and data searches have been performed by the computer at the same time. Extensive use has been made of this capability in drug analysis and drug metabolism research.

7. Amino acid analysis.

The 6AH JEOLCO Analyzer has been adapted to use the Durrum Amino Acid Analytical System. This adaption will allow a total amino acid analysis to be completed in 6 hours, a four-fold improvement in analysis rate.

Project 3A762760A872 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 128 Biochemical methodology and laboratory automation

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6503	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. SMSPN INSTR'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	01	129
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Epidemiology of Hepatitis in the Military

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	3	65
FISCAL YEAR CURRENT 76	3	60

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Epidemiology; (U) Hepatitis; (U) Drug Abuse

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To define and study the prevalence, incidence, and variables of hepatitis transmission in medical care provider and line military populations. To apply this information to the design of hepatitis prevention and control programs.

24.(U) Contemporary epidemiologic methods are employed. Multidisciplinary collaborative approaches are utilized and new methods developed as required.

25.(U) 74 07-75 06 Analysis of demographic and occupational data from the study of the prevalence of Hepatitis B antigen and antibody in Army health care personnel and association of those data with serologic results were completed. Two-year questionnaire and serologic followup of the 1972 portion of this cohort was completed. This study is complementary to work described under DA OB 6513, Work Unit 176, entitled "Mechanisms of Transmission of Hepatitis Viruses." Analysis of data from investigations of hepatitis outbreaks at Fort Riley, Kansas and Camp Zama, Japan, was completed. A one-year prospective study of Hepatitis B antigen and antibody acquisition by personnel newly assigned to Fort Hood was completed. Analysis of data from a one-year followup of Hepatitis B antibody positive personnel remaining at Fort Hood was completed. (This study is complementary to work described under DA OB 6513, Work Unit 176, entitled "Mechanisms of Transmission of Hepatitis Viruses." A protocol for the study of chronic hepatitis following Hepatitis B infection was developed. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 74-30 Jun 75.

*Applicable to contractor use at discretion of agency.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 129 Epidemiology of hepatitis in the military

Investigators:

Principal: LTC Herbert E. Segal, MC
Associate: COL Taras Nowosiwsky, MC; LTC James A. Ferguson, VC; MAJ Gilbert R. Irwin, MC; SSG Michael C. Callahan; L. Charlene Evans

1. Hepatitis B Antigen and Antibody in Army Health Care Personnel

A. Prevalence Study at the Academy of Health Sciences, Fort Sam Houston, Texas

Army Medical Department Officer personnel enrolled at the Academy of Health Sciences, Fort Sam Houston, Texas, between July 1972 and June 1974 participated in the study as did enlisted personnel enrolled from July 1973 to June 1974. Officers, including physicians (MC), veterinarians (VC), dentists (DC), nurses (ANC), medical administrators and allied health scientists (MSC), and dietitians and physical therapists (AMSC), were attending the Basic Officer Course at the Academy of Health Sciences upon their entrance onto active duty. More senior personnel representing those with career commitments were taking the Officer Advanced Course. Enlisted men and women, representing a wide range of medical occupational specialties (e.g. laboratory, dental, pharmacy, social work), were attending the Basic or Advanced Courses in their specialties. Students enrolled in the Physician's Assistant Course also participated in the study. These latter are picked from among senior enlisted personnel with substantial experience in health care related fields.

During each of the course orientations, personnel completed a questionnaire, giving name, social security number, course and class numbers, military occupational specialty, grade, sex, year of birth, place of birth, home of record, histories of hepatitis, transfusion, or positive HB_sAg tests, and previous assignment(s) overseas. The officer questionnaire additionally requested the year and type of professional degree and number of subsequent months in medical or surgical specialties. During the orientation, one of the investigators was present to clarify the questionnaire requirements and the

voluntary nature of participation. Academy technicians drew a venous blood specimen from each subject completing the questionnaire and used a portion of the specimen for blood typing. The remaining serum was frozen and transported to the Walter Reed Army Institute of Research for serologic studies.

Sera were tested for Hepatitis B surface antigen (HB_SAg) using the Ausria I kit (Abbott Laboratories), with specificity for HB_SAg confirmed as previously described. In addition, sera HB_SAg positive by counter-electrophoresis (CEP) were subtyped. Antibody to the surface antigen (anti-HB_S) was determined by the passive hemagglutination (PHA) test described by Vyas. Serologic evidence of recent or past hepatitis B virus infection (HB_SAg or anti-HB_S) was then correlated with the demographic and occupational variables listed in the questionnaire. Data were adjusted for age only when noted.

Completed questionnaires together with usable serum specimens were obtained from 5,537 officer and 1,966 enlisted personnel. Ninety-five percent of these officer personnel, including all the physicians (MC) attended the Basic Officer Course. Only veterinarians (VC) and medical administrators and allied health scientists (MSC) were well represented in the Officer Advanced Course. Eighty-six percent of the enlisted personnel attended the Basic Course in their occupational specialty. Incomplete questionnaires, which were encountered occasionally, were utilized as fully as possible. Two hundred forty-eight otherwise usable questionnaires (3.2%) were not included in the analysis because the vials containing the corresponding serum specimens were broken in shipment. Refusal to participate was rare.

Serologic evidence of Hepatitis B infection (HB_SAg or anti-HB_S) was found in 384 (5.1%) of personnel studied (Tables 1a and 1b). Thirteen of the 19 sera HB_SAg positive by CEP were subtypable: ten were Adw and Ayw. Officers in Corps engaged in routine direct patient care (MC, DC, and ANC) had a higher seropositivity rate than officers in predominantly non-patient care oriented Corps, 5.7% versus 2.9% ($X^2=19.9$, p 0.0001). HB_SAg or anti-HB_S positive enlisted personnel were more commonly found among those in clinical or laboratory specialties than among those in support specialties, but this difference was not statistically significant ($X^2=1.48$, p=0.22). Many of the newly enlisted personnel (31.8%) apparently did not have or did not know their specialty designations and are listed as "unknown."

Table 1a Serologic Results for Officers

Corps	No. Tested	No.(%)HB_SAg+	No.(%)Anti-HB_S+
MC	1534	12(0.8)	130(8.5)
VC	82	0	1(1.2)
DC	849	7(0.8)	24(2.8)
ANC	1470	5(0.3)	44(3.0)
MSC	1503	6(0.4)	37(2.5)
AMSC	105	0	4(3.8)
Total	5543	30(0.5)	240(4.3)

Table 1b Serologic Results for Enlisted Personnel

Specialty	No. Tested	No.(%)HB_SAg+	No.(%)anti-HB_S+
Clinical	852	6(0.7)	59(6.9)
Laboratory	60	1(1.7)	4(6.7)
Support	428	0	25(5.8)
Unknown	626	2(0.3)	17(2.7)
Total	1966	9(0.5)	105(5.3)

The prevalence of both HB_SAg and anti-HB_S generally rose with advancing age (Table 2) in both the officer Corps and the enlisted specialty groups. Controlling for age did not eliminate the higher anti-HB_S prevalence of physicians (MC).

Table 2 Age-Specific Rates of Seropositivity

Age Group	No. Tested	No.(%)HB_SAg+	No.(%)anti-HB_S+
19	652	2(0.3)	16(2.5)
20-24	2512	9(0.4)	59(2.3)

Continued

Table 2 Age-Specific Rates of Seropositivity

Age Group	No. Tested	No.(%) HB_SAg+	No.(%) anti-HB_S+
25-29	2376	14(0.6)	93(3.9)
30-34	1495	10(0.7)	131(8.8)
35-39	192	1(0.5)	34(17.7)
40	49	1(2.0)	7(14.3)

A history of either hepatitis or blood transfusion generally increased with advancing age. Males more likely were HB_SAg or anti-HB_S positive than were females (0.6% and 5.6% versus 0.3% and 2.0%); the lower prevalence of anti-HB_S in females did not disappear when age was controlled. More females, by history, received transfusions (4.6% compared with 4.0%); males reported more hepatitis (4.3% versus 2.8%). Personnel born in one of the eight most populous states (used here as an indicator of "urban" residence) were more often HB_SAg or anti-HB_S positive than those born in the remaining states ("rural") (Table 3), but this difference is not statistically significant (X^2=1.29, p=0.26). A significantly higher infection rate was obtained for personnel born outside the United States compared with that for all native-born personnel (X^2=4.22, p=0.04).

Table 3 Seropositivity by Place of Birth

Place of Birth	No. Tested*	No.(%) HB_SAg+	No.(%) Anti-HB_S+
Urban	1874	12(0.6)	92(4.9)
Rural	2609	11(0.4)	114(4.4)
Outside U.S.	217	4(1.8)	14(6.5)

*1973-4 Data only

Two hundred and eighty-six personnel (3.9%) reported a history of hepatitis; these included 4.3 percent of officers in Corps engaged in direct patient care, 2.8 percent of officers not so engaged, 4.9 percent of enlisted personnel in clinical and support specialties, 3.3 percent of those in laboratory specialties, and 2.9 percent of those without a listed specialty designation ("unknown"). Two hundred eighty-nine personnel (4.0%) reported receiving a blood transfusion. This procedure was more common among officer personnel in predominantly non-patient care oriented Corps (5.0% versus 3.1%) and more common in enlisted personnel with clinical or support specialties (6.3% and 6.6% respectively) than in those with laboratory or unknown specialties (1.7% and 3.5% respectively).

Both a prior history of hepatitis and previous blood transfusion were associated with seropositivity (Table 4). Rearrangement of the hepatitis and serologic data demonstrates that 85% (324/381) of HB_SAg or anti-HB_S acquisitions were asymptomatic (or not recollected).

Table 4 Association of Hepatitis and Transfusion History with Seropositivity

	No. Tested	No.(%) HB_SAg+	No.(%) anti-HB_S+
Hepatitis*			
Yes	285	9(3.1)	48(16.8)
No	7107	28(0.4)	296(4.2)
Transfusion**			
Yes	278	1(0.4)	23(8.3)
No	6860	36(0.5)	318(4.6)

* $X^2 = 130.2$, p 0.001

** $X^2 = 5.94$, p=0.015

Thirty-eight personnel reported a history of a positive test for HB_SAg; serologic evidence of Hepatitis B infection was found in 17 (45%) of them. Of personnel with previous overseas assignments, 9.6% had serologic evidence of Hepatitis B infection; 4.5% of personnel without prior service overseas had such evidence. This difference was not eliminated by controlling for age. Among personnel who had served overseas, the seropositivity rate was higher for officers in

non-patient care oriented Corps than for those in direct patient care oriented Corps (57% versus 26%) and higher for enlisted support personnel than for enlisted personnel in clinical and laboratory specialties (77% versus 60%).

All officer personnel divided their professional experience into months in medical and surgical specialty areas. Dentists were included among the surgical specialists. The majority of personnel studied, being very recent graduates, had little or no post-degree experience in either specialty area. A somewhat greater proportion of personnel with one or more months in surgical specialties reported a history of hepatitis overall (7.1% versus 5.3%) and for all experience cohorts except 1-17 months (Table 5) (X^2=3.10, p=0.078). Personnel with one or more months in surgical specialties were positive for HB_sAg and anti-HB_s more commonly, 1.0% and 8.7% versus 0.5% and 5.6% respectively (Table 6) (X^2=5.81, p=0.016). Rates of anti-HB_s positivity rose with increasing professional experience among both the medical and surgical specialists.

Table 5 Hepatitis History and Professional Experience

	Hepatitis			
Months in Specialty	Medical Specialties No. responses	No.(%) Hepat.	Surgical Specialties No. responses	No.(%) Hepat.
0	2390	84 (3.5)	2799	87 (3.1)
1-17	716	38 (5.3)	464	21 (4.5)
18-35	260	13 (5.0)	149	13 (8.7)
36-53	465	18 (3.9)	236	16 (6.8)
54	156	7 (4.5)	314	27 (8.6)

Table 6 Seropositivity and Professional Experience

	Seropositivity					
	Medical Specialties			Surgical Specialties		
Months in Specialty	No. Tested	No.(%) HB_sAg+	No.(%) anti-HB_s+	No. Tested	No.(%) HB_sAg+	No.(%) anti-HB_s+
0	2330	9(0.4)	79(3.4)	2789	8(0.3)	87(3.1)

Continued

Table 6 Seropositivity and Professional Experience

	Seropositivity					
	Medical Specialties			Surgical Specialties		
Months in Specialty	No. Tested	No.(%) HB_SAg+	No.(%) anti-HB_S+	No. Tested	No.(%) HB_SAg+	No.(%) anti-HB_S+
1-17	778	4(0.5)	42(5.4)	457	2(0.4)	20(4.4)
18-35	262	2(0.8)	21(8.0)	148	3(2.0)	12(8.1)
36-53	456	3(0.7)	26(5.7)	240	2(0.8)	33(13.8)
54	156	0	16(10.3)	310	6(1.9)	43(13.9)

Serologic evidence of Hepatitis B infection in physicians was examined for those specialties represented by more than 25 officers (Table 7). Personnel in these specialties were comparable with respect to age with the exception of those placed in "general medicine" who were younger and lacked specialty training. General surgery appears to confer the highest risk, psychiatry the lowest. In addition to those in general surgery, practitioners of obstetrics/gynecology, otolaryngology, orthopedic surgery, radiology, and pathology have a risk of infection in excess of "all physicians."

Table 7 Seropositivity in Selected Professional Specialties

Specialty	No. Tested	No.(%) HB_SAg+	No.(%) anti-HB_S+
General Medicine	221	0	10(4.5)
Obstetrics/Gynecology	101	0	13(12.9)
Anesthesiology	36	0	3(8.3)
Pediatrics	83	0	5(6.0)
Otolaryngology	48	1(2.1)	4(8.3)
Psychiatry	63	0	2(3.2)

Continued

Table 7 Seropositivity in Selected Professional Specialties

Specialty	No. Tested	No.(%) HB_SAg+	No.(%) anti-HB_S+
Internal Medicine	418	2(0.5)	30(7.2)
General Surgery	196	3(1.5)	33(16.8)
Orthopedic Surgery	75	1(1.3)	7(9.3)
Radiology	65	0	7(10.8)
Pathology	49	2(2.1)	4(8.2)
All Physicians	1534	12(0.8)	130(8.5)

Of 1,160 general duty nurses, 4 (0.3%) were HB_SAg positive and 20 (2.4%) were anti-HB_S positive; of 124 medical surgical nurses of similar ages, 1 (0.8%) was HB_SAg positive and 7 (5.6%) were anti-HB_S positive. The number of dental specialists was not sufficient for comparison; the largest specialty group (oral surgery) contained only 18 personnel. Of these none were HB_SAg positive and one (5.6%) was anti-HB_S positive.

B. Followup Incidence Study of the 1972 Academy of Health Sciences Cohort

A cohort of 2730 Army Medical Department officers, including nurses (ANC), physical therapists and dietitians (AMSC), dentists (DC), physicians (MC), administrators and allied scientists (MSC), and veterinarians (VC), inprocessed at the Academy of Health Sciences from July through October 1972 and was studied for the presence of HB_SAg and anti-HB_S. Antigen and antibody prevalences were 0.4% and 4.4% respectively in this overall population, with rates varying greatly by Corps. Most of these personnel were eligible for release from active duty between July and October 1974. The members of this cohort were located and those remaining on active duty were sent a mailing which requested the completion of a questionnaire and submission of a serum specimen for HB_SAg and anti-HB_S testing. The questionnaire data have not been analyzed. Serologic testing is in progress and preliminary results on acquisition of Hepatitis B seropositivity (HB_SAg or anti-HB_S) are reported here.

A listing of the name, social security account number, and class number of each officer enrolled in the cohort was obtained from existing project records. The duty stations of these personnel were determined with the cooperation of OTSG locator. Two months before the second anniversary of entrance onto active duty, a mailing was made to each officer listed as still on active duty. Included in this mailing were a letter to acquaint the officer with the purpose of the study and request participation; a questionnaire to assess assignment(s), job description, and selected personal health experiences; an instruction sheet to assist local laboratory personnel to properly process the serum specimen; and serum collection and storage tubes with mailing materials. The mailing was made in three parts, for those scheduled to leave active duty in July, in August, or in September-October 1974.

Questionnaire responses were coded for computer analysis. Sera were tested for HB_SAg by radioimmunoassay (RIA) and for anti-HB_S by passive hemagglutination (PHA). The characteristics of respondents will be compared with those of nonrespondents. The rates of HB_SAg and anti-HB_S positivity were calculated and compared with those obtained two years previously. The demographic and occupational characteristics of officers who have acquired antigen or antibody will be studied with the intent of identifying high risk groups.

The proportion of officers who responded and submitted a usable serum specimen is given, by Corps, in Table 1. By the time of study, The Surgeon General's Office locator had "no record" of 555 personnel (21% of the cohort); these had previously been released from active duty. Six hundred seventy-nine of the remaining officers (32.7%) submitted a completed questionnaire and serum specimen.

Table 1. Followup Response Rates by Corps

Corps	Total Eligible	No Record	Total Mailings	No (%) Responders
ANC	569	120	449	203 (45.2)
AMSC	48	12	36	16 (44.4)
DC	460	108	352	85 (24.1)

Continued

Table 1. Followup Response Rates by Corps

Corps	Total Eligible	No Record	Total Mailings	No (%) Responders
MC	846	63	783	228 (29.1)
MSC	646	246	400	121 (30.3)
VC	61	6	55	26 (47.3)
Total	2630	555	2075	679 (32.7)

The response rate was highest for the nurses and lowest for dentists. Response rates decreased as the study progressed, probably because of an early release policy, put in effect after the first mailing. This was especially true for physicians and dentists, more than 80% of whom were given early outs reportedly to permit the Army to meet Congressionally set manpower levels. If physician and dentist participation had continued at the levels seen in the first part of the mailing (41% and 30.8%, respectively) 88 additional physicians and 22 additional dentists would have participated.

One of 679 sera submitted (0.1%) was positive for HB_sAg. The HB_sAg positive officer was a physician-pathologist (Major, MC) who performed primarily veterinary pathology examinations. None of the 9 personnel who were HB_sAg positive in 1972 responded to the mailing. Nine personnel were confirmed anti-HB_s positive (1.7%); nineteen others remain to be confirmed and are not considered further here. Of the former, two (0.4%) represent new acquisitions. These occurred in a physician-general medical officer (Major, MC) with duty station in Europe and an obstetrical/surgical nurse (1LT, ANC) with assignments in Texas, Southeast Asia, and California. Taking HB_sAg and anti-HB_s data together, three of the 678 personnel (0.4%) acquired Hepatitis B infection during their two-year assignment. The conversion rate for physicians was 0.9% (2/228), for nurses 0.5% (1/203) and for the other Officer Corps zero.

Additional officers who were anti-HB_s positive in 1972 lost their seropositivity. The number of such officers has not yet been tabulated.

2. Epidemiologic Investigations of Hepatitis Outbreaks

A. Hepatitis B Outbreak at USAH, Camp Zama, Japan

Cases of acute viral hepatitis among enlisted personnel of the Medical Company, United States Army Hospital Camp Zama (USAHCZ), Japan, began to occur during mid-March 1974. By early May, sixteen of the eighty enlisted personnel (20%) living in Medical Company quarters had experienced illnesses compatible with a diagnosis of hepatitis. Interviews were conducted with these personnel to elicit possible common experiences and sources of infection. Although no plausible explanation for an outbreak of Hepatitis A (infectious hepatitis) emerged, gamma globulin was administered to all enlisted personnel resident in Medical Company barracks on 19 or 26 April. A hospital-wide screening program to detect biochemical abnormalities in liver function was carried out on May 2nd and again on 14 May; all personnel with abnormalities were hospitalized for evaluation. Urine screening for drugs of abuse was performed on 6 May. Staff physicians suspected the outbreak might be one of Hepatitis B (serum hepatitis) related to the use of illicit drugs. Because of the paucity of data to support any specific etiologic diagnosis, consultation from the Epidemiology Consultant Service (EPICON) was requested on 22 May.

Conduct of the Consultation:

Two consultants arrived at Camp Zama on 31 May and began with a review of available records. The hepatitis and drug-use experiences of the Command for the previous 16 months were abstracted from the Command Health Reports. Information on USAHCZ admissions and dispositions for hepatitis, drug usage, and viral diseases possibly confused with hepatitis was gotten, for January through May 1974, from Registrar's records. Data from laboratory records on liver function studies and counter electrophoresis (CEP) determinations for Hepatitis B surface antigen (HB_sAg) for the previous 17 months were examined, as were the results of the biochemical screening program. Charts of all patients hospitalized with a diagnosis of hepatitis and interviews conducted with these patients were reviewed.

Patients still available were re-interviewed by the consultants. Results of urine screening programs for drugs of abuse were obtained from the Staff of the Drug and Alcohol Team. Serum specimens for Hepatitis B surface antigen (HB_sAg) and antibody (anti-HB_s) testing were collected from all enlisted personnel. A sample of the gamma globulin remaining

from that used on 19 and 26 April was also available for testing. Arrangements were made to rebleed enlisted personnel resident in Medical Company barracks six weeks and six months following the initial bleeds. The consultants completed their initial studies on 6 June 1974.

All sera were tested at the Walter Reed Army Institute of Research. Hepatitis B surface antigen (HB_sAg) testing was by radioimmunoassay (RIA). Those specimens also HB_sAg positive by counterelectrophoresis (CEP) were subtyped. The passive hemagglutination (PHA) method was used for testing for antibody to the surface antigen (anti-HB_s).

Command Health Report (CHR) data indicated that hepatitis had not occurred during the first half of 1973 but had occurred sporadically during the latter half of the year. Two cases had been reported for July and one each for August and October of 1973. Sixteen cases had been reported for the period January through April 1974. Two of the four 1973 cases and 11 of the 16 1974 cases occurred in enlisted personnel assigned to USAHCZ. The cases had not been reported by type.

After examining all records thought to be pertinent, thirty-three enlisted and two officer personnel were considered to be "cases" of hepatitis as they had historical, clinical, and/or laboratory evidence compatible with that diagnosis. Twenty-four of the cases, or 68.5%, occurred in young male enlisted personnel residing in Medical Company barracks and will be discussed further. Those cases represent a case rate among barrack occupants of 29.6 percent (24/81). The remaining eleven cases were excluded from additional consideration on the basis of grade and place of residence different from the others and/or liver biopsy data incompatible with viral hepatitis. These eleven cases represent attack rates among enlisted personnel not resident in Medical Company barracks of 7.1 percent (9/127) and among officer personnel of 2.7 percent (2/74). Six of these eleven were identified through the biochemical screening program.

A majority of barrack residents who were classified as cases (13/24) first came to medical attention through clinic visitation. Five of the 24 were identified by the biochemical screening program and two through the abnormal result of self-testing for liver function found in laboratory records. Four cases were hospitalized directly. The

chronology of cases and methods of identification are shown in Figure 1. Eleven of the cases (45.8%) were HB_sAg positive by CEP when tested at various intervals after onset of illness or after identification as cases. Review of admissions for other viral diseases and admissions with drug diagnoses did not contribute additional cases.

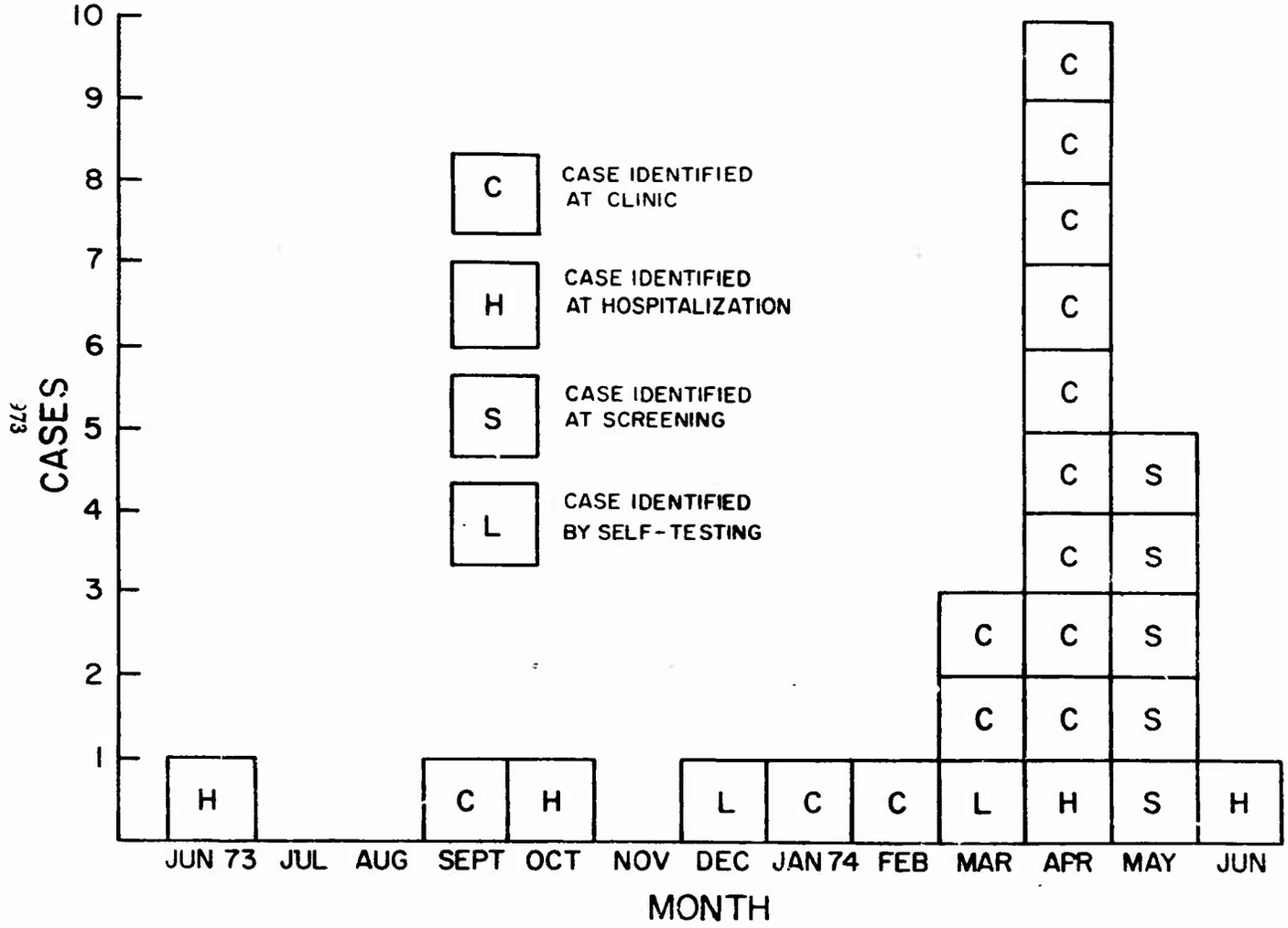
An estimate of the prevalence of use of illicit drugs was made from re-interviews and from results of urine screening programs. Twenty-nine enlisted male occupants of Medical Company barracks (35.8%) were considered to have used heroin intravenously on one or more occasions. A separate review of the personnel roster with one such individual resident in the barrack for 18 months, provided the information that 32 percent (26/81) of residents were either definite or highly probable heroin users. Using both these sources to identify drug users, a history of heroin use was associated with the probability of being a hepatitis case (Table 1). Twenty-two of 30 personnel with a history of use (73.3%) acquired case status, in contrast to the rate among presumed non-users of 3.9% (2/51).

Table 1. Association of History of Heroin Use and Identification as a Case

		History of Heroin Use		
		YES	NO	
Identification as a Case	YES	22	2	24
	NO	8	49	57
		30	51	81

Re-interviews with drug using personnel ill with hepatitis suggested that January-February 1974 was a period of heavy intravenous drug use. Three of these personnel admitted needle sharing during the latter months of 1973; others denied sharing earlier than January 1974. Of the former three, one had abnormal liver function studies in December 1973, a positive HB_sAg test in April, 1974 and a liver biopsy diagnosis of chronic persistent hepatitis; a second had a

FIG. I OCCURRENCE OF CASES WITH METHOD OF IDENTIFICATION



hospitalization for viral hepatitis in October 1973, a positive test for HB_sAg in January 1974, and a liver biopsy diagnosis of persistent viral hepatitis. Two of three admitted needle sharing with the HB_sAg positive (index) case identified in June 1973. One additional man who denied intravenous drug use prior to January 1974 had recorded abnormal liver function studies that month.

Interviews with these personnel gave investigators the impression that the cessation of U.S. military operations in Vietnam had resulted in considerable and progressive scaling down of local activities. The impact of this on the personnel of USAHCZ seemed to have been severe. The very marked drop off in patient load, the severe cut-backs in funding for recreational activities, and the unfavorable dollar to yen exchange apparently provided for very low morale among the enlisted personnel housed in USAH barracks. The investigation of their off duty environment suggested that the over-all social climate was very oppressive and devoid of the usual challenges and incentives.

Aliquots of sera of cases positive for HB_sAg by CEP were still available and were retested by RIA; all were confirmed as positive. Sera from cases which were HB_sAg negative by CEP were not routinely saved and were not available for re-testing. A serologic survey for both HB_sAg and anti-HB_s among all enlisted personnel of the Medical Company was carried out at the completion of initial studies. Participation rates were 80.2% for barracks residents and 70.8% for others. The results (Table 2) indicate that while evidence of past Hepatitis B infection was common to both groups, the occurrence of active disease was concentrated among the barracks personnel.

Table 2. HB_sAg and Anti-HB_s Positivity Among Enlisted Medical Company Personnel, USAHCZ

	Barracks Residents		Others	
Serologic Test	No. Tested	No. (%) Positive	No. Tested	No. (%) Positive
HB_sAg	65	8(12)	91	1(1)
Anti-HB_s	65	13(20)	91	17(19)

These serologic data identified one additional HB_sAg positive and eight anti-HB_s positive personnel not previously considered to be cases. The HB_sAg positive and three of the anti-HB_s positive personnel were among those identified as drug users. None of these individuals were ill and all had normal liver function studies on screening. If these four drug users are included as cases, the number of Hepatitis B virus infections attributable to the outbreak is 28 or 34 percent of barracks personnel and 86.6 percent of identified drug users.

A serologic follow-up limited to the barracks residents was carried out six weeks and six months after the initial investigation. Five of the eight HB_sAg positive personnel were rebled at six weeks and one at both six weeks and six months. None of these were either HB_sAg or anti-HB_s positive. Eleven of the anti-HB_s positive were present for followup. Anti-HB_s persisted in eight of these, became undetectable in two at the six weeks followup and in one at the six month followup. Sera from thirty-one enlisted personnel assigned to USAHCZ subsequent to the initial investigation were also collected and tested during the followup period. None were found to be HB_sAg or anti-HB_s positive.

Nine HB_sAg positive sera were subtypable; these were all Ayw. The gamma globulin tested was anti-HB_s positive, with a titer of 1:32.

B. Hepatitis Outbreak at Fort Riley, Kansas

On 13 May 1974, a telephonic request for the Epidemiology Consultant (EPICON) Service assistance was received from LTC Richard O. Proctor, MC, Area Epidemiologist, U.S. Army Health Services Command on behalf of the Commander, Fort Riley MEDDAC. The request related to a sudden occurrence of cases of viral hepatitis in a group of military families. Approval for this investigation was granted on the same day.

BACKGROUND

Fort Riley, KS is an Army installation with approximately 17,400 assigned active duty personnel, 16,200 dependents and 2,400 DA civilian employees. On-post housing is available for 2,080 military families while over 7,000 reside in surrounding civilian communities. Unaccompanied military personnel reside on post in barracks and BOQs. The major unit assigned to Fort Riley is the 1st Infantry Division -

"Big Red One." Medical care is provided by the Fort Riley MEDDAC/Irwin Army Hospital.

Since 1972, military cases of viral hepatitis at Fort Riley were sporadic, with an annual rate of less than 7 per 1,000 average strength (Table 1).

Table 1

Reported Occurrence of Cases of Viral Hepatitis in Military Personnel, Fort Riley, KS

Year	Calendar Quarter	No. Cases	Rate*	Average Strength
1972	1st	27	6.7	16,179
	2nd	22	5.3	16,744
	3rd	31	6.7	18,490
	4th	25	5.6	17,770
1973	1st	25	5.9	17,037
	2nd	26	5.7	18,273
	3rd	27	5.7	18,801
	4th	20	4.3	18,519
1974	1st	11	2.5	18,073
	2nd	19	4.3	17,510

* Expressed as number of cases per 1,000 average strength per year.

In order of frequency, the cases were diagnosed as Unspecified, type B or type A viral hepatitis (Table 2).

Table 2

Reported Distribution of Diagnoses in Military Cases of Viral Hepatitis, Fort Riley, KS

	All Cases		A		B		Unspecified	
Year	No.	%	No.	%	No.	%	No.	%
1972	105	100	20	19	42	40	43	41
1973	98	100	18	18	22	22	58	59

No statistical information was available pertaining to viral hepatitis in dependents. It was "guessed" that cases were occurring at an annual rate of approximately 1 per 1,000 dependents.

The first case in the present outbreak was diagnosed on 12 April in a noncommissioned officer. The second case, his wife, was diagnosed on 17 April. By 10 May, seven additional cases were added to the list. All cases occurred in families of noncommissioned officers that resided on-post. In view of the negative Hepatitis B surface antigen (HB_sAg) test results in patients, immune serum globulin (ISG) was offered to all close contacts. As a result of meticulous contact tracing by the community health nurse, 173 doses of ISG (.01 ml per pound of body weight) were administered prior to the EPICON team's arrival

INVESTIGATION

Two EPICON investigators arrived at Fort Riley on 14 May 1974. They began their studies with a review of available medical and environmental sanitation records. On epidemiologic grounds it was immediately established that the outbreak was not due to exposure to a common vehicle, such as the community water and food sources.

On the same day, three additional cases of hepatitis were reported, bringing the total to 12. Of these, six were hospitalized and 6 were treated and followed through the outpatient clinics. All cases were called Hepatitis A based on the presence of characteristic signs and symptoms, elevated bilirubin and SGOT levels, and negative results on HB_sAg testing.

The investigators re-interviewed all reported cases and/or their parents in case of children. All cases denied recent past contact with known cases of hepatitis, participation in special group events, consumption of shellfish, or any form of parenteral inoculation. The factor in common to all was their mutual acquaintance and, with the exception of one family, a residence close-by (Fig 1).

The breakthrough in the investigation came when it was established that the second case diagnosed in this outbreak in fact became symptomatic 30 days prior to the first case. The occurrence of cases is summarized in Table 3, which includes 2 additional cases that were reported later during the follow-up period.

Fig 1

Geographical Distribution of Cases of Hepatitis A
Among Family Housing Areas. Fort Riley, KS
March-August 1974

(Note houses marked as A,B,C,D & E)

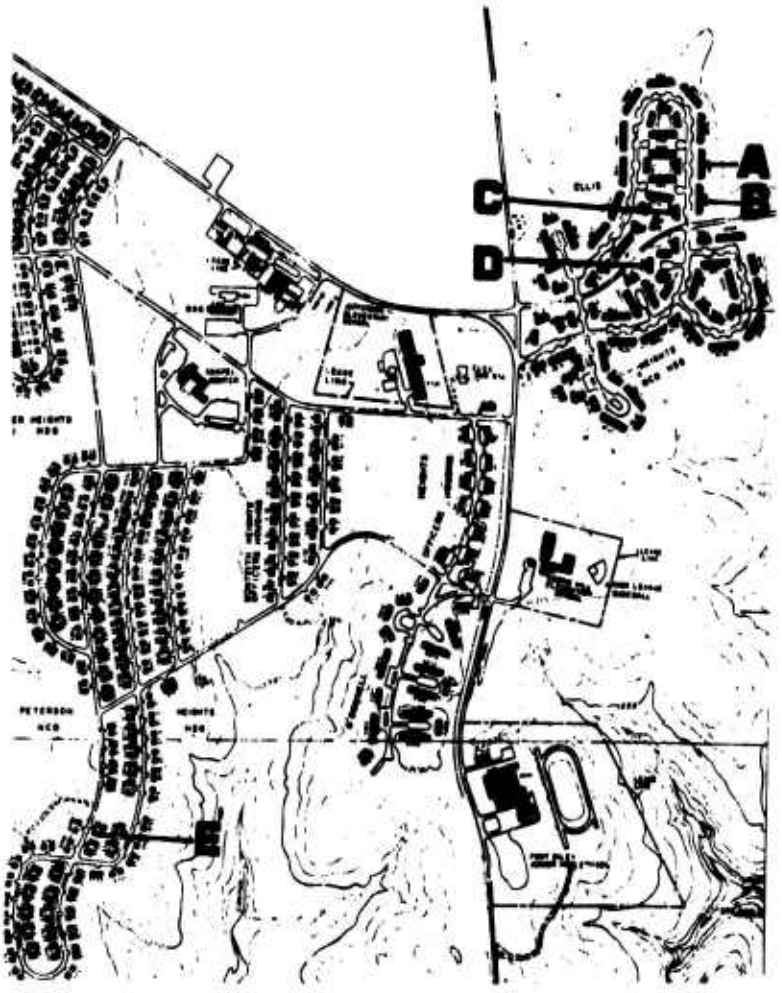


Table 3

Hepatitis A in Military Families
Fort Riley, KS, March - August 1974

Case	Age	Sex	Residence*	Date of Onset
1-B.M.	31	F	A-8	11 March
2-J.M.	31	M	A-8	10 April
3-L.B.	8	F	D-1	10 April
4-S.M.	3 1/2	F	E-1	18 April
5-A.B.	2	F	D-1	19 April
6-D.S.	25	M	B-1	27 April
7-F.M.	7	M	E-1	30 April
8-E.M.	5	M	E-1	1 May
9-C.B.	5	M	B-8	6 May
10-P.O.	25	F	B-3	6 May
11-T.M.	7 1/2	F	B-5	7 May
12-K.H.	27	F	B-2	8 May
13-M.R.	25	F	C-1	29 May
14-B.R.	2	F	C-1	8 August

* Refer to Figs 1 & 2.

During the course of the investigation Mrs. B.M. (Case 1) emerged as the key figure in the outbreak. The composite picture was one of a very active and outgoing woman, who visited her friends almost daily, who arranged coffees for new women arrivals to the area and who, on occasion, gave a birthday party for another child. She usually served home baked cakes on such occasions. On the larger community scale, she played piano once weekly in the kindergarten and she

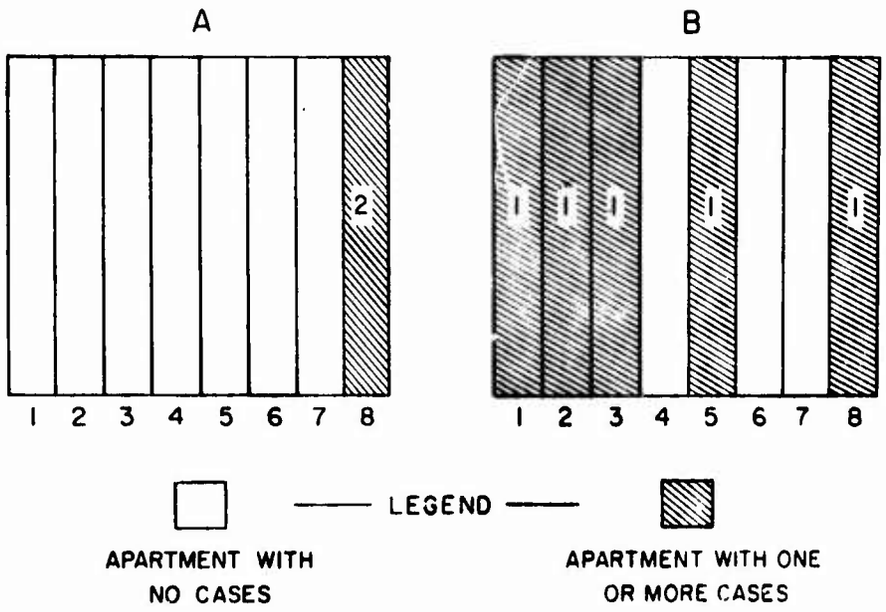
participated in all activities of a community weight watchers club and her church. Despite "feeling ill" during the latter part of March she continued with her usual social activities. However, by mid-April all these activities were halted because of increasingly more severe symptoms. At the time of hospitalization (17 April) her SGOT was reported 5,360 and the total bilirubin 7.0.

Following is a summary of some epidemiologic notes by location of residence (Fig 2).

Fig 2

Distribution of Cases of Hepatitis A
in Two Adjacent Apartment Houses

(Refer to houses A & B, Fig 1)



Building A - Apts 1-7: No cases of viral hepatitis.

Apt 8: Mrs. B.M. (Case 1), SGT J.M. (Case 2) and 2 children. Both children had ISG on 17 April, the parents had none. No reported close association with other inhabitants of building A.

Building B - Apt 1: SGT D.S. (Case 6; No ISG), wife (ISG 20 April) and 1 child (ISG 6 May). Wife was a friend of Mrs. B.M. Husband (D.S.) disclaimed any association with Mrs. B.M. and the eating of any of her cakes.

Apt 2: Mrs. K.H. (Case 12; No ISG), husband (ISG 13 May) and 1 child (ISG 13 May). Mrs. K.H. was well acquainted with Mrs. B.M. and on several occasions had cake in Mrs. B.M.'s apartment, the last time early in April.

Apt 3: Mrs. P.O. (Case 10; No ISG), husband (no ISG) and 2 children (both had ISG on 17 April). Mrs. P.O. was a very close friend of Mrs. B.M. She attended her parties and had eaten cup cakes baked by Mrs. B.M. on 24 March and probably later.

Apt 4: No cases in the family consisting of husband (no ISG), wife (ISG 15 May) and a retarded child (ISG 26 April). Because of the child, the parents lived in relative seclusion.

Apt 5: T.M. (Case 11; ISG on 26 April) father, mother and one sibling (ISG on 26 April). Parents had no ISG. Mrs. B.M. baby-sat on 2 occasions during March. Both children attended a birthday party given by Mrs. B.M. on 24 March.

Apt 6: No cases among husband, wife and 3 children. Children had ISG on 1 May, parents none. Only casual association with Mrs. B.M.

Apt 7: No cases in the only black family in the building. None had ISG. Both adults were usually away during the day and their older children were occupied elsewhere. None had any association with Mrs. B.M.

Apt 8: C.B. (Case 9; ISG on 26 April), father, mother and 1 sibling (ISG 26 April). Parents had no ISG. Mother was a close friend of Mrs. B.M. C.B. played

regularly with the son of Mrs. B.M. and had attended Mrs. B.M.'s parties. C.B.'s younger brother had not attended the parties.

Building C - Apt 1: Mrs. M.R. (Case 13; no ISG), B.R. (Case 14; ISG on 21 May) husband (ISG on 14 May) and son (ISG on 30 April). Mrs. M.R. was a very close friend of Mrs. B.M. and participated in most social affairs held at Mrs. B.M.'s home. Mrs. M.R. and her child B.R. were on a farm in Iowa 12-14 April, 8-29 May and 6-28 June, otherwise resided at Fort Riley.

Apts 2-4: No cases of viral hepatitis.

Building D - Apt 1: L.B. (Case 3; no ISG), A.B. (Case 5; no ISG) and both parents (ISG on 30 April). Although the family had no social ties with any individuals listed here, the mother babysat once weekly through early part of April, in her own home, for parents residing in A-8 (Mrs. B.M.), B-5 and B-8. Mrs. B.M. was reported to be especially friendly to the children L.B. and A.B. while visiting the household. L.B. had attended the birthday party at Mrs. B.M.'s on 24 March and had brought home a cup cake for A.B.

Apts 2-4: No cases of viral hepatitis.

Building E - Apt 1: S.M. (Case 4; no ISG), F.M. (Case 7; ISG on 26 April), E.M. (Case 8; ISG on 26 April) and both parents (ISG on 30 April). The family had very close relations with residents of B-1. The children had occasional contact with Mrs. B.H. whose apartment (A-8) was adjacent to B-1.

Apts 2-4: No cases of viral hepatitis.

Serotesting for HB_sAg was performed at the Irwin Army Hospital on all hepatitis cases using the counter electrophoresis (CEP) technique. Sera on all patients and their families were also tested for HB_sAg at the Walter Reed Army Institute of Research by radioimmunoassay (RIA). All test results were negative. The search for asymptomatic cases among the family members of patients was unproductive. Although some mild deviations in liver chemistries were observed, they were insufficient to make the diagnosis. The immune serum globulin was administered to a total of 264 close contacts to cases. A special effort was made to identify as many of Mrs. B.M.'s contacts as possible and to

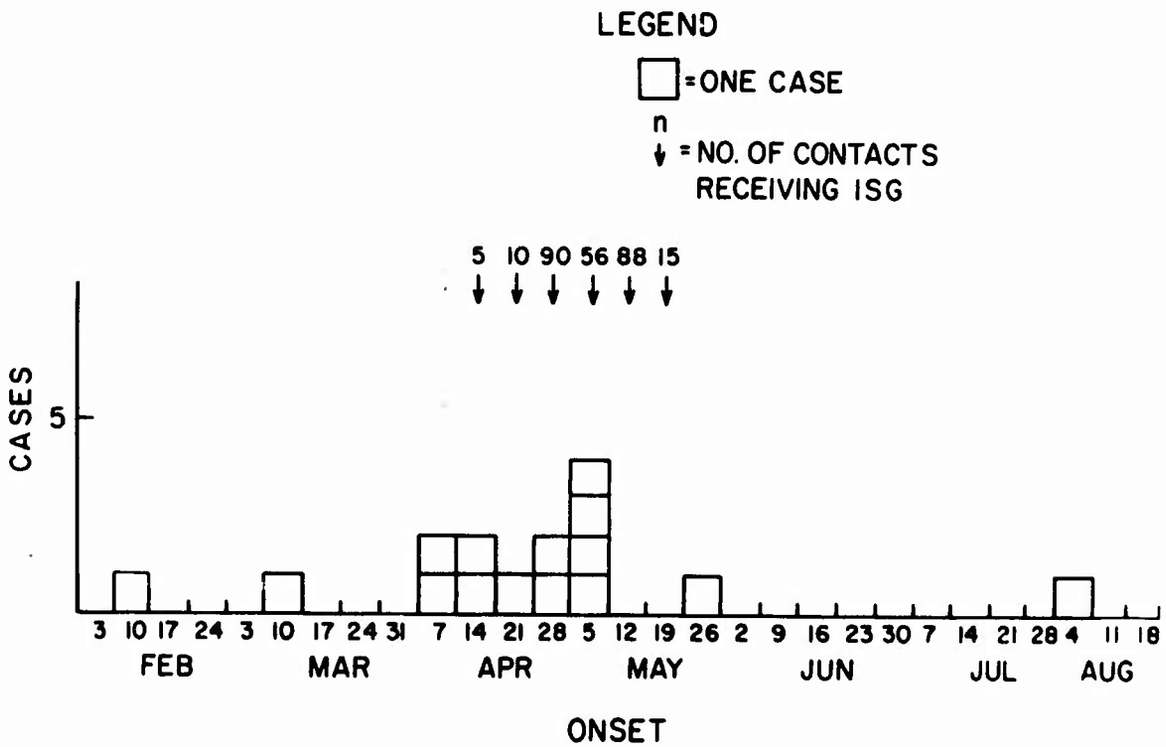
administer ISG when a reasonable risk was suspected.

The final step of this investigation was only academic. The question was - how did Mrs. B.M. become infected? On previous occasions, Mrs. B.M. had denied contact with any known case of hepatitis. On further probing, however, she remembered having cocktails in the home of a friend (D.B.) who lived in the nearby civilian community. Her story made mention of his subsequent hospitalization for reasons unknown and his release from the hospital after a few weeks. One month later he moved to another state. Although his medical records had already been transferred, the chief's of medicine file cabinet was more revealing - a copy of D.B.'s discharge summary was still on file. The digest of the summary: Hepatitis A (HB_sAg negative), disease onset 12 February. This brought the total count to 15 (Fig 3).

The EPICON team stopped their on-site investigation on 17 May not daring to look further into the past.

Fig 3

Hepatitis A Cases by Date of Onset
Fort Riley, KS, February-August 1974



SEQUELA

B.R. (Case 14), a 22 month old female child, was hospitalized with viral hepatitis at the Irwin Army Hospital on 14 August (onset 8 August). The HB_sAg test performed on admission was reported positive by CEP. However, serum obtained four days later and tested at the Walter Reed Army Institute of Research by the more sensitive RIA technique was HB_sAg negative. On her third hospital day B.R. was noted to have moderately severe pancytopenia. Over the next three weeks the pancytopenia became progressively worse, the child developed Pseudomonas aeruginosa septicemia and had to be transferred to the Fitzsimons Army Medical Center (9 September) for more intensive therapy. Despite heroic measures to sustain life, the child expired on 12 September. The primary diagnosis at the time of death was aplastic anemia.

Mrs. K.H. (Case 12) conceived at the height of her clinical illness. Although not a sequela by itself, the matter was of some concern to the investigators. Obstetrical History: LMP 8 May 1974, EDC 12 February 1975. Newborn (V.H.): DOB 17 February 1975. 7.1 pound female with physiologic jaundice. On 6 March total bilirubin was still elevated (9.4). Clinic visits: 12 May 1975 - normal growth and development; 21 July - liver chemistries, growth and development all normal.

DISCUSSION

By various means of medical documentation it was established that this was a person propagated outbreak of Hepatitis A. Transmission occurred by the oral route but the vehicle of spread was not clearly established. Epidemiological data point conclusively to the cluster's index case (Mrs. B.M.) as the prime reservoir of infection for this outbreak. She was an efficient spreader using aggressively, perhaps, several vehicles to reach susceptible hosts. In several cases, the home made cake was the leading candidate for such vehicle. This finding is reminiscent of a similar transmission pattern observed in an outbreak in Michigan in 1968 and described in THE ORANGE MAN AND OTHER NARRATIVES OF MEDICAL DETECTION, The West Branch Study by Berton Roueche (Little, Brown 1971). The acquisition of infection by cases appearing at the tail-end of the epidemic could have been from sources other than Mrs. B.M., but from the same infective pool. Because of the late disease onset in case 14 (B.R.) the question of source of acquisition remains unanswered.

Posthepatitis aplastic anemia (Case 14) was well documented by appropriate clinical and laboratory findings including bone marrow biopsy. This relatively rare complication occurs in all ages, more frequently in males than females. The mean age has been reported as 18 years and the mean interval from hepatitis through aplasia to death as 19 weeks. It is interesting to note that all reported cases that had been tested were HB_sAg negative.

3. Epidemiologic Studies of Hepatitis B at Fort Hood, Texas

A prospective study of enlisted personnel newly arrived at Fort Hood was begun in February, 1974. Each of the 2,330 personnel in this cohort was administered a demographic and health experience questionnaire and had serum collected for Hepatitis B antigen and antibody determinations. The cohort was followed quarterly by questionnaire and HB_sAg and anti-HB_s determinations. Results and discussion of this collaborative study is reported elsewhere (Project 3A161101A91C, Work Unit 105, Mechanisms of transmission of hepatitis viruses).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OR 6500	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	01	130
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Gastrointestinal Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 008800 Life Support 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PREVIOUS 75	7	150
FISCAL YEAR CURRENT 76	7	200

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL E. L.
TELEPHONE: 202-576-3551

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ASSOCIATE INVESTIGATORS
NAME: Alan N. Charney, MD, MAJ, MC
NAME: Marshall D. Kinsey, MD, MAJ, MC DA

PII Redacted

20. GENERAL USE

Foreign intelligence not considered

21. KEYWORDS (Precede EACH with Security Classification Code) (U) Diarrheal Diseases; (U) Salmonellosis; (U) Intestinal Absorption; (U) Shigellosis; (U) Intestinal Cell Surface; (U) Cholera; (U) Prostaglandins

22. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Research efforts in this department will continue to be directed toward gastrointestinal diseases of military importance. In particular, the focus is on the enteropathogenic diarrheal diseases, Salmonellosis and Shigellosis. These have critical military relevance since they represent a major factor in troop mobility.

24 (U) Studies will continue to employ several in vivo and in vitro models. These include perfusion models using rhesus monkeys and rats and in vivo rabbit ileal loop models and subcellular membrane fractions. Lymphocyte function (antibody mediated cellular cytotoxicity) will be studied in vivo.

25 (U) 74 07-75 06 The concept that symptomatic bacterial diarrhea is related to specific functional alterations in absorption and secretion by intestinal epithelial cells is being extended beyond the original cholera model. Salmonella diarrhea is not related to changes in intestinal permeability. Both Salmonella and Shigella activate adenyl cyclase and increase cyclic AMP in tissues. Prostaglandin levels in secretion appear to be increased and indomethacin, an inhibitor of prostaglandin synthesis, decreased Salmonella and Shigella mediated secretions. Na-K-ATPase, an enzyme related to water and electrolyte absorption can be increased with adrenal steroids. Lymphocytes killing of enteropathogenic organism and intestinal cell surface glycoproteins are being investigated. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 74 to 30 Jun 75.

*Available to contractors upon originator's approval

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Project 3A762760A822 Military Internal Medicine

Task 01 Military Internal Medicine

Work Unit 130 Gastrointestinal Diseases of Military Importance

Investigators
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Description

The research activities in this Department have continued to focus on intestinal fluid and electrolyte secretion, especially the biochemical and physiological mechanism of secretion associated with the enteropathogenic diarrheal diseases. In addition, pharmacologic control of intestinal fluid and electrolyte secretion using adrenal steroids and indomethacin have been studied. Two major new directions of the department include a definition of the host immunological defense mechanisms against enteropathogenic bacteria and a definition of the intestinal cell surface characteristics which may be expected to determine invasiveness or toxigenicity of individual bacterial strains.

Progress and Results

To define the probable sites of small intestinal secretion and absorption, villus cells were isolated sequentially from the villus tip to the small intestinal crypt. One enzyme known to be associated with electrolyte and probably water transport, Na^+-K^+-ATPase, was studied in isolated villus tip and crypt cells. A significant gradient of decreasing activity from the villus tip to crypt was found for Na^+-K^+-ATPase. This was true in both the jejunum and the ileum and the jejunal villus tip activity was considerably higher than that in the ileum. This study has been published (8).

To better define the relation of Na^+-K^+-ATPase to electrolyte transport, rats were treated with deoxycorticosterone acetate (DOCA) or methylprednisolone (MP). Electrolyte and water transport and potential difference (PD) were then measured in perfused segments of jejunum, ileum and colon and compared to ATPase and adenylate cyclase activity in these segments. MP increased Na and H_2O absorption and ATPase in all segments. DOCA increased ATPase and electrolyte transport in colon. Thus, since adrenal steroids were successfully used to increase both Na-K-ATPase and water and electrolyte absorption, a primary role for Na-K-ATPase in intestinal electrolyte transport is suggested. Two abstracts were submitted (9, 10) and a manuscript has been accepted for publication (1).

A paper detailing the pathophysiology of Salmonella diarrhea in rhesus monkeys has been published (11). The study included observations of intestinal transport of water and electrolytes and morphological and bacteriological studies. It was shown that the transport abnormalities in Salmonella diarrhea occur in both the large and small intestine. The degree of transport abnormalities was not necessarily associated with direct observation of bacterial overgrowth and invasion. This suggests that additional factors indirectly arising from the infectious process, such as undefined humoral messengers, may participate in secretory events. Current studies are being undertaken to find these processes. The role of permeability in the pathogenesis of salmonella diarrhea was investigated in a group of rhesus monkeys. Although animals developed diarrhea, morphological changes and water and electrolyte secretion, permeability was unaffected in infected animals. These observations suggest that permeability is not a contributing factor in salmonella diarrhea. An abstract has been published (12).

Similar studies of the pathophysiology of Shigella diarrhea and dysenteriae were carried out in rhesus monkeys. It was again found that the diarrhea resulted from small and large intestinal secretory defects. Once again the possibility of a humoral messenger was suggested by the fact that the intensity of transport abnormalities did not necessarily correlate with bacterial overgrowth of the affected site (13).

Studies have evaluated the colonic effects of the enterotoxins of V. cholera, E. Coli and Shigella Dysenteriae I. Cholera toxin causes colonic secretion in the rat at very low doses. This secretion is similar to other cyclic AMP mediated processes with water and Na secretion and an increase in bicarbonate secretion and a decrease in chloride secretion. This secretion is dose related. This colonic secretion indicates that the colon is taking part in the outpouring of fluid present in cholera and not just acting as a conduit overwhelmed by fluid from the small intestine. The remarkable sensitivity of the rat colon to choleragen indicates that this would be a good source for isolating choleragen receptors. E. Coli toxin does not cause colonic secretion. This suggests further differences in E. Coli toxin and cholera toxin. Shigella Dysenteriae I has no colonic effect (2).

Studies designed to delineate the biochemical mechanisms of Salmonella and Shigella mediated secretion were performed. These examined the activation of adenyl cyclase and the stimulation of intracellular cyclic AMP mediated by experimental salmonellosis and shigellosis. We found that the invasive enteropathogens, like the toxigenic cholera, stimulated adenyl cyclase. One study, which conclusively demonstrated a causative relationship between infection of rabbit ileum with Salmonella typhimurium, subsequent activation of adenylate cyclase and accumulation of cyclic AMP and ultimate fluid secretion was completed (3).

Studies demonstrating activation of rabbit ileal adenylate cyclase by Shiga toxin have just been completed. The positive findings of enzyme stimulation by this toxin contradict two negative reports in the literature. We have examined control and treated ileal mucosal scrapings in a large number of rabbits and have consistently found enzyme activation and accumulation of the cyclic AMP product. Studies were carried out at several incubation time intervals and with several lots of toxin harvested in various laboratories with consistent results. A manuscript is currently in preparation (4).

To further probe the mechanism of intestinal secretion in the enteropathogenic diarrheal diseases, Indomethacin, an inhibitor of prostaglandin synthesis, was used. Indomethacin completely abolished Salmonella-mediated fluid secretion in the rabbit ileal loop model and markedly reduced the secretion secondary to shigellae, cholera toxin and cholera organisms. A manuscript has been published (14).

The role of prostaglandins in pathological intestinal fluid secretion was further investigated. In preparation for the many prostaglandins assays carried out in our laboratories, we prepared our own antibody to PGB in lieu of continuing to use the commercial material. Rabbits were challenged with PGB antigen in a standard way. After six weeks, a large supply of high titer antibody was recovered.

Prostaglandin levels in rabbit ileal mucosal scrapings from control loops and loops infected with Salmonella typhimurium or treated with Shiga toxin were analyzed and compared. No differences were found between PGB and PGF levels in treated or infected tissues versus control tissues. Because previous studies had suggested a prostaglandin role in enteropathogenic fluid secretion and since others have questioned the validity of prostaglandin values obtained from homogenized tissues, we next examined prostaglandin levels in the secretory fluids generated by enteropathogenic stimuli.

We have found extremely high prostaglandin levels in the secretory fluid generated by Shiga toxin when compared with Cholera toxin fluid. Studies are currently in progress to survey the secretory fluids from Salmonella typhimurium infection and compare its prostaglandin levels to Shiga toxin and Cholera toxin stimulated secretory fluid (5).

A group of studies have dealt with the effects of bacterial overgrowth, the Blind Loop Syndrome, on small intestinal structure and function. In this study, it was shown that bacterial overgrowth resulted in alterations in active transport of sugars and amino acids by causing biochemical abnormalities in the brush border of small intestinal cells (15). Other studies in experimental blind loop syndrome considered the effects of small intestinal bacterial overgrowth. Studies showed that those bacteria caused morphologic damage to the brush border, mitochondria and endoplasmic reticulum. A manuscript has been published (16).

To investigate active destruction of bacteria by host immunocytes as a mechanism of host defense against enteric organisms, human peripheral lymphocytes were isolated. It was demonstrated that in the absence of complement, lymphocytes will kill 80% to 90% of antibody coated Shigella, Salmonella or E. Coli organisms in vitro (6). We have found no evidence of direct lymphocyte mediated cytotoxicity of these bacteria. Furthermore, one individual was studied pre and post infection with Shigella and it was shown that the patient's own lymphocytes in the presence of his own post-infection serum was capable of killing the Shigella organism with which he was infected. This phenomena is not due to either macrophages or polymorphonuclear leukocytes (6). Similar experiments with meningococci demonstrated antibody dependent cellular cytotoxicity as a potential mechanism of destruction of these bacteria (7).

To better understand the functional characteristics and surface markers of isolated subpopulations of human peripheral lymphocytes, the binding and mitogenic characteristics of six purified plant lectins were investigated (17). Binding of radioiodinated lectins to isolated human T, Null and B lymphocytes indicated that although functionally different, each lymphocyte population bound equivalent amounts of E- and L-PHA, lentil PHA, Con A, RCA-I, and WGA. Thus functionally different lymphocytes do not differ with respect to surface content of their carbohydrate structures. However, upon incubation of E and L-PHA, lentil PHA, and Con A with isolated lymphocytes subpopulations, we found that Null and B cells displayed a delayed peak of DNA synthesis compared to T cells (17). These observations then allowed us to compare the binding of these same radioiodinated lectins to lymphocytes from patients with chronic lymphatic leukemia (CLL). Because CLL is a "B cell" leukemia, it had not previously been possible to compare the binding characteristics of CLL cells to normal B cells until our demonstration that the number of binding sites was equivalent to isolated T, Null and B cells (17). We found the number of binding sites on CLL cells compared to normal B cells to be significantly lower for E-PHA, WGA, and Con A, and significantly higher for L-PHA. Thus CLL lymphocytes were found to be characterized by extensive and significant cell surface alterations detected by lectins (18).

Initial interaction between the intestine and a variety of nutrients (.e.g vitamins) and toxic factors (e.g. bacterial enterotoxins, bacterial cell surfaces) is mediated by receptors at the surface of the intestinal epithelial cell. It is likely that these receptors are glycoproteins or glycolipids. Since plant lectins are known to bind to specific cell surface oligosaccharide structures, the ability of a series of plant lectins to bind to intestinal cell surface membranes (brush borders) was investigated. Wheat germ agglutinin, ricinus communis agglutinin and E phytohemagglutinin all bound to proximal and distal guinea pig brush border with about 10^{14} binding sites/mg of brush border protein (19).

In order to investigate the influence of plant lectins on a well characterized intestinal cell surface binding reaction, the binding of intrinsic factor vitamin B_{12} complex (IF-B_{12}) was chosen. Pre-incubation of E phytohemagglutinin with distal brush border resulted in competitive inhibition of subsequent IFB_{12} binding (19). This suggests that E Phytohemagglutinin interacts specifically with the intestinal cell receptors for IFB_{12}.

A clinical study on the metabolism of deoxycholic acid in a group of patients with alcoholic cirrhosis was performed. In order to explain the low levels of the secondary bile acid deoxycholic acid in bile of patients with cirrhosis, disappearance of ^{14}C labelled deoxycholic acid from stools of patients with cirrhosis, quantitative and qualitative determination of fecal bile acids and in vitro ability of fecal bacteria to metabolize cholic acid were studied. Our in vitro data suggested that unpaired conversion of cholic acid to deoxycholic acid by intestinal bacteria best explains low deoxycholic acid in cirrhotic bile (20).

A manuscript from a previously performed study was completed and published (21) in 1974. This manuscript dealt with the functional association of hexokinase to rat liver mitochondria. It considered for the first time a kinetic relationship between an associated enzyme and the supply of substrate by respiring mitochondria. It is one of the few demonstrations of an important functional association between a substrate provider and a substrate acceptor. A review on the subject of the hexokinase acceptor theory of insulin actions and hormonal control of functional compartmentation was published (22).

Conclusions and Recommendations

The observation that the invasive organisms Salmonella and Shigella promote water and electrolyte secretion by a possible humoral mechanism have been confirmed and extended. Alterations in intestinal permeabilityhave been clearly shown to play no role in the diarrhea of salmonellosis. On the other hand, salmonella infection in the rabbit caused activation of adenylate cyclase and accumulation of cyclic AMP. In contrast to negative studies in the literature, Shiga toxin had a similar effect on adenylate cyclase. Indomethacin an inhibitor of prostaglandin synthetase completely abolished salmonella mediated secretion and decreased shigella mediated secretion. Increased levels of prostaglandins have been found in shigella induced secretions. The last two results implicate prostaglandins as possible mediators of salmonella and shigella induced secretion.

Na-K-ATPase activity seems to be closely correlated with intestinal transport of water and electrolytes. Its activity is greatest in intestinal tip cells. Modification of Na-K-ATPase activity pharmacologically is possible and may offer a means of preventing or reversing pathological intestinal secretion.

Cholera toxin has been shown to cause colonic secretion, a previously unrecognized finding which further differentiates it from E. Coli and Shigella toxin.

The ability of plant lectins to bind to the intestinal cell surface has been demonstrated and the specific ability of lectins to inhibit as well recognized binding reaction (IFB_{12}) with ileal membranes has been shown. Lectins offer the possibility of isolated and characterizing many of the intestinal cell surface receptors which mediate attachment of bacteria and enterotoxins to the gut.

The ability of human lymphocytes to kill antibody coated enteric bacteria has been identified. The isolation and characterization of subpopulation of human lymphocytes with particular surface characteristics and patterns of r-sponse is continuing.

The major aim of the Department continues to be the elucidation of the fundamental mechanism of normal and pathologic intestinal secretion and absorption. It is becoming increasingly clear that infectious dia-rhea is related to specific functional alterations in intestinal cells and not to general cell destruction. If the hormonal mediators, enzymatic activities, specific toxins or cell receptors involved in enteropathic diarrhea can be defined, specific pharmacologic reversal or alteration of these mechanisms can be hoped for. In addition, the factors involved in host immunologic resistance to enteric bacteria is being pursued with emphasis on defining the mechanisms of cell killing and the subpopulation of immunocytes involved.

Project 3A762760A822 Military Internal Medicine

Task 01 Military Internal Medicine

Work Unit 130 Gastrointestinal Diseases of Military Importance

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PROJECT 3A762758A823

MILITARY PSYCHIATRY

Task 00
Military Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6470	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GRP. MGT'R'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10 NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A823	00	030
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Military Psychiatry

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 75	4	30
	CURRENT 76	4	110

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Drug Abuse Prevention; (U) Epidemiology; (U) Military Psychiatry; (U) Demography

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The mission of this unit is to identify psychiatric, psychologic, sociologic, and organizational factors which predispose the soldier to perform ineffectively or develop psychiatric illness and to develop more effective preventive treatment techniques.

24. (U) The research methods of psychology, sociology, clinical psychiatry, anthropology, and social work are used to identify and modify factors that contribute to ineffective military performance.

25. (U) 74 07 - 75 06 The military family and adolescent dysfunctioning study describes the families of problem adolescents who seek help at an Army out patient psychiatric service. The mental health care utilization pilot study has examined patterns of mental health care and the utilization of mental health facilities by active duty personnel and their dependents. Project Home is a study of drug using Vietnam veterans. Data analysis has been completed and it is in the initial stages of write up. The Career Outcome Study is a study of the military and medical careers of urine positive and negative soldiers. A cohort selected from three basic training posts has been followed for fourteen months. Techniques of personnel and IPDS record linkage, essential to the establishment of more accurate incidence rates for various disorders, have been developed. The Social Adjustment and Multiple Interactive Determinants of Stress Study will study the way in which social factors and social support systems affect responses to chronic stress using hemodialysis and kidney transplant patients as well as control groups as participants in the study. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 74 - 30 June 75.

*Available to connectors upon originator's approval.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Investigators.

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Description

The military psychiatry work unit included the following investigations: the Military Family and Adolescent Dysfunctioning Study, Mental Health Care Utilization in a Military Population Study; Project HOME, the Career Outcome Study, the Social Adjustment and Multiple Interactive Determinants of Stress Response Study, the Demographic Analysis of the WRGH Psychiatric Patient Population Study.

Progress

1. The Military Family and Adolescent Dysfunctioning Study

a. The Military Family and Adolescent Dysfunctioning Study describes the families of problem adolescents who seek help at an Army outpatient psychiatric service. The purpose of this study is to differentiate one family from another in terms of the internal family relationships, the external social relationships, and to classify the problems described behaviorally.

b. The military family has been described as being subjected to considerably more family dislocation and separation from the father than the civilian family.[1-4] The possibility that separation, frequent moves, and changing interfamilial roles may contribute to the disorganization and dissolution of the military family is of clinical interest. Data collect from this project may contribute to the development of new primary and secondary preventive programs within Army psychiatry.

c. In order to accomplish the study objectives a number a data-gathering techniques have been employed. These techniques include questionnaires, structured and semi-structured individual and group interviews, individual diaries and structured and unstructured video taped family transactions. A self administered questionnaire pertaining to aspects of the study contained in the three major sections has been developed and currently is being tested, for feasibility and validity in further studies.

d. The study has been divided into three major sections: the family activity section, the social network section and the problem section. Sub-sections of the study direct their attention to; communications patterns among family members during structured and unstructured time periods, the assessment of the effect of recent events in the life of the family on the behavior of its members and role-behavior incompatibilities within the family.

e. While detailed data analysis will not begin until all of the research families have been interviewed and all materials have been collected (Fall 1975) a number of impressions have emerged that seem to be worthy of reporting at this time. At present our provisional impressions of the data appear to indicate that the primary problems involved in adolescent dysfunction and its consequently stressful effects on the military family are more readily referable to the social milieu of the family rather than to intrinsic pathology within the family unit. Characteristically the families have indicated stress within the family unit as generated by (1) vague and unclear patterns of communications and (2) failure of families to negotiate or re-negotiate and abide by behavioral norms consistent with psycho-social and maturational changes occuring during adolescence. The data also indicate that patterns of interaction among family members are fragmented, i.e. family members go their own separate ways. We have yet to trace out the relationship, if any, between the lack of family interaction, the structure of their social network relationships, family activities, and variations in role behaviors within family units. All subjects in the Family Research project were also administered the Schedule of Recent Events. All subjects aged 11-18 were given a special adolescent form of this instrument. Although the study is not yet completed, analysis of the data on the 9 identified patients and the 15 siblings shows that the SRE distinguishes symptomatic from non-symptomatic subjects at a significant level ($p=0.01$) and in the predicted direction, i.e. the identified patients accumulated higher scores during the 6 months prior to intake at the Child Guidance Clinic than their siblings. It was also noted that parents showed a tendency to underreport significant events reported by their children (both patient and siblings). No trend of parents reporting events that should have appeared on the childrens form exists.

2. Mental Health Care Utilization in Military Population

Description

A pilot study has been carried out at Fort George G. Meade in order to examine the patterns of mental health care and the utilization of mental health facilities by active duty personnel and their dependents. Forty seven randomly selected active duty service members were the subjects of semi-structured interviews about the use of mental health care services.

Progress

The data for the pilot study has been collected and analyzed. Among the more salient findings were that of the 162 potential users (including 47 wives and 68 children), 12 (7%) had received mental health care during the preceeding year. The sample of 47 subjects appears to have been fairly representative of the population at the post. Our average subject was 27 years old, had 2.45 dependents, and had been in the army 7 years and 6 months. At Fort Meade the average active duty person with dependents is 28.6 years old, has 2.32 dependents, and has been in the army 8 years and 11 months. Median rank (E5) and median educational level (high school graduate) were identical for the sample and population. Blacks constitute 28% of the population and 30% of our sample. Usage rate of children (under 18) of military families was found to be greater than the rate for children of non-military families as described for a national sample by N.I.M.H.[5]; while the rates for adults of military families were not greater than those of adults of non-military families. One possible explanation of this finding is that adult members of military families may be more receptive to seeking mental health care for their children then for themselves. The success of the pilot study and the nature of the findings will serve as the basis for more extensive research in both psychiatric epidemiology and patterns of care seeking and in resource utilization during the coming fiscal year.

3. Project Home

Description

Project Home has been a study of drug use among Viet Nam veterans. An original panel of 183 subjects was studied via interview and 70 were followed for a year via questionnaire.

Progress

The data involved have been analyzed and are in the initial stages of write up. In general the findings have been consistent with those of other studies of Viet Nam era veterans and drug use. Heroin users were

more likely to be enlisted, to be black, and to be of lower rank. Urine negative non-drug users were particularly distinquished by their rank (43% of non-drug users were E-5 while only 17% of Recreational users, who were urine negative), and by greater alcohol consumption (beer). Non users also expressed significantly higher regard for their Co and NCO's.

4. The Career Outcome Study

Description

A cohort of drug users who entered the Army during 1972-1973 was defined in order to study prospectively their individual military and medical careers. Since the Army draws from the civilian age group at highest risk for drug abuse, urinanalysis screening for illicit drugs was routinely done at the reception stations and all positives were to have been medically evaluated. This study was designed to assess the long range behavioral implications of urine positivicity for drugs of "abuse" at the reception station and, indirectly, to evaluate the medical evaluation procedure. After matching against the Army personnel files, the cohorts consist of 1697 individuals with positive urines and 2432 negative urine controls. The rate of matching was slightly over 80% for each cohort.

Progress

Initial demographic characterization of each cohort on age, race, AFQT, education, residence at time of induction and type of service showed minimal differences. The largest difference was in race (14.5% Black for the control and 22.2% Black for the urine positive).

As of the first eight months of service, the two cohorts were similar in terms of changes in grade, assignment and MOS, and in terms of separations. Failure to remain in the Army after eight months is equally and strongly associated in both cohorts with failure to be a high school graduate. Preliminary data indicates that the cohorts have different separation rates by their 18th month in service.

The linkage of the first year medical records from the Individual Patient Data System behavior problem sub-file has been done for these cohorts. The incidence of records for the control cohort shows contacts with the Alcohol and Drug Abuse Prevention and Control program to be in excess of 30 per 1000 for the first year and the rate for "mental diagnosis" to be in excess of 20 per 1000 in the first year. The analysis of the positive cohort is in progress. It maybe noted that less than 50% of the positives have an IPDS medical record within two months of their positive urine. The collection, arraying and analysis of data in this study will continue throughout the coming fiscal year.

5. Social Adjustment and Multiple Interactive Determinants of Stress Response

Military psychiatry has maintained a longstanding interest in and commitment to stress research.[6,7] Such research has demonstrated that it is necessary to consider multiple determinants of stress response.[8-10] The symptomatic responses to stressful events have integral relationships to both the social milieu in which an individual experiences a stressful event and the "coping style" of the individual, i.e. his or her pattern of response to such events. At present we lack systematic knowledge of the interaction of these determinants. This is particularly true insofar as our understanding of how the social milieu organizes, shapes and reinforces responses to stressful events and the coping styles of the individual respondees. This study, presently being initiated, addresses these issues utilizing a population undergoing chronic stress, i.e., renal transplant and hemodialysis patients as well as selected control groups. The goal of the investigators is the construction of a set of multiplicatively determined interactive models that will further our understanding of the relationships involved between the various factors involved in stress response. The primary patient population involved will be renal dialysis and transplant patients at WRAMC. Consideration will be given to detailing the events experienced by the subject and the symptomatic expression of stress within the population. Asesssment of the coping style of the individual and of the social supports and patterns to the individual will be undertaken. Assessment of these parameters will be made over approximately one year and final analysis will be made in terms of outcome of treatment and social adjustment. This project has been approved by WRAMC and awaits approval by WRAIR.

Progress

The basic bibliographic work, choice design and instruments to be used and the design of the research project have been completed. The project has been approved by WRAMC and the patient populations required as subjects will be made available. It is anticipated that actual work will begin in the first part of the next fiscal year contingent upon final approval of the protocol by the WRAIR.

6. Demographic Analysis of the WRGH Patient Population

Description

This study, being carried out with the collaboration of WRGH will analyze the demographic characteristics of all psychiatric patients admitted to Walter Reed General Hospital during the period 1973-74. From this data inferences may be drawn as to those demographic factors which place a soldier at higher risk to be a psychiatric casualty within the WRGH catchment area.

Progress

The Department of Psychiatry, WRGH, has provided the investigators with a summary of the COMPSY demographic data for the period in question. The arraying of this data is now under way as is a literature review and bibliographic studies.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

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2. Rothberg, J.M.: A Note on Alcoholism. New England J. of Med. 292:1137, 1975.

3. Zung, W.W.K.; Gianturco, D.; Pfeiffer, E.; Wang, H.; Whanger, A.; Bridge, T.P.; Potkin, S.G.: Pharmacology of Depression in the Aged: of gerovital-H-3 as an antidepressant. Psychosomatics Vol 15, 1974.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6456	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A823	00	031
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code): (U) Military Performance and Stress: Factors Leading to Decrements of Performance & Disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 016200 Stress Physiology 013400 Psychology 009400 Man-Machine Relat

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 07	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 75	3.5	138
CURRENT FISCAL YEAR 76	3	215

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foregin intelligence not considered

22. KEYWORDS (Provide EACH with Security Classification Code): (U) Electrophysiology; (U) Biorhythms; (U) Psychophysiology; (U) Operant Conditioning; (U) Stress; (U) Performance; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number, precede text of each with Security Classification Code.)

23. (U) Stressful environments, physiological conditions and performance demands likely to produce significant deterioration in the accomplishment of a soldier's mission are studied. The behavioral and physiological functions that contribute to deteriorated performance are identified and therapeutic and prophylactic strategies are developed.

24. (U) Using psychophysiological and operant methodology, time series analysis, and computer-based control and analysis techniques, behavioral and physiological events are isolated, analyzed, and controlled. Endogenous and exogenous factors contributing to behavioral and physiological rhythmicity and performance levels are studied under specified normal and stressful conditions.

25. (U) 74 Ø7 - 75 Ø6 Choice reaction times under varying levels of mild alcohol intoxication have been investigated yielding information on the influence of alcohol on the trading of accuracy of response when speed of response is controlled. Preliminary work on speech recognition performance under conditions of stress has begun. Controlled environment chambers for continuous performance studies have been installed and are being instrumented. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 74 - 3Ø JUN 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A823 MILITARY PSYCHIATRY

Task ØØ Military Psychiatry

Work Unit Ø31 Military performance and stress: Factors leading to decrements of performance and disease

Investigators.
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Description

The elucidation of the biological substrates of stress and performance decrements is important both to military psychiatry and to the performance of normal military missions. The basic research strategy of this work unit is psychophysiological in nature, i.e., concurrent measures of behavioral processes and physiological activity are made. Special attention is paid to stressors having their origin in continuous performance requirements, sleep deprivation, temporal disorientation, and drug use. Due recognition is given to the fact that performance is not a unitary construct, but a continuum of human activity ranging from simple motor behavior to the most complex cognitive activity. Research is directed at the experimental delineation of interactions between stressors and complex performance that are functional analogs of militarily relevant activities. These include vigilance, the integration of multiple sources of information, and decision processes. When necessary for scientific clarity, complex performances are analyzed in terms of more basic processes involving sensorimotor, attentional and mnemonic components.

Progress

1. Speed-Accuracy Trade-Off Functions in Choice Reaction Time Experiments

Choice reaction time (RT) experiments are useful for investigating the effects of stress on human performance in a number of respects. First, reaction time is an important component of many types of skilled performance. Second, by varying the complexity of the decisions required, choice RT experiments can provide sensitive measures of a wide variety of information-processing capabilities. Finally, appropriately designed RT experiments also permit inferences to be made concerning specific components of performance which may be disrupted by stress or other variables (18). Detailed information about which component or components or performance are most severely disrupted by a given type of stress and which components are relatively unaffected will

significantly enhance our understanding of stress-performance relationships.

One potential difficulty associated with choice RT experiments is the problem of trade-offs between speed and accuracy of performance. The term speed-accuracy trade-off refers to the observation that subjects are capable of trading speed for accuracy and vice versa over a wide range in choice RT experiments (11, 19). For example, when rewarded primarily for speed subjects have been found to decrease their mean RT, but only at the cost of concomitant increases in error rate. Therefore, unassessed changes in subjects' bias for speed versus accuracy may obscure the effects of stress and other independent variables.

A recently suggested solution to the problem of speed-accuracy trade-offs in choice RT experiments is the use of complete speed-accuracy trade-off functions as primary dependent variables instead of a single mean RT and error rate (11, 14, 20). In the past year we have investigated experimental procedures for generating empirical speed-accuracy trade-off functions. Two major classes of experimental design and two major computational procedures have been identified and evaluated using both experimental and computer-simulated data. Although all available procedures for generating speed-accuracy trade-off functions involve empirically untested assumptions, one procedure requires fewer such assumptions and is less sensitive to sources of experimental and statistical error. This procedure involves plotting average accuracy against average RT over a set of experimental conditions designed to manipulate subjects' bias for speed versus accuracy systematically over a wide range.

A paper describing this work has been submitted for publication. We are continuing to investigate alternative procedures for manipulating subjects' bias for speed versus accuracy in order to determine an optimal procedure which interacts minimally with the primary choice RT task.

2. Effects of Graded Doses of Alcohol on Speed-Accuracy Trade-Off in Choice Reaction Time

In partial response to the reported widespread use of alcohol in the Army (2), we have instituted work studying the effects of relatively low doses of alcohol on performance. As part of this project, the speed-accuracy trade-off methodology described in the preceding section has been used to clarify two long-standing issues concerning the effects of alcohol on choice RT performance. The first issue is whether alcohol in moderate doses (i.e., doses near the threshold for legal intoxication) produces any detectable deficit in choice RT performance. Despite numerous experiments which report increases in choice RT at moderate alcohol doses, other experiments have reported no significant effect of similar doses on choice RT (3, 7, 8, 15).

The second issue concerns the precise relationship between alcohol dose and the magnitude of the obtained deficit in choice RT performance. Such deficits have not been commonly observed at blood alcohol concentrations less than 80 mg% (mg per 100 ml of blood) (3, 17), and some investigators have suggested that lower doses may even facilitate performance in tasks such as choice RT (4, 22). Investigation of a range of low to moderate doses is necessary to determine the relation between alcohol dose and choice RT performance.

Part of the ambiguity concerning the effects of alcohol on choice RT may involve unassessed changes in subjects' bias for speed versus accuracy as discussed in the preceding section. For example, subjects might maintain a roughly constant average RT under alcohol by allowing themselves to make slightly more errors than in a control condition. The use of complete speed-accuracy trade-off functions permits changes in trade-off bias for speed versus accuracy to be distinguished empirically from changes in performance efficiency. Changes only in trade-off bias between experimental conditions are reflected in shifts in performance along a single speed-accuracy trade-off function. In contrast, a change in performance efficiency is demonstrated by a shift in performance from one speed-accuracy trade-off function to another.

Speed-accuracy trade-off functions were computed for each of five alcohol doses (0, .33, .66, 1.00, and 1.33 ml/kg body weight, respectively). Each of five subjects received each dose on a separate day in an order specified by a 5 x 5 latin square. A double-blind procedure was used so that neither the experimenter nor the subjects was aware of the dose being administered. An RT-deadline procedure with five deadline intervals (175, 225, 275, 325, and 375 msec, respectively) was used to manipulate subjects' bias for speed versus accuracy (5, 13).

The effects of alcohol on speed-accuracy trade-off functions are illustrated in Figure 1, which compares trade-off functions from the placebo and 1.33 ml/kg doses for a single subject. Each data point represents the joint RT and accuracy (information transmitted) values for a single deadline interval in each dose condition. The solid and dotted lines represent the best-fitting linear equation for each dose according to a least-squares criterion. The data in Figure 1 suggest that alcohol reduced the slope of the speed-accuracy trade-off function. This suggestion was verified statistically in the data across all subjects. Increasing doses of alcohol produced a progressive decrease in the slope parameter of the linear trade-off equations but had no significant effect on the intercept of the functions with the RT axis at zero accuracy. These results suggest that alcohol reduced the rate of growth of accuracy per unit time (i.e., the slope), but did not significantly affect the point in time at which such growth began (i.e., the intercept).

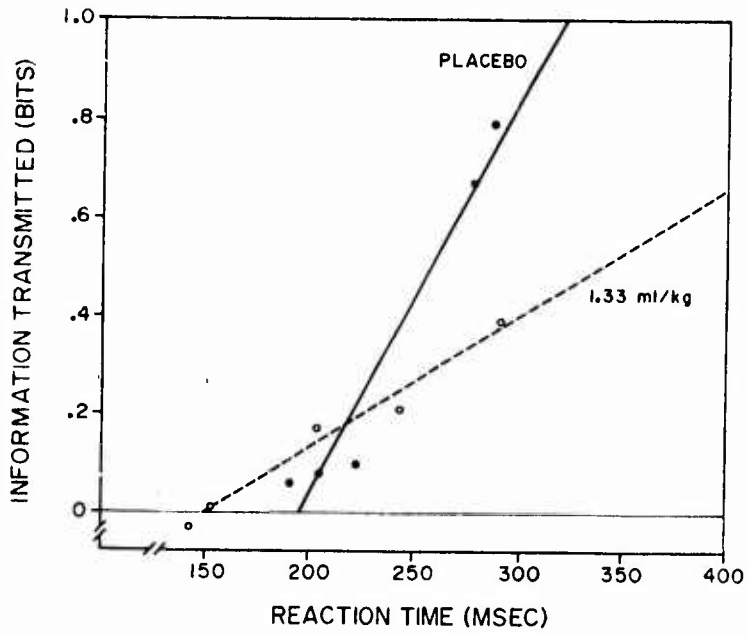

Figure 1. Illustrative speed-accuracy trade-off functions for the placebo and 1.33 ml/kg alcohol doses.

In addition to the changes in performance efficiency just described, alcohol also produced changes in subjects' trade-off bias for speed versus accuracy. Evidence for this effect was most obvious at the 1.33 ml/kg dose, where performance was least efficient according to the trade-off functions but where mean RT was faster than at any other dose. This result may reflect either a direct effect of alcohol on trade-off bias or an interaction of alcohol with the specific demands of the deadline procedure.

In summary, alcohol in the dose range from 0-1.33 ml/kg produced changes in both performance efficiency and trade-off bias for speed versus accuracy. Increasing alcohol doses produced a progressive decrease in the slope of linear equations fit to the speed-accuracy data but did not significantly alter the intercept of the functions with the RT axis. Thus, alcohol reduced performance efficiency by decreasing the rate of growth of accuracy per unit time, but did not significantly affect the point in time at which such growth began. A change in trade-off bias toward increased speed and decreased accuracy was combined with the decrease in efficiency at the highest alcohol dose. These results have implications for military tasks in which fixed levels of both accuracy and speed must be maintained. Under alcohol a man will not be able to maintain both speed and accuracy in such tasks, although he may well attempt to maintain speed and in so doing commit errors.

A paper based on these results has been submitted for publication. Additional work is planned to assess the specific psychological nature of the decrements in performance due to relatively low doses of alcohol.

3. Effects of Multiple Task Demands on Attention, Memory, and Autonomic Reactivity

A common stressor, which frequently produces decrements in performance, is the requirement to perform two or more tasks at the same time. Situational factors and our ability to allocate our attention determine whether all tasks show a decrement, whether performance on one is maintained, or some other hierarchy of tasks performed is maintained. The situation in which one task is primary and one secondary provides a means of studying the allocation of performance resources when faced with concurrent tasks. In an experiment recently completed, a serial anticipation memory task was the primary task and a simple reaction time task was the secondary task. Performance of the serial memory task was relatively uninfluenced by the concurrent reaction time task. The reaction time performance, however, closely mirrored the different phases of the serial memory task. Reaction times were maximally slowed relative to a control during the recall of items. The initial learning of the items also produced significant slowing of the probe reaction times. A detailed analysis of the temporal location of the reaction signals leading to the slowest reactions showed maximal slowing to occur when reaction signals were simultaneous with presentation of an item to be learned or recalled.

These results were interpreted in terms of selective attention within a limited capacity information processing organism (1, 10). The relative sensitivity of this task to changes in attention suggests its utility for the study of autonomic correlates of information processing. Such a study following up current work in press (9) is currently planned, as is work directed at examining the influence of alcohol on such concurrent tasks.

4. Cardiac Correlates of Performance Within a Fire Direction Center (FDC)

In collaboration with USARIEM, Natick, Mass. a FDC team was observed over multiple day sessions at sea level and altitude. The FDC team was engaged in continuous, simulated combat type problems during the sessions. Electrocardiograms were recorded by telemetry from each of the six individuals on the team. Heart rate data are currently being analyzed to determine whether any changes due to the prolonged simulated combat situation occurred in biological rhythms or in responses to brief stressors. Results obtained at sea level will then be compared to those found at 14,000 feet of simulated altitude.

5. Sensitivity and Response Bias in Speech Discrimination

A central theme in our investigations of stress and human performance is the distinction between sensitivity and response bias in tasks requiring decision and choice among alternative stimulus events. Evidence from a wide variety of experiments has demonstrated that factors related to an individual's expectations, motivations, and strategies can significantly influence performance in choice and decision tasks (1, 6, 21). One example of response bias effects was described in the alcohol-choice RT experiment above. As another example, consider how the values and costs for different types of errors might affect an individual attempting to discriminate between two alternative stimulus events. If the cost for incorrectly reporting alternative 1 were high relative to incorrectly reporting alternative 2, then alternative 1 would be reported only when the evidence is strongly in its favor. The degree to which a given stressor acts on performance primarily via response bias, sensitivity, or both has direct implications for understanding mechanisms of performance decrement and possible means for performance enhancement.

Due to the obvious importance of verbal communication in military performance, a series of experiments has been designed to investigate the roles of sensitivity and reponse bias in speech recognition performance. In the first experiment in this series, the ability to classify speech stimuli into phonetic categories has been investigated. The stimuli for this experiment were computer-generated synthetic syllables which constituted a continuum for one phoneme category to another. Subjects were presented pairs of stimuli which were either

identical or adjacent stimuli along this continuum and were required to discriminate between them in a "same-different" judgment task. An important finding from previous experiments (16, 12) was verified; namely, that discrimination was more accurate at the boundary between phoneme categories than within either category. However, the present experiment demonstrated further that the superiority of discrimination of the phoneme boundary was associated with large changes in subjects' response bias as well as with an increase in sensitivity. By demonstrating that response bias factors may influence speech recognition performance at the level of phoneme categorization, these results imply that response bias factors may be even more important in tasks involving comprehension and understanding of meaningful discourse. Additional experiments are planned to determine the degree to which the changes in sensitivity and response bias in speech discrimination may be altered experimentally by variables such as selective adaptation and extended practice.

6. Measurement Technology for Assessing the Effects of State Variables.

In the preceding year the department established and equipped a small animal laboratory for research where the use of humans would be uneconomical, impractical or unethical. The laboratory is now in operation and a first study is underway to test techniques designed to improve traditional [illegible]res for measuring the psychophysiological effects of state variab[illegible]riables that produce a change in the physiological state of t[illegible]ganism, which in turn may modify behavior, e.g., drugs, stress, disease, etc.).

The present study compares alternative methods for measuring the timing behavior changes that result from many psychoactive agents. Timing behavior (time estimation, time perception, temporal discrimination) is chosen since it is an important component of most behaviors, is sensitive to many environmental and physiological factors, and yields orderly results that generalize across species.

The traditional procedures for establishing and monitoring a temporal discrimination is the Differential Reinforcement of Low rates (DRL), a procedure in which an animal is trained to make responses at some nominally constant rate. Responses spaced more than t seconds apart are reinforced with a food pellet, while shorter inter-response times (IRTs) are not. This procedure generates a distribution of inter-response times clustered about the criterion value. The mean of this distribution is generally decreased by stimulants and increased by depressants, relative to its baseline value.

The DRL procedure is effective but has two undersirable features. First; it confounds timing responses with the non-negligible time required to retrieve and consume a food pellet. This contamination is asymetric since it occurs only for the reinforced responses, the

proportion of which is controlled by the subject not the experimenter. This artifact leads to several analytical problems and reduces both the sensitivity and face validity of the procedure. The second undesirable feature of DRL is that it generates a number of extra-long inter-response times that are off the distribution. The arithmetic mean weights these large values disproportionately and their effect is to constrain or conceal small changes in the mean while distorting its absolute value.

Both of these faults can be corrected by differentially reinforcing response duration instead of inter-response times. This alternative procedure (DRD) separates eating-time from the response measure and does not generate extra-long durations. An added advantage of DRD is that it still allows IRTs to be measured, independently of duration, yielding a bonus datum for distinguishing between rate and accuracy.

The initial comparison of the two procedures has shown the baseline duration distributions generated by DRD to be more peaked and narrow than IRT distributions of an equivalent-valued DRL. If this result holds across different interval lengths it should allow smaller effects to be detected with greater reliability. The next step planned is to compare the two procedures in terms of their relative sensitivity to standard dosages of stimulants and depressants.

Project 3A762758A823 MILITARY PSYCHIATRY

Task ØØ Military Psychiatry

Work Unit ØØ1 Military performance and stress: Factors leading to decrements of performance and disease

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6438	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A823	00	033
b. CONTRIBUTING				
c. [illegible]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Anatomical and Physiolcgical Correlates of Brain Function in Stress and Disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002400 Bioengineering 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 75	6	178
CURRENT FISCAL YEAR 76	5	587

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Neurophysiology; (U) Neuropsychiatry; (U) Neuroanatomy (U) Combat Shock; (U) Autonomic Nervous System; (U) Biomedical Engineering; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The principal research objectives are to identify the anatomical and physiological mechanisms by which the central nervous system maintains vital functions during or following physiological stress, disease or trauma in the military environment.

24. (U) The disciplines and techniques of neuroanatomy, neurophysiology, physiological psychology and neurochemistry are used.

25. (U) 74 07 - 75 06 Anatomical and physiological analyses of central nervous system mechanisms subserving muscular coordination, posture, and movement have been studied. These studies attempt to understand the role of the nervous system in recovery of function following traumatic injury or disease. A morphological basis for proposed neuroendocrine functions of the habenular nuclei was examined. Thermoregulatory functions of the hypothalamus were studied in cases following the administration of a presumed pyrogen isolated from Penicillum gilmanis. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

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Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 033 Anatomical and physiological correlates of brain function in stress and disease

Investigators.
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DESCRIPTION.

The research program of the Department of Neurophysiology attempts to: (1) provide fundamental neuroanatomical and physiological information, both basic and applied, regarding behavioral functions of the limbic system and the physiological and behavioral functions of the autonomic nervous system acting to integrate and regulate the body's vital functions, particularly as these functions may be related to the medical, surgical or psychiatric care of military patients; (2) define the environmental or physiological circumstances contributing to or causing trauma, stress, disease, and shock; (3) suggest applied methods of corrective therapy for stress avoidance and recovery from diseases and surgical or medical shock. These studies have applied physiological, neurological, and neurosurgical implications.

The knowledge and research methods of neuroanatomy, electron microscopy, neurophysiology, neuroendocrinology, physiology, tissue culture, and experimental psychology are utilized in the department's studies. In some cases the expertise of one discipline is applied to a particular research task, but in most cases, multidisciplinary approaches are utilized to study stress. The following problem areas are under study, or have been completed this year:

(1) Spinocerebellar projections;
(2) The fine structure of the nucleus cervalis centralis;
(3) The extracellular connective tissue in brain perivascular space;
(4) Epithalamic supraependymal axons: effects of neurotoxic agents and brain lesions;
(5) Functions of single neurons in the sensory-motor cortex;

(6) Systems analysis of neuron populations;
(7) Recovery of motor function after spinal injury;
(8) Recovery of function after dorsal rhizotomy: corticospinal linkage to motoneurons;
(9) Relationship between activity of cells of the vestibular nuclei and conditioned movements in cats;
(10) Synaptic transmission in the vestibular nuclei;
(11) Temperature regulation: Gilmandiantriol, a new low molecular weight putative pyrogen;
(12) Biomedical engineering and electronics in support of basic and applied research in military medicine: new instrumentation.

PROGRESS.

Spinocerebellar Projections.

Studies are continuing of relationships between spinal cord and cerebellum, with a view to establishing a better understanding of the anatomic and physiologic substrates of control of fine movement in mammals, including man.

Classically two spinocerebellar tracts have been recognized. The origin of the dorsal spinocerebellar tract has long been regarded as the n. dorsalis or column of Clarke. The origin of the ventral spinocerebellar tract has been controversial. Cooper and Sherrington (1940) claimed that large multipolar neurons of the lumbosacral spinal cord gave rise to this tract. Sprague (1951, 1953), on the other hand, disputed this contention and instead suggested that nucleus pericornualis ventralis neurons were propriospinal cells. Hubbard and Oscarrson (1962) reported a population of dorsal horn and zona intermedia neurons in the L4 and L5 segments which contributed to the ventral pathway. In contrast to the dorsal pathway, the ventral tract mediates signals from receptors located in tendons of muscles acting across different joints rather than signals from single muscle spindle organs.

Oscarrson (1964) has also reported finding cells in the cervical spinal cord which project to the cerebellum. He suggests that these neurons are physiologically equivalent to the ventral spinocerebellar tract of lumbosacral segments. The location of these cells is unknown.

There remain many unanswered questions regarding the cells of origin for the rostral spinocerebellar, the ventral spinocerebellar and the dorsal spinocerebellar tracts. This study was undertaken in order to identify the nuclei of origin for these tracts and to determine the laterality of these pathways. Anatomical identification of the nuclear groups involved may enable further physiological characterization of

spinocerebellar cell groups. Based upon evidence from prior studies in this laboratory there are reasons to believe that besides the column of Clarke and the nucleus pericornualis ventralis, there are several additional cell groups of the spinal cord which project to the cerebellum.

The nuclei of origin for the spinocerebellar projections have been studied. Cerebellectomy was performed by aspiration in anesthetized neonatal dogs. After 10 - 16 days survival, the aninals were euthanatized and perfused with physiologic saline solution followed by 10% formalin. The brain stems were cut transversely. Spinal segments were cut in either the transverse or horizontal planes. Cerebellectomy provoked striking changes in neurons of the cervical spinal cord. Large numbers of these reactive neurons occurred in the nucleus cervicalis centralis and the nucleus centrobasalis. The central cervical nucleus, identified by Rexed (1954), contained large chromatophilic neurons. These were aggregated in the centromedial part of the zona intermedia and extended from the C1 to the C4 segments. The retrograde changes observed in the nucleus centrobasalis occurred in the largest cells at the base of the dorsal horn. Reactive centrobasaliar neurons were most numerous in the caudal cervical segments. Petras (1966) demonstrated that both the central cervical and centrobasilar nuclei received numerous afferent dorsal root fiber terminations. The results of C1 hemicordotomy suggested that the central cervical nucleus gives rise to predominantly contralateral projection to the cerebellum, while the projection from the centrobasilar nucleus is mainly ipsilateral. Cerebellectomy failed to provoke changes in the nucleus cervicalis lateralis, although the same lesion produced clear-cut changes in the cranial portion of the nucleus dorsalis (Clarke's column).

The results of these experiments were compared with the results in experimental animals in which the retrograde horseradish perosidase tracing method was utilized. The enzyme was injected into the cerebellum. It was incorporated at the terminal site of spinocerebellar axons and transported in a retrograde direction, toward the cell somata. The results of these experiments confirmed previous findings using the retrograde chromatolytic method.

The Fine Structure of the Nucleus Cervicalis Centralis.

A cytoarchitectonic description of this nucleus in the cat was accompanied by an electron microscopic study of the nuclear neuropil in normal cats. Dorsal rhizotomy was performed in additional cats and survival times range between 2 to 5 days. The appearance of normal boutons were compared with early reactive boutons with the object of identifying a specific boutonal type with dorsal root afferent

fibers known to synapse in the nucleus. Three boutonal types have been identified. They occur as asymmetric synapses (1) with flattened vesicles, (2) round vesicles and (3) pleomorphic vesicles. Large darkened boutonal profiles were seen. These commonly contain flattened vesicles. Degenerated boutons range in density from dark to blackened profiles, and sometimes contain large membrane-bound flocculent debris or membranous whorls. Boutons in advanced stages of degeneration contain such a dense cytoplasmic matrix that details of structure cannot be seen.

Degenerated boutons were found in axodendritic, and more rarely in axosomatic relationship with nucleus cervicalis centralis neurons. When degenerated axodendritic boutons were found they appeared to appose a concave profile along the "receptor" surface of the dendrite. Normal boutons were in synaptic contact with dendrites presenting a rounded symmetric appearance.

The Extracellular Connective Tissue in Brain Perivascular Space: Differences in Two Strains of Rats.

The presence of extracellular perivascular connective tissue space in the medial terminal nucleus (MTN) of the accessory optic system in rats has recently been reported (Tigges, M. and Tigges, J. 1972). Except for capillaries in specialized neuroendocrine areas (i.e. median eminence, neurohypophysis, epiphysis, etc), central nervous system capillaries lack extracellular connective tissue in their perivascular spaces. Therefore, an implication of this finding was that the MTN may be involved in neurosecretory function. Because of this, we decided to investigate its occurrence in suspected neuroendocrine regions such as the medial habenular nucleus.

Eighteen Sprague-Dawley adult rats of both sexes ranging in body weight from 150-350 gms and 15 Wistar-derived adult male rats ranging from 160-380 gms body weight were used in this study. The brain regions sampled were: medial and lateral habenular nuclei, interpeduncular nucleus, and dorsomedial nucleus of thalamus.

Examination of tissues under the electron microscope revealed that extensive extracellular perivascular connective tissue in all areas observed. The connective tissue was located between the external and internal basal laminae and was characterized by collagen fibrils and pericytic cell processes. The same brain nuclei in the Spraque-Dawley strain lacked connective tissue between both laminae. The endothelia in both strains were not fenestrated.

Since the basal laminae of capillaries are susceptible to pathological changes, it is important to determine the occurrence of con-

nective tissue in rat brain capillaries prior to experimental manipulations. Our observations suggest that the presence or absence of this type connective tissue in rat CNS capillaries is strain-dependent.

Epithalamic Supraependymal Axons: Effects of Neurotoxic Agents and Brain Lesions.

The habenular complex is said to contain an intrinsic serotonergic system referred to as "epithalamic short indoleamine neurons" (Bjorklund et al, 1973) which may have an endocrine function. A collaborative project between our laboratory and Dr. Juan Saavedra, Laboratory Clinical Science, NIMH, Bethesda, was developed. The serotonin content of the habenular complex was studied before and after intraventricular administration of hydroxylated tryptamines. The serotonin content was measured by a sensitive enzymatic-radioisotopic assay (Saavedra, 1974). The serotonin content of the treated animals was reduced to 50% of that of the controls.

Ultrastructural studies have established the presence of varicose nerve fibers on the ventricular border of the ependyma. Combined cytochemistry and pharmacology have strongly suggested that an indoleamine, most likely 5-hydroxytryptamine, is contained in these terminals.

Since dihydroxylated tryptamines (DHT) and 6-hydroxydopamine (6-OH-DA) are selectively taken up by their respective terminals, we undertook a combined morphologic-pharmacologic study to determine the effects of these agents on epithalamic supra-ependymal nerves. The following injections were made bilaterally into the lateral ventricles of adult male rats: 50 µg 5,6-DHT; 50 µg 5,7-DHT (pretreated with 25 mg/kg DMI, i. p.); and 200 mg 6-OH-DA. Sham-injected and untreated animals served as controls. Scanning and transmission electron microscopy of the ependymal surface of the habenular region failed to reveal the presence of supra-ependymal fibers after treatment with DHT. These nerves were present in 6-OH-DA-treated, sham-injected and untreated animals. Tractotomy and electrolytic lesions of the habenulo-interpeduncular tract (which includes indoleaminergic fibers from raphé) resulted in the disappearance of the supra-ependymal fibers. On the other hand, lesions of the stria medullaris thalami (which also contains indoleaminergic fibers from raphé) had no effect.

Our findings provide additional support for the serotonergic nature of these fibers, which may have their origins in cells of the raphé nuclei.

Functions of Single Neurons in the Sensory-Motor Cortex.

Collaborative work with Dr. A. L. Towe (University of Washing-

ton) has continued on the neuronal circuitry of the somatic sensory-motor cortex of the cat. Current efforts involve computer analyses of 4500 individual neurons studied electrophysiologically in this tissue (a major supraspinal center for both limb movement and limb sensation). Work during the past year has centered on showing that the cortical cells of origin of the pyramidal/corticospinal tract -- cells which have extensive, apparently non-selective input sources -- are in fact subject to highly selective control, that this control is activated by stimulation of restricted areas of skin, and that it is probably mediated through cortical neurons with small peripheral receptive fields. Loss of this controlling input, which is normally superimposed on the underlying less-organized input to these motor cells, could result in major disorganization of limb movement but probably would not produce paralysis; such a mechanism could possibly account for some symptoms seen in human patients who suffer loss of oxygen supply to the cerebrum through birth trauma, cerebrovascular accident, etc. A manuscript on this work has been submitted to Experimental Neurology: Facilitatory and Inhibitory Modulation of Wide-Field Neuron Activity in Post-Cruciate Cerebral Cortex of the Domestic Cat. (A. L. Towe, C.F. Tyner, and J.K. Nyquist; 1975).

Systems Analysis of Neuron Populations.

During the past year a first stage has been reached in examining some theoretical problems in systems analysis: how does one study an extremely large collection of interacting elements when available technology generally allows the elements to be examined only one at a time and an exhaustive survey is impossible? This issue has been considered in terms of the vertebrate nervous system and the cells which comprise it; a first conclusion is that the strategies developed by taxonomists for studying natural populations of flora and fauna may be applicable to both experiment design and data analysis for single brain cells. A manuscript on this work has been submitted to Brain, Behavior, and Evolution: The Naming of Neurons: Applications of Taxonomic Theory to the Study of Cellular Populations. (C.F. Tyner; 1975).

Recovery of Motor Function After Spinal Injury.

Studies of motor behavior recovery in monkeys following brain injury (surgical descruction of the dorsal root input from a limb) have progressed satisfactorily. A chair in which a monkey can be trained to flex its arm to lift a weight is in regular use to compare the performance of normal and deafferented animals: following an auditory warning, the animal must quickly lift its arm to a minimum height and then return to rest to receive a juice reward. First results showed that the post-operative monkey could flex its arm in a fashion

generally similar to that of normal aminals, but that on repeated trials, the post-operative animal lifted its arm to rather variable heights above the required miminum while the normals lifted their arms to much less variable heights above the minimum.

Recovery of Function After Section of Dorsal Roots of the Spinal Cord: Corticospinal Linkage to Motoneurons.

Injuries of the central nervous system are frequently accompanied by disturbances of motor control. Depending upon the location and extent of the injury, different degrees of recovery may be observed following the injury. Experimentally, severe motor impairment of a single limb can be created by sectioning the dorsal roots which carry sensory information from that limb to the central nervous system. Recovery of function can be elicited by appropriate procedures. We have hypothesized that recovery of function after deafferentation of a limb by dorsal rhizotomy depends in part upon a change in the physiology of synaptic transmission between the corticospinal tract and motoneurons. Preliminary data described in the previous annual report (1974) indicates that transmission across this synapse in the chronically deafferented animal is at least as powerful as that in the normal animal, despite the absence of facilitating influences from the deafferented limb. One corollary of this hypothesis is that immediately after dorsal rhizotomy, transmission at the synapse should be reduced.

Our own observations as well as those of other workers in the field indicate that immediately after dorsal rhizotomy, the denervated limb lacks muscle tone. The contrast in muscle tone between the normal and deafferented limb is striking even as the animal is recovering from anaesthetic and persists for at least several days after the operation. This suggests a marked reduction in the average firing frequency of the motoneurons ennervating the muscles of that limb. We have begun an attempt to create a physiological model of this loss of tone which would also allow us to study transmission across the synapse between corticospinal fibers and motoneurons in normal animals, in animals immediately after deafferentation, and in animals after recovery from deafferentation.

In one experiment thus far, in an acute preparation under barbiturate anaesthesia, we have recorded resting muscle tension and twitch tension evoked by electrical stimulation of the rostral spinal cord with needle electrodes placed in the region of the corticospinal tract. After obtaining data from the intact monkey, we unilaterally cut all dorsal roots from C2 through T3, a procedure that should have abolished all sensory inputs through the dorsal roots from the limb from which we were recording muscle tension. We then repeated our measurements of resting tension and evoked twitch tension. Although we have not

yet completed analysis of this data, at the time of the experiment we observed no gross changes in muscle tension consequent to the acute rhizotomy. Inasmuch as the levels of anaesthesia we used rendered all limbs flacid, our data may indicate that the anaesthetized animal is an inappropriate model for testing our hypothesis. Once the analysis of the data from this experiment is complete, we will reassess both our hypothesis and our methodological procedure.

Relationship Between Activity of Cells of the Vestibular Nuclei and Conditioned Movements in Cats.

The role of the various descending systems of the brain in the control of movement and in recovery of motor mechanisms after injury to the nervous system are only imperfectly understood. Fibers originating from cells in the vestibular complex of the brainstem constitute one of the major descending pathways. Although the role of these vestibular pathways in mediation of vestibular reflexes has received considerable attention, little thought has been given to the possibility that these pathways may also play an important part in the mediation of purposeful movements. Currently under development is the methodology to test the hypothesis that neurons of the vestibular complex are concerned with the execution of purposeful movements as well as with vestibular reflexes. The hypothesis was generated by: (a) anatomical studies indicating that certain of the vestibular nuclei are closely linked to the spinal cord and receive inputs from various non-vestibular regions of the brain (Brodel, 1969); (b) physiological studies indicating that sensory inputs from the limbs themselves influence the activity of cells in the vestibular nuclei (Wilson et al., 1967a, 1967b, 1968; Wylie and Folpel, 1971), and that electrical stimulation of somatosensory cortex influences the activity of cells in the vestibular nuclei; (Gildenberg, and Hassler, 1971) and finally, (d) studies in decerebrate cats who display ambulatory movements have shown that cells in the vestibular nuclei discharge in phasic relationship to the movements (Orlovsky, 1972).

We are training cats to stand with each foot on one of four platforms. Vibration applied to any platform is the signal that the cat should raise the corresponding foot to touch a panel, and then return the foot to its resting position upon the platform. Correct performance of this conditioned response is rewarded with a squirt of a liquid food. Once training is complete, a plug will be mounted on the skull covering a craniotomy to be made over the cerebellum. The skull plug will allow the sterotaxic introduction of recording microelectrodes into the vestibular nuclei. At the same time, a device will be mounted on the skull to allow painless fixation of the head. After post-operative recovery we will record the activity of single cells in the vestibular nuclei while the cat performs the appropriate motor

responses. By conditioning movements of each of the four limbs, we will test the initial hypothesis, and, if the hypothesis is correct, also determine whether the activity of single cells is related only to the movement of a single limb or to the movements of several limbs. With the help of the Division of Instrumentation, we have developed a skull plug which we can stereotaxically implant, and which will serve as the primary guide for the positioning of microelectrodes in the vestibular nuclei. We have successfully used this skull plug to record from single cells in anaesthetized, acute preparations. By marking the position of the microelectrodes in the brain in acute preparations and reconstructing the tracts from the post-mortem histological material we have worked out an approach to the vestibular nuclei that avoids the teutorium, major venous sinuses and major cerebellar vessels.

Synaptic Transmission in the Vestibular Nuclei.

Recent work has shown that destruction of at least certain neural pathways is followed not only by the classically described degeneration of the terminals of those pathways but by invasion of the target cells of these degenerated terminals by terminals of other pathways which, previous to the lesion, did not occupy those target sites. This invasion of recently vacated synaptic sites by otherwise "foreign" terminals may play an important role in the recovery of function after injury to the central nervous system. The invasion also implies the existence of an unknown means of communication whereby the previously "foreign" pathways are stimulated to develop new connections with the synaptic sites left vacant by the degeneration of the terminals of the lesioned pathway. The importance of understanding the ways in which information can be transmitted between neurons lends new interest to the study of electrical coupling between neurons in the mammalian central nervous system. Electrical coupling implies the presence of a low resistance pathway between cells. Anatomical evidence for this low resistance pathway has been described in some systems; in addition the transfer of molecules with molecular weights as high as 600 from the interior of one cell to the interior of another cell has been demonstrated. The anatomical substrate underlying electrical coupling between vestibular afferents and their target cells in the vestibular nuclei (Wylie, 1973) has yet to be identified.

We have begun a study to describe anatomically the terminals of vestibular afferents by labeling them with tritium and searching for the labeled terminals with the electron microscope. After tritiated leucine was injected into the vestibular ganglion of the rat, the presence of the label was observed in the vestibular nuclei and no where else. This has been done using light microscopic autoradiography. These results suggest that the label is picked up by the cell

bodies, incorporated into proteins and transported along the fibers into the vestibular nuclei. Techniques are being worked out which will yield the most efficient labelling and we are beginning to develop the technology requisite for electron microscopic autoradiography. This will enable us to determine whether the label is being transported into the terminals themselves. If the label is found in the terminals, the vestibular terminals can then be distinguished from all other, nonlabeled terminals present in the nuclei. The study of the ultrastructure of vestibular terminals will, make it possible to determine whether or not the gap junctions present in these nuclei are between vestibular afferents and their target cells.

Temperature Regulation: Gilmandianetriol, a New Low Molecular Weight Putative Pyrogen.

When administered intravenously in a dose as small as 1.0 ug/kg, gilmandianentriol induced in most but not all trials, a long lasting fever in the rabbit. This newly discovered substance, with empirical formula $C_{16}H_{20}O_5$, was isolated from Penicillium gilmanis mold by Dr. F. A. H. Rice of the Chemistry Department at American University, might be a chemical component in the genesis of fever. Hence, we examined its effects when administered intravenously to the cat and the rat. Furthermore, gilmandianetriol was micro-injected directly into the anterior preoptic area of the rat's hypothalamus to determine whether it acts in this thermosensitive-thermoregulatory area to alter core temperature.

Ten male albino rats of the Walter Reed strain and two cats were used. Core temperature was recorded either by; 1) inserting a rectal Yellow Springs Instrument temperature probe 6-8 cm into the rectum, or; 2) implanting a temperature sensor in the peritoneal cavity of the rat. The thermistor was connected to a (Yellow Springs) telethermometer and a chart-recorder for obtaining chronic temperature records. Gilmandianetriol was injected into the rat's tail vein in dose levels of 0.75, 1.5, and 3.0 μg/kg. Similiarly, three dose levels ranging from 0.75 to 20.0 μg/kg were injected intravenously in the cat. This substance was applied directly into the rat's hypothalamus in a dose of 10 or 100 nanograms contained in a 1 μl volume of saline.

Unlike the rabbit's response, the intravenous administration of gilmandianetriol failed to induce a fever in any experiment with the rat or cat. On the other hand, a hyperthermic response of 0.5° C or greater was observed following seven of nine intrahypothalamic injections. Although all cerebral injections were directed at the anterior preoptic area of the hypothalamus, the fever generally took more than one hour to develop and lasted more than 2 hours. Therefore, it seems that gilmandianetriol elicits a rise in core temperature by an inter-

mediate mechanism, and not by a direct action on hypothalamic temperature sensitive cells. It is possible that the compound may release endogenous pyrogen following its diencephalic application.

It is important, not only for theoretical understanding, but obviously also for possible future clinical applications, to understand where in the body a specific drug exerts its specific action. Therefore, further work is in progress in an effort to detail the exact locus of action and duration of this compound's hyperthermic effect.

Biomedical Engineering and Electronics in Support of Basic and Applied Research in Military Medicine: New Instrumentation.

Some of the new instrumentation developed this year include the following:

(1) A data accumulating device was completed. It consists of a bank of analog-to-digital converters, a digital buffer and control unit, and a digital tape recorder. It transforms analog data generated during neurophysiologic experiments into digital signals which are recorded on the tape recorder. The analysis is then done at a later date on a computer at the Division of Biometrics.

(2) A thermoregulator for Petri dishes was perfected. The device holds the temperature of a culture dish under the microscope at a constant temperature within 0.2°C. It permits a researcher long observation times and the opportunity to record changes in electrical activity of membranes of cells in culture. A paper on this development is in preparation (Spector, N. H. and M. E. T. Swinnen) and will be published this year in the Handbook of Tissue Culture (Tissue Culture Association, 1975).

(3) In cooperation with the Division of Instrumentation an automatic vial injector was developed. This instrument injects a measured amount of liquid into each one of thirty two test tubes on a carrousel. One of the requirements for the electrical wiring and switching was that the system be compatible with a computer environment, i.e. not to cause any electrical inferference whatsoever. Special spark suppressors were built into the instrument. The finished device will be used at the Department of Clinical Chemistry of the Hospital.

(4) An interface between animal-subject and the computer was built. It transforms lever presses for food, water and brain stimulation into signals compatible with the computer and subsequently converts computer responses into control signals that will activate dispensers for the three above named rewards.

(5) A tactile stimulator for cats was developed. It consists of four vibrators mounted horizontally in a square, such that a cat can be positioned on them, each of its paws on one vibrator. The device will be used for classical Pavlovian behavioral conditioning, in connection with neurophysiological studies of motor function.

(6) An impedance measurement device was built to measure galvanic skin response. The device consists of a classical Wheatstone bridge circuit coupled to an oscilloscope used as a read-out display.

(7) Four digital and two analog training boxes were built. Our goal is to start electronics classes in the fall of 1975. Members of the electronics lab and selected researchers and lab technicians will attend. The training boxes will give the students an opportunity to work out practical problems in analog and digital design of electronic circuits.

(8) During the past fiscal year, a project was started in cooperation with the Division of Biochemistry, WRAIR, to develop a more efficient liquid flow analyzer. The first important step was the development of an electronic "debubbler", a component circuit that electronically, as opposed to the traditional mechanical method, removes the undesirable effects of the air bubble from the graphical read-out. In connection with this project, the Division of Instrumentation built a new, more efficient flow cell. Preliminary results indicate that the speed of sample handling has been tripled, while reagent use has been reduced by a factor of ten. One model is in use at the Clinical Chemistry Lab at the Hospital and six more units are being built. The Division of Biomedical Research at Fort Mead is in the process of building ten more units according to our specifications. Two patents were applied for, one for the Electronic Debubbler Circuit and one for the Improved Flow Cell. A paper on this latest development (A photocolorimeter flow analyzer; M.E.T. Swinnen et al), has been accepted by the Society for Clinical Chemistry and will be presented at their annual meeting in Toronto, Canada from 5 to 10 July 1975.

(9) An aluminum housing has been designed in the Department of Neurophysiology and fabricated by the Division of Instrumentation for adaptation to a commercial, 6-channel slip-ring assembly. A problem encountered in previous models was the generation of recording artifact due to intermittant contact of the slip-rings resulting from the lack of a supporting shaft structure. Incorporation of a slip-ring housing to support the shaft on the new model will vastly improve the recordings and increase the lifetime of future slip-ring assemblies. Multiple electrical leads plus a central cannula for infusion

or withdrawal of liquids from the brain are incorporated in the apparatus.

(10) An improvement in design of implantable temperature (thermistor) electrodes has resulted in a relatively long-lasting lead, with less trauma to the experimental animal. Previous models were short-lived due to ion leakage in the probes. To reduce infection and trauma, the leads are now being enclosed in smaller diameter polyethylene tubing. To alleviate the problem of short circuits, the ends of the tubing are heat-sealed. These have now been kept trouble free for more than two months while sewn in the retroperitoneal cavities, with the recording leads passed subcutaneously to the heads of rats and thence out thru swivel connectors to continuous recording devices.

(11) A commercial, low-noise, coaxial cable and slip-ring device for recording of small amplitude physiological signals in chronic animals was developed, including a counterbalanced multichannel cable and slip-ring carriage. This apparatus resulted in substantial reduction of movement artifact in recordings. Although this system was used for recording the electroencephalogram (EEG) in chronic cats, it is readily adaptable to other biomedical studies requiring electrical artifact free recording conditions. A full description of this (A. T. Pryzbylik and R. C. Howe. A low-noise cable and slip-ring assembly for recording of small amplitude physiological signals in chronic animals) has been submitted for publication.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 033 Anatomical and physiological correlates of brain function in stress and disease

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2. Martin, G. E. and Myers, R. D.: The cerebral release of catecholamines in the rat during the performance of an operant task to obtain food. Proc. Eastern Psychological Assn., 1975. (Abstract)

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A823	00	034
b. CONTRIBUTING				
c. CARDS	CARDS 114F			

11. TITLE (Provide with Security Classification Code): (U) Influence of Stress on Hormone Response, Performance and Emotional Breakdown in Military Personnel

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400 Psychology 016200 Stress Physiology 005900 Environmental Bio

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17. CONTRACT/GRANT
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FISCAL YEAR — PRECEDING	75	4	159
FISCAL YEAR — CURRENT	76	4	302

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

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Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code): (U) Stress; (U) Emotions; (U) Homeostasis; (U) Psychophysiology; (U) Neuroendocrinology; (U) Psychoendocrinology; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Principal objective is to study the integrating influences of the central nervous system in controlling and coordinating the organs of the body and their metabolic functions under environmental and emotional stresses which are likely to produce casualties due to psychiatric or psychosomatic disease in military personnel.

24. (U) This involves measurement of plasma and urinary hormone levels in humans and monkeys in a variety of acute and chronic stress situations. One important conceptual approach is that the organization of endocrine regulation can only be understood by viewing "overall" hormonal balance, or multihormonal patterns. A second approach requires intensive study of individual endocrine systems in order to establish the significance of multihormonal patterns for the individual exposed to stress. Continuous updating of endocrine assay methodology is also required.

25. (U) 74 07 - 75 06 Collaborative studies of physical stress with ARIEM (Natick) have continued. Studies of acute heat exposure in human subjects have shown no increase in plasma or urinary corticosteroid levels, urinary epinephrine or norepinephrine levels, nor in plasma prolactin, thyroxine, TSH or insulin levels even at heat levels elevating body temperature to 100.2°F. These data strongly suggest a need to reexamine the concept of nonspecificity of stress response, as formulated by Selye. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 034 Influence of stress on hormone response, performance and emotional breakdown in military personnel

Investigators.
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Description.

This program is concerned primarily with the role of the central nervous system in the control and especially the coordination of endocrine regulation. As this program has gradually developed, some general concepts have emerged which appear to have major and far-reaching implications for the field of stress research. Included among such concepts which have been particularly important in opening up productive new avenues of stress research are: 1.) The neuroendocrine apparatus represents a third effector system of the brain (along with the autonomic and skeletal-muscular systems) providing sensitive and objective reflections of central integrative processes, such as emotions and psychological defenses, which are of key importance in human performance under stress, but which have so far been extraordinarily difficult to bring under rigorous experimental investigation. 2.) There is now serious doubt concerning the validity of Selye's "non-specificity" concept in stress theory, because of considerable recent research indicating that psychoendocrine reactions are frequently and inadvertently elicited during experiments designed for the study of physical stimuli. There is a pressing need, therefore, for a thoroughgoing reevaluation of past research on neuroendocrine responses to the physical stresses, with closer scrutiny of independent variables and special attention to the possible contamination of physical stress experiments by psychoendocrine reactions reflecting attendant discomfort, pain, or emotional reactions. Our recent studies of muscular exertion, fasting, and heat, for example, indicate that erroneous conclusions concerning the effects of these stimuli upon neuroendocrine systems are likely to be drawn unless rigorous and systematic efforts are made to evaluate and to minimize interfering psychoendocrine reactions. 3.) While it has long been the prevailing practice in stress research to study isolated endocrine systems, usually one at a time, it is increasingly clear that the

many neuroendocrine systems are closely interdependent in their functions and that a key to the understanding of the principles underlying the integration of these systems lies in the study of relative changes between interacting endocrine systems, as manifested in the organization of patterns or profiles of multiple hormonal responses to various stressful stimuli. In recent years, we have learned that relatively distinctive, broadly organized patterns of hormonal changes, involving many hormones in addition to those of the adrenal systems, occur in response to various types of psychological and physical stress. A major immediate goal, therefore, is to define as conclusively as possible the characteristic hormone response profiles for various stressful stimuli, with principal emphasis on psychological stimuli, but also including exercise, heat, cold, fasting, hypoxia, infection, and other physical stimuli encountered in military stress situations. Such basic knowledge of the organization of integrative machinery is an essential foundation for more complex neuroendocrine approaches to the study of stress-related clinical and field problems concerned with such parameters as endurance, fatigue, host resistance, performance, and the pathogenesis of some psychosomatic disorders. It is clear that this approach logically must move eventually through a series of successive stages, beginning with 1.) basic definition of response profiles for the various stressful stimuli, 2.) determination of the degree of response profile specificity for diverse, discrete stimuli, 3.) evaluation of factors which determine response profile priorities when there are various natural admixtures of multiple, concurrent stimuli, 4.) evaluation of the physiological significance of differing neuroendocrine response profiles by extending the approach to the concurrent study of their metabolic or physiological concomitants or consequences, and 5.) evaluation of the degree to which both acute and chronic hormone response profiles may be adaptive or maladaptive. It is evident that each of the above projected stages in the development of this conceptual approach is in large measure dependent upon establishment of prerequisite knowledge in the preceding stage, so that the stages must generally best be pursued in logical sequence. Our efforts at present, therefore, are still largely limited to the sizeable task involved in just the first two stages of this approach. The amount of stress response profile data accumulating, however, is already quite substantial and is providing an increasingly useful basis for the clarification and revision of stress concepts, as discussed in some detail in a recent overview of our approach (Toronto Neuroscience Symposium). During the past year, a major portion of our effort has been devoted to continued collaboration in physical stress with the Army Research Institute for Environmental Medicine (ARIEM) at Natick, MA.

Developmental work has also continued on new or refined hormone assay procedures in order to provide the necessary, up-dated methodological foundation for this stress research program.

Progress

1. Organization of Neuroendocrine Responses to Psychological Stress. Profile of Acute Hormonal Responses to Capture and Chair Restraint in Monkeys: The development of highly sensitive and reliable new methods for the measurement of plasma levels of certain hormones has now made possible, for the first time, in our laboratory, the study of a relatively detailed profile of neuroendocrine responses during acute emotional reactions. The selection of hormonal indices in our research program is based primarily on the rationale that it is logical in the study of neuroendocrine organization to begin with endocrine systems that have well-established neuroendocrine linkages, that is, systems in which endocrine cells articulate with nerve cells, either via neural or neurohumoral connections. The battery of methods used in this study includes those for the measurement of plasma cortisol, epinephrine, norepinephrine, total thyroxine, thyrotropin, testosterone, growth hormone, insulin, prolactin, and glucagon. Chair restraint and capture was selected as a particularly suitable situation for response profile study, since previous research with the adrenal systems had shown it to be an especially potent, reliable, and convenient psychoendocrine stimulus. Following a period of at least one week of cage housing in a quiet, stable environment, monkeys were captured as rapidly as possible and blood samples obtained by saphenous venipuncture at intervals of 1, 3 and 5 minutes. The monkey was then installed in the restraining chair and additional samples obtained at 20, 40 and 60 minutes, 2, 4, 6, 24 and 48 hours. A series of 8 capture and restraint experiments and 12 catheter control experiments have now been completed, but hormonal analyses are only about 75% complete. From the data so far, a family of hormonal response curves is being defined, with different hormones having remarkably different dynamic characteristics. Prolactin levels show about a four-fold increase, peaking at 20 minutes after onset of restraint. Growth hormone shows greater than a fifteen-fold elevation peaking at 40 minutes. Cortisol shows about a three-fold elevation peaking at 4 hours. Thyrotropin levels show nearly a two-fold increase during the first hour, but total thyroxine rises only very slowly to about a two-fold elevation at 48 hours. Testosterone levels, after a mild and transient rise, show a slow but marked decline over a 24 hour period and remain very low for at least a week. Plasma insulin levels generally tend to be suppressed after 4 to 6 hours. Preliminary

evidence indicates that a four-fold increase in plasma glucagon levels may occur within 5 minutes after capture. In general the overall pattern of acute neuroendocrine responses observed following capture and chair restraint is closely similar to that observed earlier in conditioned avoidance experiments and chair restraint experiments which were of longer duration and dependent largely upon 24-hour urinary hormonal excretion measurements. In these earlier studies it was difficult to evaluate in some instances the extent to which such factors as altered food intake, altered sleep patterns, prolonged postural changes, or altered muscular activity might have been determinants of the neuroendocrine response pattern along with psychological factors. The present study, however, using plasma hormone measurements during the initial minutes and hours of restraint, before the above non-psychological variables become significant factors, provides a much stronger basis for interpreting these data as representing primarily a psychoendocrine response pattern. The data on prolactin and glucagon provide some of the most striking data available so far, particularly with regard to control of non-psychological variables, which indicate that these two hormones should be included among the growing assemblage of hormones which are sensitively responsive to psychological stimuli. Finally, control experiments incorporated in this study include experiments in which blood samples were taken by saphenous venipuncture on exactly the same schedule after one month of restraint and three subsequent control experiments performed in each monkey at weekly intervals in which blood samples on the same schedule were withdrawn remotely through chronic indwelling venous catheters in order to minimize psychological disturbance of the monkeys. Only very minor hormonal fluctuations have been observed in the catheter control experiments so far. In the saphenous venipuncture experiments, a pattern of response very similar to that seen with acute capture and restraint has been observed, but generally hormonal changes are of substantially smaller magnitude.

2. Organization of Neuroendocrine Responses to Physical Stress.

Profile of Acute Hormonal Responses to Heat Exposure in Human Subjects: Earlier pilot neuroendocrine studies of heat exposure in monkeys indicated that heat per se elicits a distinctive pattern of neuroendocrine responses in which the pituitary-adrenal cortical system is suppressed rather than stimulated, as was thought on the basis of early experiments in the stress field. In order to pursue these observations to a more conclusive level, a collaborative study with Dr. John Maher at ARIEM in Natick, MA, has been conducted in which neuroendocrine measurements were made before, during, and after 3-hour periods of exposure at four different ambient temperature levels: 74°F (Control), 95°F, 100°F, and 105°F in a group of

8 normal young men. Experiments were performed at weekly intervals and relative humidity was maintained constant at 50%. Measures employed to minimize or evaluate possible interfering psychoendocrine reactions were: 1.) "Sham", or pre-experimental, exposure to the experimental setting and procedures, excluding heat exposure, on an occasion during the initial week, in order to minimize well-known novelty effects during the subsequent heat experiments; 2.) Gradual temperature change from 74°F to the respective heat exposure levels over a 30-minute period, in order to avoid sudden discomfort effects apparent in our pilot monkey experiments; 3.) The use of indwelling venous catheters to avoid repeated venipuncture and the allowing of a two-hour recovery period from possible psychoendocrine reactions to I.V. catheter insertion, which were sometimes observed in earlier exercise studies in human subjects; 4.) Multiple blood samples at intervals during the hour immediately prior to onset of heat exposure in order to assess possible anticipatory psychoendocrine reactions to the impending procedures, again as were often observed in previous exercise studies in human subjects.

Hormonal measurements completed so far have yielded the following findings. First, on the basis of successive 3 hour urine samples collected before (0800-1100 hours), during (1100-1400 hours) and following (1400-1700 hours) heat exposure, it is clear that there is no 17-hydroxycorticosteroid elevation in relation to heat exposure under these conditions. At all three heat exposure levels, mean urinary 17-OHCS levels were lower than during the control experiment at the 74°F level. Urinary epinephrine and norepinephrine also consistently showed the same tendency to be mildly suppressed during all three levels of heat exposure. Urine volume was not decreased during heat exposure because of a liberal water intake schedule designed to minimize the significance of residual urine in the bladder following voiding at sample close-out times.

A large series of plasma hormonal measurements have now also been completed. There is no significant change in plasma cortisol levels during any of the heat exposure sessions, including the one at 105°F, 50% RH. There is also no significant change in total thyroxine, thyrotropin (TSH), prolactin, insulin, or testosterone attributable to heat exposure under these conditions. There is a curious, consistent, transient rise in growth hormone at about 1200 hours, during the first hour of heat exposure in all sessions. Further work will be required to clarify the significance of this change, including assessment of the possible role of observed somnolence, probably associated with heat and a semi-reclining posture, in eliciting growth hormone release, perhaps in a fashion similar to that observed in early phases of sleep at night. The fact that several subjects showed the same tendency in the control

experiment at 74°F certainly suggests the need for some caution before ascribing this growth hormone spike to heat exposure per se. These findings add substantial additional support to our growing body of data indicating that the "non-specificity" and "general adaptation syndrome" concepts formulated by Selye are probably erroneous and that neuroendocrine responses are organized on a considerably more specific or selective basis, depending upon the nature of the "stressful" stimulus in question. These results do appear to fit generally with Cannon's view of the homeostatic principle of bodily organization from which it might be predicted that levels of a thermogenic hormone such as epinephrine, for example, should not be increased at a time when homeostatic needs would presumably be best served by mechanisms promoting either increased loss of body heat or decreased production of body heat. It should be pointed out that the graded heat exposure levels used in this study produced the expected graded increases in body temperature, ranging up to a mean rectal temperature of 100.2°F at the 105°F ambient temperature level. Plans are currently in progress with Dr. Maher to extend our heat exposure studies to the study of neuroendocrine responses to both acute and chronic heat exposure. Acclimatization studies present greater technical difficulties, but are of considerable interest in relation to field stress research of heat-related medical or performance problems.

3. Developmental and Methodological Research.

Efforts are continuing to produce antibodies to cortisol and testosterone conjugates specific enough to permit the radioimmunoassay of cortisol and testosterone in monkey and human plasma samples. The development of our own supply of these antibodies will alleviate the expense of having to purchase these relatively costly materials.

A method has been developed for the radioimmunoassay of urinary cortisol metabolites. Following immunization of rabbits with BSA conjugates of 21-hemisuccinate derivatives of tetrahydrocortisol and tetrahydrocortisone antibodies were obtained which proved to be suitable for the radioimmunoassay of THF and THE in monkey and human urines. These antibodies, although of relatively low titer, proved to be reasonably specific. Each showed about 20% cross reaction with the opposite steroid. A very good coorelation was found between THE and THF levels ($r = >.95$) and also between THE plus THF and the total 17-OHCS as measured by the Glenn-Nelson method. Antibodies raised against the carboxymethoxime-BSA conjugate of THE were found to be much less specific when assaying urine samples for THE.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 034 Influence of stress on hormone response, performance and emotional breakdown in military personnel.

Literature Cited.

Publications:

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PROJECT 3A762758A824
RADIATION INJURY AND PROTECTION

Task 02
Microwave Radiation

Project 3A762758A824 RADIATION INJURY AND PROTECTION

Task 02 Microwave Radiation

Work Unit 057 Biological effects and hazards of microwave radiation

Investigators.
Principal: Edward L. Hunt, B.A.
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During the FY 75 report period, additional changes in project personnel occured and the development was undertaken of a broad range of new biophysical, physiological and behavioral lines of investigation. The earlier investigations of the frequency/polarization effects of microwave irradiation on lethality (and seizure) were continued to determine rates of body heating and whole-animal energy absorption associated with these exposure parameters. In addition, support of extramural investigations of microwave bioeffects, as a collaborative effort in some instances, was continued and expanded during this period.

1. The interaction of microwave frequency, animal size and orientation on lethality.

Investigations of the influence on lethality of microwave frequency, E-field orientation and animal size, begun in FY 74, were completed[1]. Elapsed time to induction of a terminal tonic-clonic convulsion was measured during microwave irradiation at frequencies of 710, 985, 1700, 2450, and 3000 MHz. Subjects were exposed at each frequency with the electric field (E vector) polarization plane parallel and vertical to the long axis of the animals' body. Groups of mice (25 - 35 g), small rats (100 - 125 g), and large rats (380 - 420 g) were exposed at each combination of frequency and body orientation. An anechoic chamber (16' x 16' x 32') was used for irradiation of each animal while held restrained in a plexiglass enclosure located four feet distance from the radiator. All exposures were CW at the rate of 150 mW/cm^2.

Frequency, body size and orientation significantly affected the elapsed time for induction of the convulsion. The horizontally aligned E field produced consistently shorter latencies to convulsion across all animal sizes and frequencies. In general, the animal of larger size displayed greater vulnerability (shorter convulsion latencies) at lower frequencies. For example, mice

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6484	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. CRDA'N INSTR'N	8A. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	YES NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A824	02	057
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Provide with Security Classification Code)

(U) Biological Effects and Hazards of Microwave Radiation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology 016208 Stress Physiology 005700 Electronic & Electrical Engineering

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: NA — EXPIRATION:

b. NUMBER:

c. TYPE: — d. AMOUNT:

e. KIND OF AWARD: — f. CUM. AMT.:

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	5	600
FISCAL YEAR CURRENT 76	6	609

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

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22. KEYWORDS (Provide EACH with Security Classification Code)

(U) Microwave Hazards; (U) Nonionizing Radiation; (U) Dosimetry; (U) Behavioral Effects; (U) Physiological Effects; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To establish meaningful criteria for delimiting human occupancy in an elctromagnetic (EM) environment in support of maximum operational effectiveness of Army and other military personnel with minimum medical and health risk.Delineate the interaction of radiofrequency and microwave radiation (10 MHz to 100 GHz) with biological systems. Survey and evaluate known methods and techniques of EM dosimetry and develop same for control and measurement of incident and absorbed energy where appropriate and necessary.

24. (U) Investigate each major organ system and biological process where EM effects might occur at reasonably low power intensities. Where indicated, determine the military significance of the effects and the measures necessary to obviate them. Coordinate publication of quarterly digest on biological effects of EM radiation. Scientific methods of experimental psychology, biophysics, physiology and engineering will be used primarily. Exposure parameters will be chosen for relevance to Army radiating equipment and operational requirements.

25. (U) 74 07 - 75 06. Research is conducted on: body temperature rise and lethality in rodents as a function of EM frequency, animal size and orientation to E-, H- and k-vectors of EM field; modeling with figurines by size scaling at near resonant frequencies for energy absorption in humans; effects of ambient temperature and humidity on vulnerability to EM radiation;effects on excitability using audogenic seizure tests; low-level pulsed microwave effects on blood-brain barrier; measurement of tissue dielectric properties and of energy absorption in anisotropic tissue models; absolute energy measurements of absorption by whole animal. For technical report see Walter Reed Army Institute of Research Annual Report, 1 July 74 - 30 June 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

were most vulnerable in the 1700-2450 MHz range, small rats at 985 MHz, while large rats convulsed most quickly at 710 MHz. The exposure frequency to which each size animal was most sensitive had a wavelength approximately twice the length of the animal (nose to base of tail). The field energy coupled with the animal in a manner consistent with the body acting approximately as a half-wavelength antenna.[2,3] If these results represent a general phenomenon, man might be expected to be most vulnerable to frequencies in the range of from 50-450 MHz, depending on the person's size and the circumstances of exposure.

2. Energy Absorption Modeling.

The direct measurement of radio-frequency (RF) energy absorption and distribution in man is not possible and recourse has been made by Dr. Om P. Gandhi, University of Utah, in collaborative work at WRAIR, to measurement of absorption with frequency-scaled models. In earlier work, he established a modeling system based on prolate-spheroids composed of materials that simulate the dielectric properties of particular tissues (such as would be found with homogenized brain or muscle)[2-4]. Using a stripline exposure system, measurements were made of the energy lost in the system (gained by the model or small anesthetized rodent) according to the exposed object's aspect ratio (length/width) relative to the wavelength and to the objects orientation with respect to the E vector, H vector and propagation (k) vector of the radiation.

In the present report period, this collaborative work has been extended to investigations of saline-filled and biological-phantom models using prolate-spheroids and human figurines exposed to free-space fields in our anechoic chambers. With figurines of the particular height that will provide near resonant-frequency absorption, such as a 12 cm model oriented with the E vector of a 985 MHz field parallel to the figurines long axis, extremely high rates of heating in the neck region occur (measured during exposure with a calibrated liquid crystal probe). These modeling studies will be continued to provide accurate estimates as to particularly hazardous RF frequencies for man and to provide information needed in biological experiments with animals to assess biological effects likely to be hazardous at frequencies that are appropriately scaled relative to the animal's size.

3. Whole-body energy absorption.

In small experimental animals a method for absolute measurement of absorption of microwave energy by the whole animal has been

developed,[5] used to characterize exposure systems,[6] and presently is being installed for use at the WRAIR facility. The method employs a twin-well differential calorimeter with which measurements are made of the heat content in the animal's body following a brief irradiation relative to the heat content of a control body that, except for irradiation, is identically handled. These measurements will be routinely employed to provide energy absorption measurements under a variety of exposure conditions (frequency, orientation, and geometry of the exposure field and species and size of animal).

The mechanical and electrical design of the twin-well differential calorimeter has been completed and the initial unit was fabricated using in-house facilities. A proportional type heater control was designed and built which maintains a jacket temperature surrounding the wells within + 0.05° C. The measurement system displays the temperature of each calorimeter well and the difference between the well temperatures. The area under the differential temperature curve, during recovery of equilibrium following addition of heat to one of the wells, is calibrated in units of joules and is employed for the dosage measurement of irradiated animal bodies.

Construction has begun on two additional calorimeters at Walter Reed and of two units in collaboration with the University of Utah. These additional calorimeters are designed for much larger animals than the first unit, which has capacity for measurement with an animal of 100 gm weight or less.

One of the initial series of measurement with the whole-animal calorimeter will be undertaken to specify irradiation dosages in the lethality studies that were recently completed (cf 1, above).

4. Determination of dielectric properties of tissue.

The dielectric properties of biological tissues fundamentally determines the energy absorption by the tissue from the electromagnetic radiation transmitted through it. These dielectric parameters vary with the radiation frequency and the tissue composition and temperature. Estimation of these parameters is technically difficult to achieve. In the investigations undertaken during this report period, initially in collaboration with R.C. Lin, Wayne State University, use was made of a comparison method.[7] An automatic network analyzer was used to measure accurately the scattering parameters over a wide frequency range of tissue samples relative to a sample of physical material for which dielectric parameters are known.

A special sample holder was developed for use with the microwave network analyzer which allowed precise positioning of the sample under analysis. The design of the holder also facilitated making samples of very uniform thickness. Control of these two parameters were necessary to make accurate scattering measurements. The validity of the holder-measurement system, and calculation procedure was verified using plexiglass as the comparison material of known dielectric properties. Excellent correlation was found between the measured data and published information. Measurements were then done on other dielectric materials, including bone.

Conceptual design was started on another sample holder that will permit analysis of smaller samples, broader frequency range and will be waterproof. The latter characteristic is important because it is desirable to be able to immerse the holder in a constant temperature bath, for estimating the temperature parameter.

5. Thermographic evaluation of candidate radio-frequency (RF) transparent temperature probes.

A critical need for biophysical and biological investigations of microwave irradiation effects is that of obtaining temperature measurements at critical sites within biological systems during exposure. For maximal utility, such a temperature probe would first need to be transparent to the radiation, then sensitive with a short time constant, readily calibrated and stable, rugged and small in size. Although development of several candidate probes have been undertaken, none as yet have met all the criteria. A comparative investigation of several has been started to determine initially the transparency characteristics.

A micro-integrated circuit (MIC) transducer system and integrated bridge for bolometric thermometry, had been used in the earlier development at WRAIR of an RF transparent thermistor temperature probe.[8] This MIC electrode has undergone further development at the National Bureau of Standards and the Bureau of Radiological Health by the use of a 4 terminal resistance measurement to replace the bridge network. During the present report period, two samples of this version of the WRAIR electrode were evaluated as a thermometric device and both were found to be unusable due to a fabrication error or degradation of the electrode. RF evaluation by means of infrared pyrometry demonstrated that transmission line heating and thermal diffusion occurred. The further refinement of this electrode has been undertaken.

Another candidate is the liquid crystal fiber optic probe developed at the University of Utah. This probe is largely unsuitable

as a thermometric device due to its calibration drift, long thermal time constant, hysteresis, frank mixture instability and its relatively large size. Thermographic evaluation demonstrated good RF properties up to about 250 mW/cm^2 at S band where some differential absorption appeared.

Thermographic evaluation of candidate transmission lines included several varieties composed of carbon loaded polytetrafluorethylene (PTFE) or optic fiber (OF) bundles. Heating in the PTFE line was related to its lineal resistance. Heating in the OF was much lower than the PTFE, but its small displacement currents did produce measurable (about 0.75° C) heating. Nodal structure in the heating patterns were observed for all transmission lines.

6. Measurement of microwave propagation properties of simulated biological tissues.

Biological systems typically are anisotropic and, in such materials, microwave radiation transmission is non-uniform and, to date, largely undetermined, although available energy for absorption in local tissues of interest is determined by such non-homogeneous transmission. The necessary instrumentation has been developed during this reporting period making target transmission measurements using focused and nonfocused aperatures at frequencies ranging from 2200 MHz to 12GHz. Transmission measurements through dielectric materials were made with crystal detectors and the microwave network analyzer. Anisotropic dielectrics used to represent biological heterogeneity were also studied.

Phase and attenuation measurements of transmitted energy were made at 2450 MHz for isotropic and anisotropic targets. Reflected energy magnitudes and phase information were also obtained. Estimation of transfer functions will be attempted with the use of time domain sampling techniques for very wide band pulses.

7. Colonic temperature changes during microwave exposure.

The animal's core temperature classically has been used to indicate physiological status during and following various experimental treatments. The colonic temperature of rats increases during microwave exposure, as would be expected, but the magnitude of this effect has been found to be dependent on microwave frequency, E-field orientation, and size of the subjects in a manner parallel to previously obtained lethality data (cf.1, above). Exposures during which the E vector was aligned with the long axis of the subject's body consistently resulted in higher temperatures than did exposures with the E vector vertical. Differential frequency effects were most pronounced during exposures with the E

vector horizontal. Mice (25-30g) showed larger temperature increases at 1700 and 2450 MHz than at 710 MHz. Small rats (100-125 g) and large rats exhibited highest temperature increases at 710 MHz. The colonic temperature changes are suggestive of a differential absorption which is determined by frequency, E field orientation, the dimensions of the experimental subject and, as with the lethality investigation, an enhanced rate of absorption at the animal's resonant-frequency.

8. Combined stress - The effects of temperature and humidity on time to convulsion.

Thermal loading of the organism by microwave irradiation constitutes the primary type of hazard presently used to establish protection guidelines. However, an empirical analysis of the consequences of such loading under various ambient conditions of temperature and humidity, which would modify the animal's capacity to dissipate heat, has not been made. Such investigation was undertaken to determine the effects of variations in ambient air temperature and humidity on the vulnerability of rats to microwave radiation. Elapsed time to tonic-clonic convulsion was used as the measure of vulnerability to irradiation at 150 mW/cm^2 with 2450 MHz CW microwaves, oriented with the E field vertically aligned with respect to the long axis of the animal's body. The rats were individually exposed while confined in a holder constructed of styroform and plexiglass rods, designed to provide maximum ventilation and exposure to the ambient conditions. Exposures are being made with ambient temperatures ranging from 62 to 94° F, and relative humidities ranging from below 30% to above 70%. The data obtained to date indicate that vulnerability increases monotonically with a temperature-humidity index (THI = 1.44T+ 0.1 RH + 30.6). With the THI of 138.9 (70° F, 75% RH), for example, median latency to convulsion was 999 sec, and with a THI of 171. (94° F, 55% RH) it was 530 sec, representing a decrease of 47%. This evidence to date indicates that the prevailing ambient temperature and humidity conditions strongly influences vulnerability to microwave irradiation.

9. Sensitivity to audiogenic seizure following microwave irradiation.

During the current reporting period investigations were started to verify purported central nervous system (CNS) effects of microwave irradiation on excitability. An audiogenic seizure sensitivity test was employed on progeny from a selectively breed line of sound-sensitive rats being developed from the Walter Reed colony. In two experiments using high intensity, short duration exposures,

the high intensity sound test was imposed promptly (2 min) after the termination of a 30-min irradiation with 2450 MHz, CW microwaves with the E vector parallel to the long axis of the rat. The results of the first experiment, using 50 mW/cm^2 irradiation, were not significant. However, the second study, which was conducted with exposure for 30 min at 75 mW/cm^2, produced a highly significant reduction in audiogenic response. Recovery of responsiveness within 4 days was indicated in all rats for which a sham irradiation treatment was imposed following the microwave treatment. The present data indicate that audiogenic convulsive responses can be attenuated by a single, 30-min, CW exposure and that this effect does not depend on pulsed irradiation or the repeated exposures used by previous investigators. The extent to which longer durations of exposure at lower rates of irradiation can also produce alterations in sensitivity is being investigated.

10. Changes in blood-brain barrier produced by microwave irradiation.

In collaboration with K. Oscar of the American University, a program was begun to document changes in the blood-brain barrier (BBB) produced by microwave irradiation. A quantitative, radioactive isotope method (Oldendorf) was used successfully to verify the previously reported increased BBB permeability of rats. With the Oldendorf technique, two radio-active indicators are injected simultaneously into a common carotid artery of the rat. One indicator is the test substance which is labeled with ^{14}C. The second indicator is tritiated water which is highly diffusable into brain tissue and serves as the standard to control for general circulatory condition and profusion artifacts. The experimental measure provides a quantification of the uptake of the test substance relative to the tritiated water. Several sets of observations with a frequency of 1.2 GHz revealed significant increases in BBB permeability with both pulsed and continuous wave energy at average power density levels considerably below the 10 mW/cm^2 level. Future work will be aimed at systematically assessing the influence of various microwave parameters (including carrier frequency and pulse modulation parameters), duration of effect and location and size of barrier fenestrations.

11. Microwave ocular effects.

An evaluation was undertaken of a cavity exposure system designed by Stanford Research Institute for use potentially in chronic irradiation investigations of ocular effects in monkeys. A Tri-Service research group, working with SRI investigators and in collaboration with Prof. A.W. Guy's group, at the University of

Washington, Seattle, investigated cavity fields and thermographic evidence of absorption distribution. In collaboration with Dr. Charles Bonney, WRAIR personnel conducted a study to measure intraocular temperatures after brief, high power exposures. Selective retrolental heating (maximum cataract hazard) was studied by simultaneous, dual channel temperature measurements in the posterior chamber of the eye. Intraocular temperature measurements were made in the prototype cavity exposure facility at 2450 MHz. Measurements were also made in a plane-wave far-field at 2450 and 4975 MHz with varying source-subject geometries. It was found that both frequencies deposited energy in the eye.

These measurements were designed to survey the methdology and the results will be used primarily to form programmatic judgments concerning the future course of the Tri-Service program on ocular effects of microwave irradiation. Since it could be expected that the higher frequency exposure would have less systemic effects, it was recommended that higher frequency operation of the exposure facility should be explored. This could be accomplished with a multifrequency pilot study to determine exposive parameters needed to produce prompt lenticulr opacifications, and by investigations of intraocular temperature measurements with indwelling RF transparent probe s during exposure, when such a probe is successfully developed.

12. Development of high-power microwave exposure system for brain enzyme inactivation.

This reports only the engineering developments in the design and fabrication of brain enzyme inactivation systems for mice and rats. This work has been undertaken in collaboration with neurochemists in the Department of Neuroendocrinology (cf., Work Unit 112) and, using prototypical exposure arrangements[9], the utility of the method for fixation and analysis of brain enzymes has been demonstrated[10].

Analysis of the operation of a commercially developed inactivator system operated at 2450 MHz indicated that problems with component errors and waveform stabilization made it unreliable for use. Installation of appropriate control circuits for use of a Varian source, operated at 2450 MHz at a power level of 5kW is nearly completed. Preliminary evidence indicates that this system will provide relatively uniform energy deposition in the brain of the mouse.

Some preliminary design work and testing has been undertaken to provide means for achieving uniform energy deposition in the

brain of the rat, which is of larger size than the mouse's and consequently provides greatly altered loading characteristics for the waveguide applicator. In consultation with Dr. Om Gandhi, University of Utah, a circularly polarized waveguide applicator, operated at 2450 MHz, is being designed for the rat. In addition, a standardly polarized waveguide cavity has been designed and fabricated for use with the rat to operate at a lower frequency, 985 MHz. When in initial testing, this applicator was powered with a 2kW CW source, it appeared that relatively uniform brain coagulation in the rat could be achieved. The utility of this prototype system will be tested for comparison with the higher frequency, 2450 MHz, and, by thermography and thermometry, for uniformity of energy loading. The relatively low power level of this prototype system necessitates relatively long durations of exposure and limits its utility for analysis of highly labile enzyme systems.

13. Microwave bioeffects current-awareness digest

The publication of a quarterly digest "Biological Effects of Electromagnetic Radiation" was undertaken late in FY74 under a grant to Franklin Institute Research Laboratories (ARO No. DAHC04-74-G-0132) with the support of several agencies (Army, Navy, Air Force, FDA, EPA). During this report period, the Franklin Institute has prepared the first four issues of the digest and authorization for printing has been obtained. Arrangements with the Armed Forces Radiobiology Research Institute are being made for the printing of previous and current issues. This publication will be distributed free of charge to scientists and other workers in this field as a means of bringing together citations and abstracts of the diverse literature and to provide a method of informal communication.

14. Support of Extramural Research

WRAIR has made available the use of its microwave exposure facilities in previous years to extramural researchers to the extent that such use does not interfere with the WRAIR research program. The need for this use, as well as for internal program use, has greatly increased and, consequently, this has necessitated an increased level of oversight, support and collaboration by WRAIR personnel in this extramural use of facilities. In furtherance of a joint Tri-Service effort to coordinate microwave bioeffects research, a formal agreement with the Armed Forces Radiobiology Research Institute has been established and two Navy projects, operated by AFRRI personnel, are under review in accordance with this agreement. Preliminary experimental exposures

have been undertaken on both projects, one on lens opacification and biochemistry and the other on brain neurochemical effects. In addition, Dr. Varma of Howard University, Dr. Albert of George Washington University, Dr. Rozzell of the Office of Naval Research, and Mr. Janes of EPA have been provided facilities use with minimum expenditure of support and collaborative effort. In other researchers, however, active collaboration as well as advice and service has been provided, such as with Dr. Rioch of the Institute for Behavioral Research on investigations of developmental effects following in utero irradiation, Mr. Oscar of American University on investigations of irradiation effects on the blood-brain barrier, Dr. Gandhi of the University of Utah on modeling of energy deposition and Dr. Cleary of Virginia Commonwealth University on behavioral and biochemical effects of irradiation.

With the increased use of the facilities, in addition to meeting an increased need for advice and engineering support, it is anticipated that the burdens of review and scheduling will be greatly increased, to ensure an adequate standard of research and efficient facilities use.

Project 3A762758A824 RADIATION INJURY AND PROTECTION

Task 02 Microwave Radiation

Work Unit 057 Biological effects and hazards of microwave radiation

Literature Cited.

Reference:

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7. Lin, J.C., and Jacobi, J.H.: Computer-controlled measurement of microwave properties of biomaterials. Proc. Microwave Power Symposium 1975 (U. of Waterloo, Waterloo, Ontario, Canada) International Microwave Power Institute, Edmonton, Alberta, Canada pp. 265-271, 1975.

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Publications:

1. Gandhi, O.P.: Polarization and frequency effects of whole animal absorption of RF energy. Proc. IEEE, 62:1171-1174, Aug 1974.

2. Gandhi, O.P.: Strong dependence of whole animal absorption on polarization and frequency of radio-frequency energy. In Biological Effects of Nonionizing Radiation (P.E. Tyler, ed.), Annals New York Academy Sciences, 247:532-538, Feb 1975.

3. Lin, J.C., Grove, H.M., and Sharp, J.C.: Comparative measurement of dielectric properties of fresh mammalian tissues. Proc. Conference on Precision EM Measurements, London, England, 1974.

4. Lin, J.C., and Jacobi, J.H.: Computer-controlled measurement of microwave properties of biomaterials. Proc. Microwave Power Symposium 1975 (U. of Waterloo, Waterloo, Ontario, Canada) International Microwave Power Institute, Edmonton, Alberta, Canada, pp. 265-271, 1975.

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PROJECT 3A762759A829
MALARIA PROPHYLAXIS

Task 00
Malaria Investigations

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6535	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A879	00	308
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biological Evaluation of Anti-malarial Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
00 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	2.95	250
FISCAL YEAR CURRENT 76	2.95	250

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

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20. PERFORMING ORGANIZATION

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Div of Medicinal Chemistry
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TELEPHONE 202-576-2732
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME:
NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Biology; (U) Chemistry; (U) Pharmacodynamics; (U) Drug Metabolism; (U) Toxicology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To conduct in-house and contract studies in biology specifically related to the design, development and exploitation of new antimalarials for military use.

24. (U) Close supervision will be maintained by providing guidance and an integrated evaluation of productivity, and by the redirection and coordination of objectives as dictated by feedback from clinical studies of candidate antimalarials.

25. (U) 74 07 - 75 06 Compounds are tested by screening for prophylactic and suppressive antimalarial activity in approximately 25 test systems at six different laboratories. The primary suppressive screen using P. berghei infected mice screened 8,166 compounds during this report period. Of these, 596 were considered to have antimalarial activity. Secondary testing which ascertains estimated therapeutic indexes, duration of drug action, activity by different routes of administration and other various evaluations in in vivo and in vitro systems were conducted on 392 compounds. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 308 Biological evaluation of antimalarial drugs

Investigators.
Principal: LTC Kenneth E. Kinnamon, VC
Associate: CPT Lyle L. Ketterling, MSC

Introduction

Compounds are screened for prophylactic and suppressive antimalarial activity by employing approximately 25 test systems at six different laboratories.

Progress

a. Primary Screens

The primary screen chosen was one which would allow the Army Malaria Research Program to evaluate an optimum number of compounds for antimalarial activity at a reasonable cost. This is accomplished within the minimum amount of time that will yield clinically effective compounds.

1. Suppressive Testing

This system is based on comparing test compound responses by monitoring mean survival times of mice infected with Plasmodium berghei KBG 173 malaria and untreated controls. Compounds considered active produce significant increases in the mean survival times of the treated animals when compared with the untreated controls. ICR/HA Swiss mice are given a standard inoculum of Plasmodium berghei KBG 173 on day zero and 72 hours later are given a subcutaneous injection of the test compound in peanut oil. Untreated controls have a mean survival time of 6.2 days. Treated animals are observed for 60 days. Survivors at the end of this time period are considered cured. The minimum effective response for a test compound is an increase of 100% in the mean survival time. During this report period 8,166 compounds were tested. Of these, 596 were found to be active.

2. Prophylactic Testing

The test system employing White Leghorn cockerels has been discontinued due principally to lack of correlation with subsequent mammalian testing, including man. A system utilizing mice for prophylactic testing is expected to be in operation by 1 January 1976.

b. Secondary Rodent Testing

1. Suppressive

Approximately two percent of the compounds tested through primary screening are considered active enough for secondary testing using mice as animal models. Secondary testing of compounds includes determining potency with reference to quinine, activity by different modes of administration, activity against drug resistant strains of murine malaria, estimated therapeutic indexes, duration of action, antagonism by para-aminobenzoic acid (PABA), and synergism and/or combination of compound combinations. During this report period approximately 195 compounds were tested.

2. Prophylactic

Secondary testing in mice is performed by the Peters Laboratory. The test differentiates between prophylactic and residual suppressive activity of test compounds. Drug action on EE stages or erythrocytic forms is reflected by differences in a pre-2% patency period between control and treated sporozoite-inoculated animals. Residual drug action on erythrocytic forms and EE stages is assessed through cross-inoculation in parallel series of groups with infected red blood cells.

Testing at the Most Laboratory demonstrates direct drug action on exoerythrocytic (EE) stages of Plasmodium berghei in rats. EE forms develop in the liver of rats within 40-45 hours when inoculated with 125,000 to 250,000 sporozoites. These are quantitated after histological preparation. Elimination or reduction of the EE forms indicates compounds with prophylactic activity.

Approximately 92 compounds have been tested by these laboratories during this report period.

c. In vitro Testing

1 Antifolic Acid Assay

The test system assesses interference by test compounds with folic acid metabolism. Test organisms are three bacterial species which require a form of folic acid in order to grow. Compounds are tested for growth inhibitory properties against these organisms. Approximately 75 compounds were tested during this report period.

d. Subhuman Primate Testing

1. Suppressive

a) Simian Malaria; Simian Host

This test determines the maximum tolerated dose and antimalarial efficacy of compounds. In determining drug tolerance, animals are observed clinically during the time increased doses of the drug are given. Clinical observation is supplemented by hematologic and pathologic findings. Efficacy of the compound is determined by testing the capability of different compound dosages to eliminate or reduce malarial infection in monkeys inoculated with simian parasites. The animals are clinically observed and are also followed by hematologic and pathologic evaluation. Monkeys ascertained free of parasites after 30 days are splenectomized and monitored to day 60. If no recrudescence of parasitemia occurs by day 60 the animal is considered cured. Approximately 20 compounds were tested during this report period.

b) Human Malaria; Simian Host

The test system assesses the in vivo response of compound treated monkeys infected with human malaria. Animals infected with either falciparum or vivax malaria and treated with antimalaria compounds are evaluated via blood films to 90 days after the last treatment. Animals not parasitized at the end of 90 days are considered cured. The test system is continuing but at a reduced throughput due to difficulty in obtaining Aotus monkeys.

2. Prophylactic - Radical Curative

Due to the retirement of Dr. Leon Schmidt in 1976 prophylactic - radical curative testing in Rhesus monkeys will be accomplished subsequently by the SEATO Laboratory, Bangkok, Thailand rather than at The Southern Research Institute, Birmingham, AL. The test system at the SEATO Laboratory is expected to be fully active by 1 September 1975.

Summary

During the reporting period 8,166 compounds were screened in the Primary Suppressive System. Of these compounds, 596 were active. Secondary testing was conducted on 392 compounds by in vivo and in vitro means.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6536	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10 NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	309
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Determination of Pharmacological Effects of Antimalarial Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	4.5	300
FISCAL YEAR CURRENT 76	4.5	300

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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NAME: Rozman, Dr. R. S. DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicity, (U) Biotransformation; (U) Antimalarial Drugs; (U) Preclinical Pharmacology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objectives are to develop and exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purposes of these studies are to provide a basis for predicting human response and to fulfill requirements for submission of IND for clinical trials of new antimalarials for military personnel in malarious areas.

24. (U) The approach is to study both the effects of antimalarial drugs on healthy animals and the fate of these drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The effects of antimalarial drugs are being studied in infected animals. The handling of antimalarial drugs by diseased animals is being studied to determine the effects of malaria upon pharmacokinetics. This is in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).

25. (U) 74 07-75 06 Technical management continued for 14 contracts in pharmacology. Two new IND applications and 15 supplements were written. Pharmacokinetic investigations using radioactively labelled drugs in healthy mice were carried out for WR 184,806 and WR 180,409. Pharmacodynamic evaluations in anesthetized dogs were compared for WR 184,806 and WR 142,490. The mouse oral efficacy and toxicity and the effects of microsomal modifiers on these were intensively studied for WR 2975, WR 181, 023, WR 211,536 and WR 211,537. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 309 Determination of pharmacological effects of antimalarial drugs

Investigators.
Principal: Melvin M. Heiffer, Ph.D.
Associate: Dr. R. Rozman, Dr. A. Einheber, LTC L. Miner, 1LT J. Grindel, Dr. H. Chung, M. Wilson, P. Tilton, SP4 R. Keller, PFC C. Oyler, PFC H. Gillum

1. Description.

The thrust of the pharmacological studies carried out by the department continue in two broad areas. First is the effect of the body on the system on the drug, i.e. absorption, distribution, biotransformation and excretion. Second is the effect of the drug on the body or system. A considerable overlap exists between the two areas.

2. Absorption, distribution and excretion in mice.

The absorption, distribution and excretion of 2 new antimalarials were studied in mice using radioactive compounds.

a. WR 184,806-^{14}C:

Studies on 2,8-bis(trifluoromethyl)-4-[1-hydroxy-3(N-t-butylamino)propyl-1-^{14}C]quinoline phosphate were performed using female ICR mice weighing between 25-30 gm.

Table 1 is a summary of the data showing the routes of excretion of total radioactivity derived from the parent drug after oral administration of 10 mg/kg. For this study 2 groups of 4 mice were given a single oral dose by gavage, placed in glass Roth metabolism cages, and their urine and feces collected and monitored for 120 hr. One cage was also attached to a series of traps designed to capture volatile radioactive compounds. As these data demonstrate, the principal route of excretion was via the feces, wherein 71.97% of the total radioactive dose had been excreted by 120 hr. The urinary output accounted for an additional 26.50% of the drug-derived radioactivity, with the expired air showing only trace amounts. Table 2 summarizes the excretory data after intraperitoneal administration of 10 mg/kg. Here also, the principal

route of excretion was via the feces wherein 43.35% of the total radioactivity had been excreted by 72 hr. The urinary output accounted for an additional 19.45% of the radioactivity. The absolute rate of excretion of radioactivity after intraperitoneal administration of drug was slower than after oral administration, since by 72 hr 62.8% was excreted after I.P. administration but 93.7% was excreted after oral administration. However, the fraction of radioactivity in the urine, when compared to the total excreted during the 72 hr period, was 31% and 27% after intraperitoneal and oral dosing respectively.

The elimination t 1/2 after oral administration as calculated from the urinary excretion of parent drug was 11 hr whereas after intraperitoneal administration it was 21 hr.

In a selective tissue distribution study (Table 3) 4 mice per time period were given a single oral dose and then sacrificed at the selected time interval. The tissues were removed, pooled and homogenized in methanol. The lungs, liver, skeletal muscle, small intestine and residual carcasses contained substantial percentages of the total dose of radioactivity derived from WR 184,806-^{14}C. From these data it was calculated that at least 67% of the total radioactivity had been absorbed by 2 hours. Thin layer chromatographic analysis was performed on precoated silica gel F_{254} plates using n-butanol:acetic acid:water (66:17:17 v/v/v) as the development solvent. Of these tissue homogenates (Table 4) the principal radioactive component in lungs, liver, kidneys, GI tract and residual carcasses was tentatively identified as WR 184,806-^{14}C by comparison with a reference standard. This inference was further substantiated by the use of the inverse isotope dilution technique on the 8 hr lung and liver homogenates.

The plasma concentration curves for WR 184,806-^{14}C and total drug-derived metabolites are shown in Figure 1. Each point represents a pool of 4 mice which were anesthetized with diethyl ether and bled via a cutdown of the femoral artery. The pooled blood was separated into plasma and red blood cells by centrifugation; the separated fractions were extracted with ethyl acetate:ethanol (3:1 v/v). The extracts were analyzed by thin layer chromatography followed by scanning of the plates for radioactivity with a Varian-Berthold 6000 Radioscanner as with the tissue extracts. The plasma level of WR 184,806-^{14}C peaked at 2-4 hr and again at 7-10 hr at 0.43 μg/ml with an apparent elimination t 1/2 of 13.5 hr, a value which agreed well with that found from urinary excretion (11 hr). The total metabolites had peak levels at 7 and 12 hr of 1.55 and 1.37 μg/ml, respectively.

The red blood cell concentration curves for WR 184,806-^{14}C and total metabolites are shown in Figure 2. WR 184,806-^{14}C had a peak level at 5-6 hr of 1.62 μg/ml while the total metabolites peaked at 3 and 7 hr at 1.85 and 2.09 μg/ml, respectively. The RBC/plasma ratio of parent drug was greater than 1.5 for the first 48 hr.

b. WR 180,409-^{14}C:

The threo epimer of α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol-^{14}C phosphate was purified and studied after oral administration to both male and female albino ICR mice.

WR 180,409-^{14}C·HCl from Monsanto Research Corporation was converted with alcoholic sodium hydroxide into the free base which was crystalized from water. The crystals were treated with methanolic phosphoric acid; the resultant drug was purified from ethyl acetate:heptane.

The radioactive drug was suspended in 0.2% methyl cellulose and 0.4% Tween 80 in deionized water and 20 mg/kg was administered by oral intubation to fasted mice. The mice were then housed 4 to a modified Roth glass metabolism cage. The mice were allowed water ad libitum and food was not allowed until 4 hr after dosing.

Urine and fecal samples and appropriate trap rinsings were collected 12 and 24 hr post dosing, and every 24 hr thereafter.

Fecal samples were homogenized in absolute methanol and extracted in glass columns with methanol. The eluates were evaporated to dryness in a round-bottomed flask on a rotary evaporator and suspended in deionized water.

At the end of 240 hr post dosing, the animals were sacrificed and the carcasses homogenized in absolute methanol. The resulting homogenates were treated like fecal homogenates from there on.

The results of the excretion study are shown in Figures 3 and 4. Figure 3 shows the daily and cumulative percent dose excreted via urine and feces by the female mice. Approximately 72% of the dose was excreted via feces and 7% via urine. The excretion of radioactivity in urine and feces peaked at the time period between 48 and 72 hr. Approximately 4% of the dose was recovered in the carcasses. A total of 83% of the administered dose was recovered from the female mice.

Figure 4 shows the daily and cumulative percent dose excreted via urine and feces by the male mice. About 84% of the dose was excreted via feces and 5.5% of the dose excreted via urine. Like the female mice, the excretion of radioactivity in urine and feces peaked at the time period between 48 and 72 hr. Approximately 4.5% of the administered dose was recovered in the carcasses. A total of 94% of the administered dose was recovered from the male mice.

One male mouse was dosed as described and placed into a small glass metabolism cage which was connected to acid and base air scrubbing columns to determine possible elimination via expired air. Samples of expired air trapping solutions were taken at scheduled time periods for 192 hr. A total of 0.32% of the administered dose was recovered in the expired air after 192 hr. Peak levels of radioactivity in the expired air were found at the time period between 48 and 72 hr (Table 5).

3. Cardiovascular and pulmonary pharmacology of WR 184,806·H_3PO_4 in dogs.

a. Background:

This investigation was conducted to observe the acute cardiovascular and pulmonary responses to the candidate antimalarial drug, WR 184,806·H_3PO_4, after intravenous administration to anesthetized dogs.

b. Methods:

Nine adult beagle dogs weighing between 7.3 and 14.5 kg were anesthetized with intravenous sodium pentobarbital, 30 mg/kg of body weight. Both femoral veins were cannulated with polyethylene catheters, one for administering drugs and the other for administering maintenance doses of the anesthetic. For the latter purpose a Harvard infusion pump was employed at a rate of 0.029 to 0.078 mg/kg/min in order to maintain a uniform level of anesthesia throughout the experiment. Two other polyethylene catheters were inserted into the abdominal aorta via the femoral arteries for measurement of arterial pressure. Needle electrodes were positioned for recording both a lead II and a precordial electrocardiogram. Heart rate was determined by measurement of the intervals between R waves with a conventional cardiotachograph. An oral endotracheal tube was inserted and attached to a pneumotachometer for recording the respiratory rate and depth. Body temperature was continuously monitored rectally with the thermistor probe of a telethermometer

and was maintained near normal by the application of external heat as needed. All measurements were made with a Hewlett-Packard-Sanborn 7700 series recorder.

The chemical compound used in these experiments was from bottle number BD99078, Lot AH. The drug was dissolved in 5-10 ml of 5 percent dextrose in water. A few drops of phosphoric acid were added to facilitate dissolution of the drug in the vehicle.

The first phase of the study involved 3 dogs which received WR 184,806. H_3PO_4 intravenously in doses ranging from 0.5 to 64 mg/kg of body weight. In each experiment the drug was administered over a 1 to 2 min period in a series of increasing doses at 1/2 to 1 hr intervals. After each dose the responses were recorded continuously until they returned to control levels or until death ensued.

The second series of experiments involved 3 dogs which were bilaterally vagotomized and pretreated with atropine sulfate, 1 mg/kg, one hr prior to the experiment. Each dog received 8 mg/kg of WR 184,806·H_3PO_4 injected intravenously over a 2 min period, followed by an additional dose of 8 mg/kg infused intravenously over a 30 min period. Dose response curves to epinephrine, norepinephrine, isoproterenol and angiotensin were obtained before treatment and during the 30 min infusion. Prior to obtaining the dose response curves, several 1 µg/kg doses of epinephrine were administered until a uniform response was obtained.

The third phase of the study involved 3 dogs which were also bilaterally vagotomized and pre-treated with atropine sulfate, 1 mg/kg. They were opened at the chest via a midline incision while respiration was being maintained with a Harvard respirator. An electromagnetic flow probe was attached to the ascending aorta. The chest was then closed and the animal allowed to resume spontaneous respiration. Responses were observed to the intravenous infusion of WR 184,806·H_3PO_4 in doses of 4, 8 and 16 mg/kg. Dose response curves to epinephrine were obtained before and after the 8 and 16 mg/kg doses. Then a dose of 0.5 mg/kg of propranolol was given intravenously over a 5 min period and 15 min later the dose of 16 mg/kg of WR 184,806·H_3PO_4 was repeated.

c. Results:

The effects of drug treatment on arterial blood pressure, heart rate and respiratory rate are shown in Table 6. The values shown were taken at the time of peak effect which occurred 3 to 5 min after administration of the drug. Since each animal served as

its own control, the post-treatment values are expressed as the percent change from control readings taken just prior to each dose. Control values at the end of each experiment were not significantly different than at the beginning (Table 7). Immediately after administration of the drug there was a small, dose related decrease in systolic pressure and a more pronounced decrease in diastolic pressure. Both values promptly returned to control levels within 5 to 10 min. After the 32 mg/kg dose (63.5 mg/kg total accumulated dose) both the systolic and diastolic blood pressure fell to approximately 50 percent and 75 percent of control values respectively, but then promptly returned to normal levels within 10 min. One animal was subsequently challenged with 64 mg/kg and suffered an acute death within 5 min.

There was little or no change in heart rate after drug doses of 0.5 to 2 mg/kg and a slight increase after doses of 4 and 8 mg/kg of body weight. After doses of 16 and 32 mg/kg the heart rate was decreased by 7 to 13 percent, but it returned to the control rate within 20-30 min.

At higher doses the drug produced a marked increase in the respiratory rate which appeared to be dose related. As the rate increased, the depth of respiration decreased.

The changes in blood pressure and heart rate in response to catecholamines and angiotensin are shown in Table 8. The vasopressor responses to epinephrine, norepinephrine and angiotension appeared to be slightly attenuated, whereas the vasodepressor response to isoproterenol was not significantly altered following administration of WR 184,806·H_3PO_4. In each experiment there was a moderate bradycardia produced after drug treatment. Comparable responses were observed when similar measurements were obtained in 3 open-chest animals challenged with epinephrine before and after administration of the drug (Tables 9 and 10).

As shown in Table 11, the increase in ascending aortic blood flow produced by epinephrine was significantly enhanced after administration of WR 184,806·H_3PO_4. The drug produced very little direct effect on aortic blood flow.

There were no significant alterations in the electrocardiogram even after the highest drug doses.

d. Discussion:

The intravenous administration of WR 184,806·H_3PO_4 to anesthetized dogs was found to produce an immediate hypotension,

bradycardia and an increased respiratory rate. These responses returned to normal within 5 to 10 min and no other significant effects on the cardiovascular or respiratory systems could be observed, except at toxic dosage levels (63.5 mg/kg).

Since these effects were also observed in atropinized and bilaterally vagotomized animals, the reduced heart rate, depressor response and increased respiratory rate were independent of the parasympathetic nervous system. The moderate and transient effects observed after a total intravenous dose of 63.5 mg/kg suggest that relatively large oral doses of this drug would be tolerated in man with only minimal effects on the cardiovascular and respiratory systems.

4. Cardiovascular and pulmonary pharmacology of WR 142,490·CH_3SO_3H in dogs.

a. Background:

The antimalarial efficacy of WR 142,490·HCl has been established in man using the oral route of administration. However, it would be most advantageous to have available in the clinic an intravenous dosage preparation for treating those patients in whom oral forms of medication are contraindicated. Thus, this study was undertaken to obtain animal data which could serve as a basis for predicting the acute cardiovascular and pulmonary effects to be expected in man after administration of an intravenous dosage form of the drug, WR 142,490·CH_3SO_3H.

b. Methods:

Six adult beagle dogs weighing between 8.0 and 10.9 kg and 5 mongrel dogs weighing between 14.5 and 21.4 kg were utilized as experimental animals as described above.

The medicinal chemical used in experiments 61 through 64 and 67 through 71 was from lot AJ, bottle number BE 16485. The drug was dissolved in equal parts of propylene glycol, U.S.P. and water for injection, U.S.P. The intravenous doses administered ranged from 10 mg/kg over a 15 min period to 10 mg/kg/min for 5 min (50 mg/kg total dose).

The drug material used in experiments 51 and 52 was from lot AG, bottle number BE 08670. The drug was prepared at a concentration of 25 mg/ml in equal parts of propylene glycol and water, and was subsequently diluted to 3 mg/kg with 5% dextrose

in water. A total of 15 mg of WR 142,490·CH_3SO_3H per kg of body weight was administered over a 30 min period to dog No. 52 and over a 60 min period to dog No. 51.

c. Results:

Immediately after the start of the drug infusion, there was a dose related decrease in both systolic and diastolic arterial blood pressure (Tables 12 and 13). The pressure continued to decline during the 4 min injection period and then it slowly returned to control levels.

As shown in Table 14, the drug produced a dose dependent decrease in heart rate in each experimental animal. This bradycardia developed within the first min after the drug infusion was started and was greatest after 2 to 5 min. Thereafter the rate in the majority of animals returned to within 85 percent of control values within 2 hr.

The drug caused a marked increase in the respiratory rate which appeared to be dose-related (Table 15). As the rate increased, the depth of respiration decreased. The rate increase was greatest after 3 to 4 min and in 2/3 of the animals was still significantly elevated after 2 hr.

Dog No. 63, which received a dose of 72 mg/kg of body weight over a 4 min period, died 5 min after the injection was completed.

The results of a slow intravenous infusion of 10 or 15 mg of drug per kg of body weight on blood pressure, heart rate and respiratory rate are shown in Tables 16 and 17. Neither the 60 min infusion, the 30 min infusion nor the 15 min infusion caused significant changes in these parameters.

The effect of rapid infusion (4 min) of WR 142,490·CH_3SO_3H on the duration of the P-R interval and the Q-T interval of the electrocardiogram and the heart rate is shown in Tables 18 and 19. As shown in Table 18, the drug treatment produced a dose-related increase in the duration of both of these segments of the electrocardiogram. The maximum increase in duration occurred 5 and 15 min from time zero for the P-R and Q-T intervals, respectively. Dog No. 70 (Table 19) showed a similar pattern except for loss of the P-wave during the 15 and 20 minute time intervals. Typical tracings of the electrocardiogram taken at each time period are shown

in Figures 5 and 6. In addition to the prolonged P-R and Q-T intervals, the drug appeared to induce nonspecific changes in the morphology of the T wave. The notched T wave which appeared within one min after time zero did not revert to a normal pattern during the 2 hr course of the experiments.

d. Discussion:

It was observed that the intravenous administration of WR 142,490 methanesulfonate to anesthetized dogs at a rate of 10 to 15 mg/kg/min for 4 to 5 min produced a dose-related hypotension, increased respiratory rate and both an inotropic and chronotropic effect on the heart. The blood pressure and respiration returned to control levels within 30 min to 1 hr, whereas the heart rate was about 75 percent of control values after 2 hr. There was electrocardiographic evidence of impaired pace-maker function and intraventricular conduction, as well as prolongation of the P-R and Q-T intervals. These experiments suggest that WR 142,490·CH_3SO_3H would be well tolerated in intravenous doses of up to 20 mg/kg administered slowly over a 5 to 10 min period, whereas higher doses or more rapid administration could lead to serious adverse effects on the cardiovascular and respiratory systems.

5. Studies on the oral efficacy and toxicity of candidate antimalarials and the effect of modification of host drug-metabolizing capacity on these parameters.

a. Background:

On the basis of the rationale and methodology set forth in the previous 2 annual progress reports, we have continued investigations of candidate antimalarials of the 8-aminoquinoline class in mice, and, whenever pertinent, have used primaquine (WR 2975) as a standard for comparison.

b. Determination of the acute oral LD-50 (7-day endpoint) of WR 181,023 (4-methylprimaquine) in control mice and in mice pretreated with phenobarbital or SKF 525-A:

We previously reported that WR 181,023, on single oral dosing, was about half as toxic as primaquine, judging from LD-50 (7-day endpoint) determinations, and that its blood schizontocidal activity (blood-induced infection) was greater.

From comparisons of the effects of phenobarbital or SKF 525-A pretreatment on the oral efficacy and acute oral toxicity of

WR 181,023 and primaquine, we concluded that the metabolic handling of these 2 agents by the mouse was different. For example, phenobarbital or SKF 525-A pretreatment altered the blood schizontocidal activity of primaquine but not that of WR 181,023. Moreover, while phenobarbital pretreatment markedly reduced the lethal toxicity of primaquine, it did not significantly alter the lethality of an otherwise acute oral LD-50 of WR 181,023. If anything, lethal toxicity tended to be slightly greater after phenobarbital pretreatment.

To evaluate this tendency more systematically, we concurrently determined the acute oral LD-50 (7-day endpoint) of WR 181,023 in control mice and in mice pretreated with phenobarbital or SKF 525-A. The results in Table 20 indicate that neither of the pretreatment regimens had a significant effect on the LD-50. However, in conformity with our previous observations, the LD-50 after phenobarbital was somewhat lower than for the other two groups, i.e., lethal toxicity was again slightly higher. Consequently, we sought to find a possible basis for this trend.

c. Gross signs of hepatotoxicity after WR 181,023 and the effects thereon of phenobarbital or SKF 525-A pretreatment:

In the course of our studies, we have observed noteworthy differences in the acute toxic manifestations and gross morphological alterations induced in mice by large single oral doses of primaquine and WR 181,023. For example, primaquine causes overt swelling of the mouse's snout and tongue, while WR 181,023 does not. Gross striated muscle lesions are seen in 7-day survivors or in late dying mice only after primaquine. In contrast, WR 181,023, unlike primaquine, produces a variable incidence of gross abnormalities of the liver, including apparently necrotic changes.

Because of the tendency of WR 181,023 to cause a somewhat greater lethality after phenobarbital pretreatment, we tallied the incidence of WR 181,023-related gross liver changes noted on autopsy of mice killed one week after they had received an otherwise acute oral LD-50 (710 mg salt/kg) of WR 181,023. In the 5 experiments reviewed, controls had been studied concurrently with phenobarbital and SKF 525-A pretreated mice.

Table 21 shows that while gross liver abnormalities due to WR 181,023 were present in all 3 groups, their incidence and severity were clearly greater in mice pretreated with phenobarbital.

It seems reasonable to consider, therefore, that the somewhat greater lethality of WR 181,023 in phenobarbital pretreated mice may be related to an exacerbation of its hepatotoxicity. Detailed histopathological evaluation would prove helpful in this regard. However, it is well documented, e.g., that phenobarbital pretreatment exacerbates the hepatotoxic sequelae of carbon tetrachloride in rodents.

It thus appears that WR 181,023 may normally undergo chemical transformation in the mouse's liver. The metabolite(s) produced might be highly reactive, with a potential for causing damage at those sites in the liver where it is produced.

While reviewing our autopsy findings incident to the above study, we noted that, irrespective of pretreatment, four 7-day survivors that had received single oral doses (440, 710 or 850 (2 mice) mg salt/kg) of WR 181,023 had a mass of water-clear gelatinous matter overlying the mesentery in the vicinity of the pancreas. We could only speculate that this might reflect a pancreatitis, with a possible local escape and action of digestive enzymes from the pancreas. Recently, C.C. Lee et al., (1975) reported that daily oral treatment with 12 mg base/kg of WR 181,023 for 28 consecutive days, a lethal regimen, produced fatty degeneration of the islets of Langerhans in 3 of 4 rhesus monkeys studied. Thus, it is possible that WR 181,023 in large single oral doses may have an adverse effect on the mouse's pancreas as well, but this remains to be demonstrated histologically.

Among other pathological changes reported by Lee with subacute oral doses of WR 181,023 that proved lethal, monkeys exhibited fatty degeneration and centrolobular necrosis of the liver and fatty degeneration of the kidney. The liver changes in monkeys are clearly relevant to the gross liver findings we have reported for mice (Table 21). It is noteworthy that, irrespective of pretreatment regimen, we also encountered an incidence of definitely abnormal coloration (pale orange-brown) of the kidneys in mice 7 days after large single oral doses of WR 181,023. The pathologic significance of this, if any, remains to be established.

d. Comparison of the oral parasitemia-suppressing activities of primaquine (WR 2975) and its dextro-(WR 211,536) and levo-(WR 211,537) enantiomers and of the effects thereon of modification of host drug-metabolizing capacity:

Primaquine has served effectively as a tissure schizontocide since the Korean War. However, as with all 8-aminoquinoline

antimalarials so far tested, primaquine usage in man has been associated with problems of drug intolerance. Recently, primaquine, a racemic mixture without optical activity, has for the first time been chemically resolved into its optically active dextro-and levo-enantiomers. Since optical isomers of various drugs are known to have different pharmacological and toxicological properties, this engendered the hope that the clinically useful properties of primaquine might ultimately be shown to belong principally, if not exclusively, to one of its optically active forms. If so, then the clinically adverse and the clinically useful properties of primaquine might be separable. Consequently, we undertook to learn whether non-infected or P. berghei-infected mice respond differently to the racemate and its dextro-and levo-isomers.

Table 22 presents a comparison of 12 groups of mice that were studied in parallel. The data shown were pooled from two identical experiments since the results from each were similar.

The results in Table 22 may be summarized briefly as follows. First, all 3 agents, given once in equal dosage by gavage, exhibited parasitemia-suppressing activity (Group I vs Groups IV, VII and X). Second, parasitemia suppression after WR 211,537 (Group X) was significantly less than after either WR 2975 (Group IV) or WR 211,536 (Group VII). However, a significant difference between the latter two treatment groups was not demonstrated. We have not yet been able to account for this difference, although a number of possible explanations exist. Third, the pretreatment regimens of phenobarbital or of SKF 525-A alone had no significant effect on the course of P. berghei infection (Group I vs Groups II and III). Fourth, phenobarbital pretreatment significantly reduced the efficacy of all 3 agents (Group V vs Group IV; Group VIII vs Group VII; and Group XI vs Group X). Fifth, SKF 525-A pretreatment significantly increased the efficacy of all 3 agents (Group VI vs Group IV; Group IX vs Group VII; Group XII vs Group X).

The findings that modification of host drug metabolism by both pretreatment regimens significantly affected the oral efficacy of all 3 agents, and that phenobarbital stimulation and SKF 525-A inhibition decreased and increased efficacy, respectively, suggest that: the liver ordinarily processes these agents; this processing serves to terminate their parasitemia-suppressing activity which is, therefore, due to the administered parent chemicals and not to metabolic by-products; and finally, medications known to alter drug metabolism may affect the activity of these agents.

It is conceivable that the parent chemicals may account for blood schizontocidal activity while their metabolites may account for tissue schizontocidal activity. This possibility merits investigation since the liver, as the primary locus of tissue forms of the malaria parasite, is a primary target of tissue schizontocidal chemotherapy.

e. Comparison of acute toxic swelling, cumulative mortality and incidence of gross skeletal muscle lesions in mice given equal single oral doses of WR 2975 (racemate) or its optical isomers, WR 211,536 (+) and WR 211,537 (-):

In previous studies, we have ascertained that oral administration of single high doses of primaquine to mice results in characteristic acute toxic manifestations within a few hr after challenge and also in certain morphological changes which can be detected by gross examination a few days after challenge. Acute overt toxicity is mainly in the form of erythema and progressive swelling of the snout (including lips) and tongue (S/T swelling). The gross morphological changes that are seen on autopsy of 7-day survivors or in mice suffering delayed deaths several days after challenge consist of varying degrees of necrosis of the tongue and/or diaphragm. We have also observed that lethality after oral administration of single toxic doses of primaquine can be divided into 2 categories: acute death occurring within one hr after challenge, often in less than 30 min; and delayed death occurring well after the onset of S/T swelling (which begins about 3 hr after challenge), usually between 6 to 24 hr postchallenge. The incidence of acute deaths increases as the dose of primaquine is increased, especially above the LD-50 (7-day endpoint).

Table 23 shows the results of the first 3 experiments (Experiments A, B, and C) we conducted to compare the toxicity of primaquine and its optically active isomers. The results indicate that the levo-isomer produced neither S/T swelling nor diaphragm or tongue necrosis after any of the 4 doses tested, which ranged from lethal to subletnal (Groups XI and XIII) levels. In contrast, the dextro-isomer produced both S/T swelling and necrosis of the diaphragm and tongue. In fact, swelling appeared somewhat earlier and was more severe than after WR 2975 (e.g., compare S/T swelling between Groups V and IV or Groups VII and VIII).

All but one (Group VI) of the deaths produced by the levo-isomer occurred within 1 hr after oral challenge and usually before 30 min. Thus, the acute form of death was characterisitic of this agent. Those animals surviving beyond 1 hr ultimately survived 7 days and appeared in excellent health. Lethal toxicity was also

somewhat less than for the dextro-form or the racemate. In contrast, the dextro-isomer exhibited the greatest lethal toxicity of all 3 agents. Deaths, at the doses tested, never occurred within 1 hr postchallenge but were invariably of the delayed type.

In summary, this initial study of the comparative toxicity of the optical isomers of primaquine has revealed that each of the isomers, within the dose range studied, has a distinctive toxicological profile. Moreover, the toxicological profile of the racemate appears to reflect, at the doses studied, an admixture of the differential toxicological properties of its enantiomeric components. The dextro-component (WR 211,536) appears responsible for the production of the acute toxic manifestations, the skeletal muscle lesions and the delayed deaths, whereas the levo-components (WR 211,537) appears to account only for the acute toxic deaths seen with higher doses of the racemate.

f. Comparison of the acute oral LD-50 (7-day endpoint) of WR 2975 and its levo-(WR 211,537) and dextro-(WR 211,536) isomers:

According to the LD-50 (7-day endpoint) data in Table 24, the least toxic among the 3 agents was the levo-form, WR 211,537, (Group II) and the most toxic was the dextro-form, WR 211,536 (Group III). The latter was more than 3 times as toxic as the racemate and more than 4 times as toxic as the levo-isomer. The LD-50's of the optical isomers were significantly different from each other and from that of the racemate. If we consider that the acute oral LD-50 of the optically inactive racemate (310 mg salt/kg) is, in fact, comprised of equal portions (155 mg/kg) of the dextro- and levo-forms, and that the LD-50 of the dextro-form is only 96 mg salt/kg (Group III), then it seems that the co-administration of the levo-component in the racemate somehow tends to reduce the lethal toxicity of the associated dextro-component (155 mg salt/kg).

Table 25 shows the cumulative mortality and the incidence of skeletal muscle lesions in 7-day survivors after all doses of the respective agents in the course of determining the LD-50's in Table 24. In support of our initial observations in Table 23, it is apparent that the levo-isomer produced neither delayed deaths nor skeletal muscle lesions and that 98% of all deaths that occurred by the end of day 7 resulted within the first hr after oral challenge.

In contrast, both the racemate and the dextro-isomer produced delayed deaths and a similar incidence of skeletal muscle lesions, but only the dextro-form caused no acute deaths. Moreover,

it is evident that mice given the dextro-isomer suffered a greater incidence of delayed deaths between the second and seventh days postchallenge (33%) than did mice given the racemate (9%).

Since the levo-isomer (WR 211,537) produced deaths almost exclusively within the first hr of the 7-day observation period, the LD-50 for the 1 hr and 7-day endpoints is the same for this agent. The mechansim of death after this agent, therefore, appears to be different than for the other 2 agents.

g. Comparison of the acute oral LD-50 (1 hr endpoint) of WR 2975 and its levo-(WR 211,537) and dextro-(WR 211,536) isomers:

In view of the foregoing findings, we thought it would be useful to determine the single oral dosages of WR 211,536 and WR 2975 that would suffice to produce a mortality of 50% within the first hr after challenge and to compare these LD-50's (1 hr endpoint) with the LD-50 of WR 211,537.

Table 26 shows the results of this study. The least toxic of the agents was clearly the dextro-isomer. For this isomer, the LD-50 (1 hr endpoint) (Table 26) was over 6 fold that of its LD-50 (7-day endpoint) (Table 24). Whereas according to the 7-day LD-50's (Table 24) the 3 agents, in order of decreasing toxicity, were WR 211,536 > WR 2975 · WR 211,537, the corresponding order according to the 1 hr LD-50 was reversed. Although the 1 hr LD-50's of WR 211,536 and the other two agents were significantly different from each other, the 1 hr LD-50's of WR 211,537 and WR 2975 were not.

Since the 1 hr LD-50 of the racemate (423 mg salt/kg is comprised of ca 212 mg salt/kg of each of the isomers, it would seem that the racemate's dextro-component synergizes adversely with its levo-component to potentiate the latter's acutely lethal toxicity. It appears, therefore, that in the case of the single oral 1 hr LD-50 and acute lethality (Table 26), the racemate's dextro-component may potentiate the lethal toxicity of its levo-component, whereas in the case of the single oral 7-day LD-50 and delayed lethality (Table 24) it appears that the racemate's levo-component may antagonize the lethal toxicity of its dextro-component.

h. Effect of prior stimulation (phenobarbital) or inhibition (SKF 525-A) of drug metabolizing enzymes on the toxic reactions of mice to an otherwise acute oral LD-50 (7-day endpoint) of WR 2975, WR 211,536 or WR 211,537:

Table 27 summarizes the data obtained from 9 groups of mice that were studied in parallel and subjected to the experimental and control manipulations indicated.

i. WR 2975 (racemate):

The results (Table 27) show that WR 2975 when given to control mice (Group I) produced severe S/T swelling, both acute and delayed mortality, and a high incidence of diaphragm and/or tongue lesions in 7-day survivors.

Phenobarbital pretreatment (Group II) completely protected mice against all of these consequences of WR 2975 challenge, whereas SKF 525-A pretreatment (Group III) did not. SKF 525-A pretreatment, however, did clearly delay the onset and rate of development of S/T swelling and prevented acute deaths without affecting overall 7-day mortality.

ii. WR 211,536 (dextro-isomer):

Table 27 shows that the results (Group IV to VI) after WR 211,526 were almost identical in all respects to those after WR 2975, except for the following: in control mice, the time of onset of S/T swelling was somewhat earlier and its peak severity was greater after WR 211,536 (Group IV) than after WR 2975 (Group I); WR 211,536 did not cause any acute deaths in control mice (Group IV); and while phenobarbital pretreatment prevented S/T swelling in all 20 mice given WR 2975, it did the same (Group V) for 18 of 20 mice given WR 211,536 (the two mice that did react to WR 211,536 exhibited only a minor degree of swelling which did reflect partial protection by phenobarbital pretreatment).

iii. WR 211,537 (levo-isomer):

Table 27 shows that, irrespective of pretreatment regimen (Groups VII to IX), WR 211,537 produced neither S/T swelling nor skeletal muscle lesions. While we did not expect these adverse phenomena to occur in control mice (Group VII), we could not be sure that prior modification of host drug metabolism would not alter this picture.

Seven of eight control mice (Group VII) died within 1 hr after challenge with WR 211,537. The eighth mouse became severely ill during the first hour post challenge and showed no improvement until it died between 6 and 24 hr post challenge. Both phenobarbital (Group VIII) and SKF 525-A pretreatment (Group IX) protected mice against the lethal toxicity of WR 211,537.

The results of phenobarbital or SKF 525-A pretreatment on the various parameters of WR 2975-associated toxicity evaluated in this study are in agreement with our previous findings. By focusing on these parameters, we have also been able to discern that some of the toxic sequelae of WR 2975 are specifically ascribable to either one or the other of its constituent isomers (Tables 23, 25 and 27). In this study (Table 27) we have learned how the pretreatment regimens affect the isomer-specific toxic sequelae. While phenobarbital pretreatment protected mice against the toxic effects of the racemate and the dextro-and levo-isomers, SKF 525-A pretreatment had slight but demonstrable impact on the effects of the former two agents but a very strong effect on the latter agent as it obviated its acutely lethal effects.

Recently we submitted formalin-fixed tongues and diaphragms for histopathological evaluation from mice surviving 7 days after large oral doses of WR 2975, WR 211,536 and WR 211,537 to Dr. Loren Kintner (under the auspices of Dr. C. C. Lee) of Midwest Research Institute, Kansas City, Mo. His findings have substantiated our gross findings, viz., that only WR 2975 and WR 211,536 produce striated muscle lesions and that pretreatment with phenobarbital, but not with SKF 525-A, protects mice against doses of WR 2975 that otherwise induced muscle lesions.

Several pertinent possibilities must eventually be tested if one is to fully elucidate the mechanisms. We do not know at present whether the dextro- and levo-isomers are absorbed into the bloodstream from the gastro-intestinal tract to an equal extent. Nor do we know how the unchanged optical isomers or their possible metabolites are handled by the kidney, and hence to what extent these may be eliminated. While we cannot dismiss that SKF-525-A is accomplishing its protective role, wholly or in part, via inhibition of drug-metabolizing enzymes of the mouse, neither can we dismiss the reports of extrametabolic effects of this agent, e.g., its reputed effects on gastric emptying in rats which may lead to decreased drug absorption. In addition, since SKF 525-A pretreatment obviates acute lethality due to the levo-isomer even though this regimen increased this isomer's parasitemia-suppressing potency, further differentiation of mechanism interrelations would be desirable.

6. Development of new antimalarial drugs.

a. Background:

The Department of Pharmacology is also charged with the responsibility of writing Notice of Claimed Investigational Exemption for New Drug (IND) submissions. These include planning

and designing the experiments, and assembling, evaluating, coordinating and correlating the data required for both the initial submission and all supplementary submissions for each drug. The data must be continuously monitored and evaluated from both in-house and contract sources, as well as proprietary and open literature sources.

b. Investigational New Drug submissions:

Two new IND applications were written. They were WR 184,806·H_3PO_4 and WR 142,490·CH_3SO_3H.

Fifteen supplements to IND submissions were written. They were for 11 single drugs and 4 combinations.

c. Technical monitoring of contracts necessary for data generation:

Fourteen active contracts were closely guided by the Department. These ranged from pharmacological areas such as toxicology, drug metabolism and bioavailability of the drugs to those of their formulation, and development of methods to determine blood levels of drugs.

Table 1

The Percent of the Dose of Total Radioactivity Derived from WR 184,806·H_3PO_4-^{14}C Recovered in the Urine, Feces and Expired Air of Mice after Oral Administration of 10 mg/Kg[a]

Sample	Percent ^{14}C Recovered						
	8 hr	24 hr	48 hr	72 hr	96 hr	120 hr	Total
Urine	7.97	8.02	8.08	1.54	0.67	0.22	26.50
Feces	10.61	24.56	24.95	7.92	2.66	1.27	71.97
Expired Air[c]	0.01	0.01	0.02	0.01	0.01	N.S.[b]	0.06
Total	18.59	32.59	33.05	9.47	3.34	1.49	98.53

[a]Average of two groups containing four mice each.

[b]N.S. = No Sample.

[c]One group of four mice only.

Table 2

The Percent of the Dose of Total Radioactivity Derived from WR 184,806·H_3PO_4-^{14}C Recovered in the Urine and Feces of Mice after Intraperitoneal Administration of 10 mg/Kg[a,b]

	Percent ^{14}C Recovered				
Sample	8 hr	24 hr	48 hr	72 hr	Total
Urine	1.70	5.55	5.60	6.60	19.45
Feces	0.65	13.30	19.70	9.70	43.35
Total	2.35	18.85	25.30	16.30	62.80

[a] Average of two groups containing four mice each.

[b] The remaining radioactivity was recovered from the carcasses (37.2%).

Table 3

The Percent of the Dose of Total Radioactivity Derived from WR 184,806·H_3PO_4-^{14}C Recovered from Selected Organs, Excreta and Carcass of the Mouse[a]

Tissues	Percent ^{14}C Recovered		
	2 hrs[c]	8 hrs[c]	24 hrs[c]
Gall bladder + bile	0.05	0.08	0.10
Lungs	7.17	8.94	5.34
Heart	1.07	0.54	0.36
Submaxillary salivary glands	0.74	0.85	0.50
Liver	9.57	11.20	6.18
Spleen	0.84	1.11	0.43
Kidneys	3.04	2.76	1.40
Skeletal muscle[d]	16.20	15.90	6.50
Stomach	2.43	1.31	0.40
Small intestine	11.53	11.67	4.80
Cecum	0.64	1.16	0.59
Large intestine	1.97	2.16	1.02
Intestinal contents	32.11	17.28	15.01
Feces	0.46	9.12	30.75
Urine	0.26	2.44	16.57
Residual carcasses[e]	21.55	24.89	12.91
Total Recovery (%)	109.63	111.41	102.86

[a] WR 184,806·H_3PO_4-^{14}C was administered orally by gavage at 10 mg/kg.
[b] Methanolic homogenates.
[c] Hours postdose at sacrifice, four mice per period.
[d] Calculated as 25% of total body weight.
[e] Calculated skeletal muscle value is not included.

Table 4

TLC Analysis of the Total Radioactivity in Selected Tissues after Oral Administration of WR 184,806·H_3PO_4-^{14}C to the Mouse[a]

	Percent of ^{14}C in the Sample					
	2 hrs[b]		8 hrs[b]		24 hrs[b]	
	D[c]	M[d]	D[c]	M[d]	D[c]	M[d]
Gall bladder + bile	15.15	84.85	13.70	86.30	12.70	87.30
Lungs	91.30	8.70	85.00	15.00	73.00	27.00
Heart	91.80	8.20	100.00	0.00	35.30	64.70
Submaxillary salivary glands	73.60	26.40	81.80	18.20	100.00	0.00
Liver	85.70	14.30	74.00	26.00	56.50	43.50
Spleen	100.00	0.00	94.70	5.30	53.80	46.20
Kidneys	72.80	27.20	63.00	37.00	77.00	23.00
Stomach	72.70	27.30	77.00	23.00	60.00	40.00
Stomach contents	94.00	6.00	88.20	11.80	13.50	86.50
Small intestine	85.20	14.80	80.40	19.60	50.80	49.20
Small intestine contents	70.70	29.30	51.40	48.60	32.60	67.40
Cecum	86.50	13.50	51.60	48.40	34.10	65.90
Cecum contents	46.10	53.90	17.25	82.75	10.65	89.35
Large intestine	99.00	trace	68.80	31.20	50.00	50.00
Large intestine contents	64.50	35.50	28.70	71.30	15.15	84.85
Feces	72.70	27.30	39.50	60.50	21.10	78.90
Urine	45.00	55.00	57.90	42.10	64.00	36.00
Carcasses	89.70	10.30	83.00	17.00	70.20	29.80
RBC	36.70	63.30	42.10	57.90	20.30	79.70
Plasma	14.25	85.75	13.60	86.70	7.06	92.94

[a] WR 184,806·H_3PO_4-^{14}C was administered orally by gavage at 10 mg/kg.
[b] Hours post dose at sacrifice, four mice per time period.
[c] The area of radioactivity with the R_S value closest to the standard for WR 184,806-^{14}C.
[d] All radioactive peaks not included under [c].

Table 5

Percent Dose of WR 180,409-$^{14}C \cdot H_3PO_4$ Recovered In Mouse Expired Air[a]

Time Period[b]	% Dose
0-12	0.028
12-24	0.010
24-48	0.002
48-72	0.135
72-96	0.088
96-120	0.007
120-144	0.030
144-168	0.012
168-192	0.008
Total	0.320

[a] 20 mg/kg of drug given orally by gavage to a male mouse.

[b] Hours postdose.

Table 6

Percent Changes in Blood Pressure, Heart Rate, and Respiratory Rate in Response to a Series of Increasing Intravenous Doses of WR 184,806·H_3PO_4[a]

	Dose (mg/kg of body weight)							
	0.5	1	2	4	8	16	32	64
Percent change in Blood pressure systolic / diastolic	-2±4 / -5±5	-3±6 / -7±6	-6±2 / -13±3	-5±2 / -21±2	-8±7 / -30±5	-21±5 / -50±5	-48±2 / -71±1	[b]
Percent change in heart rate	1±1	3±4	1±3	7±3	6±4	-7±6	-13±10	[b]
Percent change in respiratory rate	-5±6	4±9	9±8	21±3	20±3	86±33	356±62	[b]

[a] Each value is the mean ± S.E.M. for 3 dogs (No. 50, 53, 57).

[b] One dog received this dose and died within 5 minutes.

Table 7

Pre-treatment Control Values in Dogs prior to administration of WR 184,806·H_3PO_4

Dog No.	Sex	Body Weight (Kg)	Blood Pressure (mmHg)	Respiratory Rate (cpm)	Heart Rate (bpm)
50	M	9.5	170/110	22	200
53	M	14.5	150/100	16	182
57	F	8.6	155/100	18	195
54	M	9.5	140/90	11	173
55	F	8.2	115/75	12	134
56	M	9.8	165/105	10	179
58	F	7.7	145/55	11	169
59	F	8.2	160/60	13	160
60	F	7.3	145/60	11	170

Table 8

Percent Change in Blood Pressure and Heart Rate Response to Graded Intravenous Doses of Epinephrine (Epi), Norepinephrine (NE), Isoproterenol (Iso), and Angiotensin (Angio) after Intravenous Administration[a] of WR 184,806·H_3PO_4 in the Dog.[b]

Drug	Dose (μg/kg)	Percent Change in Blood Pressure		Percent Change in Heart Rate
		Systolic	Diastolic	
Epi	0.25	-6.17±2.90	-6.67±6.34	-3.23±1.34
	0.50	-14.17±9.96	-1.53±6.61	-6.53±1.59
	1.00	-14.27±7.90	+4.17±12.21	-11.03±5.09
NE	0.25	-10.90±5.45	-0.57±3.68	-3.33±1.06
	0.50	-9.37±11.66	+0.73±11.13	-8.97±4.10
	1.00	-14.67±4.15	+6.93±19.24	-17.03±13.99
Iso	0.125	-2.6±2.27	-0.97±1.43	-1.87±1.53
	0.25	-2.47±4.70	-9.60±7.87	-9.83±6.79
	0.50	-1.50±8.50	-9.10±1.56	-10.57±9.38
Angio	0.2	-21.33±13.81	+3.87±12.27	-11.00±13.16
	0.4	-19.97±12.90	-2.63±4.15	-14.07±20.13
	0.8	-20.60±17.24	-0.67±17.56	-11.07±11.16

[a] After rapid intravenous injection of 8.0 mg/kg and during slow (30 min) infusion of an an additional 8.0 mg/kg of WR 184,806·H_3PO_4

[b] Each value is the mean ± S.E.M. for 3 dogs (No. 54, 55, 56).

Table 9

Changes in Blood Pressure (mmHg) in Response to Graded Intravenous Doses of Epinephrine (Epi) Obtained before and after Intravenous Doses of WR 184,806·H_3PO_4

		Before WR 184,806		After 8 mg/kg		After 16 mg/kg		After 0.5 mg/kg Propranolol and 16 mg/kg WR 184,806	
Drug	Dose (μg/kg)	sys[a]	dia[a]	sys[a]	dia[a]	sys[a]	dia[a]	sys[a]	dia[a]
Dog 58 Epi	0.25	15	15	10	15	5	10	5	5
	0.50	30	15	20	15	15	25	10	10
	1.00	35	25	35	25	25	25	30	20
Dog 59 Epi	0.25	10	15	5	15	5	15	b	
	0.50	15	20	20	20	25	20		
	1.00	40	25	35	30	30	35		
	2.00	70	40	50	40	55	40		
Dog 60 Epi	0.25	25	25	10	15	5	5	b	
	0.50	45	25	25	20	20	15		
	1.00	80	45	50	25	30	20		
	2.00	105	65	75	35	50	25		

[a] Systolic pressure = "sys", diastolic pressure = "dia".

[b] Animal died.

Table 10

Changes in Heart Rate in Response to Graded Intravenous Doses of Epinephrine (Epi) Obtained before and after Intravenous Doses of WR 184,806·H_3PO_4

Drug	Dose (μg/kg)	Before WR 184,806	After 8 mg/kg	After 16 mg/kg	After 0.5 mg/kg Propranolol and 16 mg/kg WR 184,806
Dog 58	0.25	18	14	12	no change
Epi	0.50	32	24	20	"
	1.00	52	40	39	"
Dog 59	0.25	6	-2	5	a
Epi	0.50	12	11	10	
	1.00	44	24	16	
	2.00	64	59	37	
Dog 60	0.25	2	0	0	a
Epi	0.50	15	0	0	
	1.00	36	13	3	
	2.00	58	40	22	

[a] Animal died.

Table 11

Percent Changes in Aortic Blood Flow in Response to Graded Intravenous Doses of Epinephrine (Epi) Obtained before and after Intravenous Administration of WR 184,806·H_3PO_4.[a]

Drug	Dose (μg/kg)	Control (Before WR 184,806)	After 8 mg/kg	After 16 mg/kg	After 0.5 mg/kg Propranolol and 16 mg/kg WR 184,806
Epi	0.25	12±8%	11±4%	14±1%	[b]
Epi	0.50	24±14	35±8	32±5	[b]
Epi	1.00	36±22	58±19	59±17	[b]
Epi	2.00	48±26	78±13	81±17	[b]

[a] Each value is the mean ± S.E.M. for three dogs (No. 58, 59, 60).

[b] Dogs No. 59 and 60 died within 10 minutes and dog No. 58 showed no changes in flow.

Table 12

The Effect of Intravenous Administration of WR 142,490·CH_3SO_3H on the Blood Pressure (mmHg) in the Dog[a]

Dog Number	Time 0 (Control)	Minutes after Start of Infusion								
		1 (15 mg/kg)	2 (30 mg/kg)	3 (45 mg/kg)	4 (60 mg/kg)	5	15	30	60	120
61	140[b]/95[c]	125/65	120/55	115/50	105/45	120/50	145/95	140/95	145/100	145/100
62	145/85	120/55	85/30	70/20	60/20	65/20	100/55	135/80	150/95	155/105
64	120/85	80/40	35/10	25/10	25/10	30/15	45/30	100/75	120/80	120/80

[a] Drug infusion started at time 0 at a rate of 15 mg/kg/min for 4 minutes.

[b] Systolic pressure.

[c] Diastolic pressure.

Table 13

The Effects of Intravenous Infusion[a] of 10 mg/kg/min of WR 142,490·CH_3SO_3H for 4 Minutes on the Blood Pressure, Heart Rate, and Respiratory Rate in Dog No. 70. Total Dose Administered was 40 mg/kg

	Minutes after Start of Infusion											
	0[a] (Control)	1	2	3	4	5	10	15	20	30	60	120	240
Blood Pressure (mmHg)	145[b]/100[c]	100/60	60/30	55/30	50/30	45/30	60/40	35/25	45/30	170/120	135/95	140/100	140/105
Heart Rate (bpm)	143	171	120	105	103	102	94	60	60	170	130	130	120
Respiratory Rate (rpm)	15	0	18[d]	18[d]	18[d]	18[d]	18[d]	66	18[d]	66	48	36	27

[a] Infusion started at time 0.
[b] Systolic pressure.
[c] Diastolic pressure.
[d] Artificially respired.

Table 14

The Effect of Intravenous Administration of WR 142,490·CH_3SO_3H on the Heart Rate (bpm) in the Dog[a]

Dog Number	Time 0 Control	Minutes after Start of Infusion								
		1 (15 mg/kg)	2 (30 mg/kg)	3 (45 mg/kg)	4 (60 mg/kg)	5	15	30	60	120
61	224 (bpm)	236	219	195	179	166	160	150	150	152
62	209 (bpm)	199	147	134	128	121	126	152	163	179
64	169 (bpm)	150	89	95	112	123	115	134	152	145

[a] Drug infusion started at time 0 at a rate of 15 mg/kg/min for 4 minutes.

Table 15

The Effect of Intravenous Administration of WR 142,490·CH_3SO_3H on the Respiratory Rate (rpm) in the Dog[a]

Dog Number	Time 0 Control	Minutes after Start of Infusion								
		1 (15 mg/kg)	2 (30 mg/kg)	3 (45 mg/kg)	4 (60 mg/kg)	5	15	30	60	120
61	23 (rpm)	30	40	54	81	75	24	27	24	30
62	22 (rpm)	45	72	78	75	69	57	54	36	36
64	20 (rpm)	25	b	75	75	63	42	51	60	69

[a] Drug infusion started at time 0 at a rate of 15 mg/kg/min for 4 minutes.

[b] Respiration was so rapid and shallow that it was impossible to determine a rate.

Table 16

The Effects of Slow Intravenous Infusion of 15 mg/kg of WR 142,490·CH_3SO_3H on the Blood Pressure, Heart Rate, and Respiratory Rate of the Dog

	Time (min)									
Dog No. 52 (30 min Infusion)[a]	0 Control	5	10	20	30	40	50	60	70	80
Blood Pressure (mmHg)	155[b]/105[c]	150/105	155/105	160/105	155/100	160/110	165/115	170/115		
Heart Rate (bpm)	188	188	188	184	184	184	180	180		
Respiratory Rate (rpm)	11	9	8	9	10	11	10	9		
Dog No. 51 (60 min Infusion)[a]										
Blood Pressure (mmHg)	195[b]/110[c]	190/110	195/110	195/110	195/110	195/110	195/110	195/110	200/115	200/115
Heart Rate (bpm)	132	132	132	130	130	128	128	128	128	128
Respiratory Rate (rpm)	48	49	49	44	44	43	43	46	43	39

[a] Infusion started at time 0.
[b] Systolic pressure.
[c] Diastolic pressure.

Table 17

The Effects on Blood Pressure, Heart Rate, and Respiratory Rate in Dog No. 70 of 10 mg/kg of WR 142,490·CH_3SO_3H Administered over a 15 Minute Period

	Time (min)										
	0[a] (Control)	1	2	3	4	5	10	15	20	30	40
Blood Pressure (mmHg)	140[b]/105[c]	140/105	145/105	140/105	145/105	145/105	140/105	140/100	145/110	145/110	145/105
Heart Rate (bpm)	113	111	115	125	122	122	120	120	115	115	120
Respiratory Rate (rpm)	25	25	25	30	33	39	51	54	49	46	35

[a] Infusion started at time 0.

[b] Systolic pressure.

[c] Diastolic pressure.

Table 18

The Effect of Intravenous Administration of WR 142,490·CH_3SO_3H on the Duration of the P-R and Q-T Intervals and the Heart Rate.[a]

	Minutes after Start of Infusion									
	Time 0 Control[b]	1 15mg/kg	2 30mg/kg	3 45mg/kg	4 60 mg/kg	5	15	30	60	120
Duration of P-R Interval (sec)	.083 ±.008	.084 ±.007	.099 ±.014	.108 ±.008	.123 ±.008	.125 ±.018	.120 ±.018	.111 ±.013	.096 ±.014	.092 ±.011
Duration of Q-T Interval (sec)	.193 ±.023	.196 ±.022	.211 ±.032	.223 ±.031	.223 ±.030	.224 ±.028	.225 ±.025	.213 ±.012	.212 ±.011	.211 ±.022
Heart Rate (bpm)	201 ±28	195 ±43	152 ±55	141 ±50	140 ±50	140 ±35	134 ±24	145 ±10	155 ±7	159 ±18

[a] Each value is the mean ± S.E.M. for 3 dogs (No. 61, 62, 64).

[b] Drug infusion started at time 0 at a rate of 15 mg/kg/min for 4 minutes.

Table 19

The Effect of Intravenous Infusion of WR 142,490·CH_3SO_3H on the P-R and Q-T Intervals and the Heart Rate in Dog No. 70

	Time (min)												
	0 Control[a]	1 10 mg/kg	2 20 mg/kg	3 30 mg/kg	4 40 mg/kg	5	10	15	20	30	60	120	240
Duration of P-R Interval (sec)	.080	.080	.100	.104	.120	.128	.140	b	b	.140	.104	.104	.104
Duration of Q-T Interval (sec)	.240	.208	.240	.240	.244	.240	.260	.284	.276	.244	.240	.240	.248
Heart Rate (bpm)	143	171	120	105	103	102	94	60	60	107	130	130	120

[a] Drug infusion started at time 0 at a rate of 10 mg/kg/min for 4 minutes.

[b] No P wave in evidence.

Table 20

Determination of the Acute Oral LD-50 (7-Day Endpoint) of WR 181,023 (4-Methylprimaquine) in Control Mice and in Mice Pretreated with Phenobarbital or SKF 525-A[a]

Group	Pretreatment i.p.	No. of Mice Used	LD-50[c] mg salt/kg	95% Confidence Limits mg salt/kg
I	Vehicle (Control)	90	729	671-794
II	Phenobarbital	90	634	581-692
III	SKF 525-A	50	722	643-810

[a] WR 181,023 was prepared in MCT (0.2% methyl cellulose and 0.4% Tween 80 in 0.9% sterile saline). The injection volume for all doses was 1% of the body weight (BW).

[b] Mice received: 1) a daily injection of phenobarbital sodium, 100 mg/kg, in sterile water or 2) water alone(control), 1% BW, for 3 days prior to oral dosing, or 3) one injection of SKF 525-A, 50 mg/kg in 0.9% sterile saline, 1 hr before oral dosing.

[c] None of the differences among the three groups is statistically significant.

Table 21

Incidence of Antimalarial-Related Gross Abnormalities of the Liver of Mice Surviving 7 Days after an Acute Oral LD-50 (7-Day Endpoint) of WR 181,023 and the Effects Thereon of Phenobarbital or SKF 525-A Pretreatment (Pooled Results of Five Experiments)

Group	Pretreatment[a], i.p.	No. of Mice Used	No. of Survivors Autopsied	Percent of Mice With: Liver Abnormalities: Frank Lesions[b]	Liver Abnormalities: Abnormal Color Only[c]	Liver Abnormalities: Total	Normal Appearing Livers
I	Vehicle (Control)	80	41	12 (5/41)	42 (15/36)	49 (20/41)	51 (21/41)
II	Phenobarbital	80	25	72[d] (18/25)	100 (7/7)	100 (25/25)	0 (0/25)
III	SKF 525-A	80	36	22 (8/36)	57 (16/28)	67 (24/36)	33 (12/36)

[a] See footnote b in Table 20.
[b] Includes mice with granulated (speckled) livers, and those with necrotic appearing focal (slight), focal diffuse(moderate) or confluent extensive (severe) lesions which were usually a dull yellow.
[c] Includes mice with livers that appeared mottled or had an abnormal dull brown color, exclusive of those with frank lesions.
[d] Difference from Group I and from Group III is statistically significant, $p<0.05$, by Chi-square with Yates' correction.

Table 22

Comparison of the Oral Parasitemia-Suppressing Activities of Primaquine (WR 2975) and Its Dextro- (WR 211,536) and Levo- (WR 211,537) Enantiomers and of the Effects Thereon of Phenobarbital or SKF 525 525-A Pretreatment in Mice with Blood-Induced P. berghei Infection

Group	Pretreatment[a]	Median % Parasitemia[b] (No. of mice)				
		Day of Infection				
		Before Oral Rx	After Oral Rx			
		3	6	8	10	14
		A. Vehicle Control p.o. on Day 3[c]				
I	Vehicle (Control)	2.4 (19)[d]	49.6 (19)	51.2 (11)	35.7 (6)	47.2 (4)
II	Phenobarbital	1.8 (20)	49.8 (20)	65.6 (9)	42.8 (5)	55.4 (3)
III	SKF 525-A	1.6 (20)	42.4 (19)	45.6 (9)	42.4 (5)	--- (2)
		B. WR 2975 p.o. on Day 3[e]				
IV	Vehicle (Control)	1.9 (20)	0.0+[f] (20)	5.2[f] (20)	25.9[f] (16)	52.8 (12)
V	Phenobarbital	1.8 (20)	6.4[g] (20)	11.6[g] (19)	31.6 (19)	64.0[g] (16)
VI	SKF 525-A	1.8 (20)	0.0[gh] (20)	0.4[gh] (20)	21.7[h] (20)	38.4[h] (9)
		C. WR 211,536 p.o. on Day 3[e]				
VII	Vehicle (Control)	2.0 (20)	0.0+[fi] (20)	2.0[fi] (20)	26.1[fi] (17)	45.6[i] (7)
VIII	Phenobarbital	1.8 (20)	4.4[j] (20)	10.8[j] (20)	32.6 (20)	62.6[j] (17)
IX	SKF 525-A	1.6 (19)[d]	0.0[jk] (19)	0.0+[jk] (19)	8.0[jk] (19)	23.6[k] (7)
		D. WR 211,537 p.o. on Day 3[e]				
X	Vehicle (Control)	2.2 (20)	11.8[fgj] (20)	14.7[fgj] (20)	38.2[gj] (20)	61.2[j] (17)
XI	Phenobarbital	1.6 (20)	35.8[l] (20)	38.8[l] (13)	42.4 ((9)	61.4 (8)
XII	SKF 525-A	1.2 (20)	7.2[lm] (20)	12.0[m] (20)	33.6 (20)	60.6 (17)

Table 22 (Continued)

[a] Pretreatment regimens consisted of: a daily i.p. injection of phenobarbital sodium, 100 mg/kg, in sterile water or of water alone (controls), for three days (Day 0, 1, 2) before oral dosing on Day 3; or a single i.p. injection of 50 mg/kg of SKF 525-A in 0.9% saline on Day 3, one hour before oral dosing.

[b] On Day 0, all mice were inoculated i.p. with ca 500,000 parasitized RBC of the drug-sensitive KBG 173 strain. Percentage parasitemia is routinely based on the examination of 250-300 RBC on a Giemsa-stained thin blood film. When this initial examination reveals no parasites, the blood film and its replicate are thoroughly scanned. Percentage parasitemia is designated 0.0+% if this secondary search reveals parasites and 0.0% if it does not. Values are not presented for fewer than three mice. The Mann-Whitney rank test was used for all statistical comparisons. Statistical significance was set at $p \leq 0.05$.

[c] The agent vehicle, in which all the antimalarials were dissolved, was 0.4% Tween 80 and 0.2% methyl cellulose in 0.9% sterile saline (MCT). The volume of all injections was 1% of the body weight.

[d] One mouse is excluded from the study as its parasitemia was 0.0% on Day 3 (and subsequently).

[e] Each agent is the diphosphate salt of which 57% is the base compound. The dose of 11.4 mg base/kg used for all agents was chosen because we had predetermined that this dose of WR 2975 is appropriately suppressive but subcurative in all mice tested.

[f] Difference from Group I is statistically significant (ss).
[g] Difference from Group IV is ss.
[h] Difference from Group V is ss.
[i] Difference from Group IV is not ss.
[j] Difference from Group VII is ss.
[k] Difference from Group VIII is ss.
[l] Difference from Group X is ss.
[m] Difference from Group XI is ss.

Table 23

Comparison of Acute Toxic Swelling, Cumulative Mortality, and Incidence of Skeletal Muscle Lesions in Mice Given Equal Single Oral Doses (Day 0) of WR 2975 (Racemate) and Its Optical Isomers WR 211,536 (+) and WR 211,537 (-)

Group[a]	Agent[b] and Dose (mg salt/kg) p.o.	Mean Degree of Overt Swelling of Snout/Tongue[c] (No. of Mice)				Cumulative Mortality, %				% Incidence of Gross Lesions of Diaphragm and/or Tongue in 7-day Survivors (No. of Survivors)
		Time postchallenge				Time postchallenge				
		Day 0				Day 0		Day 1	Day 7	
		3 hr	4 hr	5 hr	6 hr	1 hr	6 hr			
I	WR 2975 (320)	1.2/0.2 (9)	1.3/0.6 (9)	2.1/2.0 (9)	2.4/2.2 (9)	10	10	40	50	80 (5)
II	WR 211,536 (320)	2.8/2.0 (10)	2.9/3.4 (10)	3.0/3.8 (10)	3.0/3.9 (10)	0	10	80	100	-- (0)
III	WR 211,537 (320)	0.0/0.0 (6)	0.0/0.0 (6)	0.0/0.0 (6)	0.0/0.0 (6)	40	40	40	40	0 (6)
IV	WR 2975 (320)	0.6/0.0 (10)	1.4/0.6 (10)	2.6/2.2 (10)	2.9/2.5 (10)	0	0	60	60	50 (4)
V	WR 211,536 (320)	2.2/1.7 (10)	2.6/3.0 (10)	3.0/3.7 (10)	3.0/3.8 (10)	0	0	100	100	-- (0)
VI	WR 211,537 (320)	0.0/0.0 (7)	0.0/0.0 (7)	0.0/0.0 (7)	0.0/0.0 (7)	20	30	30	30	0 (7)

Table 23 (Continued)

VII	WR 2975 (160)	1.6/0.9 (10)	2.3/1.6 (10)	2.9/1.8 (10)	2.4/1.4 (10)	0	0	0	0	50 (10)
VIII	WR 211,536 (160)	2.6/2.6 (10)	2.9/3.5 (10)	2.9/3.8 (9)	2.8/3.8 (9)	0	10	50	90	100 (1)
IX	WR 211,537 (160)	0.0/0.0 (8)	0.0/0.0 (8)	0.0/0.0 (8)	0.0/0.0 (8)	20	20	20	20	0 (8)
X	WR 211,536 (80)	1/8/2.0 (10)	2.8/2.8 (10)	2.6/3.2 (10)	2.3/3.0 (10)	0	0	0	10	67 (9)
XI	WR 211,537 (80)	0.0/0.0 (10)	0.0/0.0 (10)	0.0/0.0 (10)	0.0/0.0 (10)	0	0	0	0	0 (10)
XII	WR 211,536 (40)	0.3/0.4 (5)	0.4/0.6 (5)	0.6/1.0 (5)	0.5/0.4 (5)	0	0	0	0	0 (5)
XIII	WR 211,537 (40)	0.0/0.0 (5)	0.0/0.0 (5)	0.0/0.0 (5)	0.0/0.0 (5)	0	0	0	0	0 (5)

[a] Ten mice were used per group for Groups I through XI. Five mice each were used for Groups XII and XIII.

[b] For Groups I to III (Experiment A), agents were dissolved in sterile pyrogen-free water. For Groups IV to IX (Experiment B), and X to XIII (Experiment C), agents were dissolved in 0.2% methyl cellulose and 0.4% Tween 80 in sterile 0.9% saline (MCT). Each of the agents is the diphosphate salt of which 57% is the base.

[c] Each mouse was picked up by the scruff of the neck and examined under a surgical lamp. Swelling of the snout (including lips) and the tongue were scored independently on an arbitrary scale of 1,2 or 3 for the former and 1,2,3 or 4 for the latter, always by the same observer. Scores intermediate between integral scores were denoted at intervals of 0.5. The absence of swelling was scored "0".

Table 24

Comparison of the Acute Oral LD-50's (7-Day Endpoint) of WR 2975 (Racemate) and Its Optical Isomers WR 211,537 (-) and WR 211,536 (+)

Group	Agent[a]	No. of mice used	LD-50 mg salt/kg	95% Confidence Limits mg salt/kg
I	WR 2975	90	310	279-344
II	WR 211,537	125	394[b]	349-444
III	WR 211,536	78	96[bc]	85-108

[a] Each of the agents is the diphosphate salt, 57% of which is the base. Each agent was prepared in MCT (0.2% methyl cellulose and 0.4% Tween 80 in 0.9% sterile saline) and all doses were given orally once in a volume equivalent to 1% of the body weight.

[b] Difference from Group I is statistically significant, $p < 0.01$.

[c] Difference from Group II is statistically significant, $p < 0.01$.

Table 25

Pooled Results on Mortality and Skeletal Muscle Lesions Incident to Determination of the Acute Oral LD-50's (7-Day Endpoint) in Table 24

Agent	Total No. of Mice Used	Overall Cumulative Mortality, as % of No. Used (Cumulative Mortality as % of the Total No. of Deaths by Day 7)					% Incidence of Gross Lesions of Diaphragm and/or Tongue in 7-day Survivors (No. Of Survivors)
		Day 0		Day 1	Day 2	Day 7	
		1 hr	6 hr				
WR 2975[a]	90	4 (9)	6 (12)	40 (84)	40 (91)	48 (100)	70 (47)
WR 211,536[b]	78	0 (0)	5 (8)	26 (42)	41 (67)	62 (100)	57 (30)
WR 211,537[c]	125	38 (98)	39 (100)	39 (100)	39 (100)	39 (100)	0 (76)

[a] Ten groups received single oral doses ranging from 160 to 583 mg salt/kg.

[b] Ten groups received single oral doses ranging from 40 to 166 mg salt/kg.

[c] Ten groups received single oral doses ranging from 40 to 640 mg salt/kg.

Table 26

Comparison of the Acute Oral LD-50's (1 Hour Endpoint) of WR 2975 (Racemate) and Its Optical Isomers WR 211,537 (-) and WR 211,536 (+)

Group	Agent[a]	No. of mice used	LD-50 mg salt/kg	95% Confidence Limits mg salt/kg
I	WR 2975	75	423	344-520
II	WR 211,537[b]	125	394[c]	349-444
III	WR 211,536	59	593[d]	520-676

[a] All agents were prepared in MCT. All doses were given in a volume equivalent to 1% of the body weight.

[b] The data for WR 211,537 are those from Table 24.

[c] Difference from Group I is not statistically significant.

[d] Difference from Group I and from Group II is statistically significant.

Table 27

Effect of Prior Stimulation (Phenobarbital) or Inhibition (SKF 525-A) of Drug Metabolizing Enzymes on the Response of Mice to an Otherwise Acute Oral LD-50 (7-Day Endpoint) of WR 2975, WR 211,536 or WR 211,537

Group[a]	Pretreatment[b] i.p.	Mean Degree of Overt Swelling of Snout/Tongue[c] (No. of Mice) Time postchallenge: Day 0				Cumulative Mortality, % Time postchallenge: Day 0		Day 1	Day 7	% Incidence of Gross Lesions of Diaphragm and/or Tongue in 7-day Survivors (No. of Survivors)
		3 hr	4 hr	5 hr	6 hr	1 hr	6 hr			
		A. Challenge (Day 0): WR 2975,[d] 310 mg salt/kg, p.o.								
I	Vehicle (Control)	0.7/0.5 (18)	2.4/1.4 (18)	2.6/2.0 (18)	2.6/2.6 (18)	10	10	20	30	79 (14)
II	Phenobarbital	0.0/0.0 (20)	0.0/0.0 (20)	0.0/0.0 (20)	0.0/0.0 (20)	0	0	0	0	0 (20)
III	SKF 525-A	0.0/0.0 (20)	1.1/2.5 (20)	0.9/1.2 (20)	2.4/1.8 (20)	0	0	20	45	73 (11)
		B. Challenge (Day 0): WR 211,536,[d] 96 mg salt/kg, p.o.								
IV	Vehicle (Control)	2.6/2.9 (20)	2.7/3.3 (20)	2.8/3.7 (20)	2.8/3.5 (20)	0	0	10	30	57 (14)
V	Phenobarbital	0.1/0.0 (20)	0.3/0.1 (20)	0.3/0.1 (20)	0.3/0.0 (20)	0	0	0	0	0 (20)

Table 27 Continued

VI	SKF 525-A	0.1/0.2 (20)	1.5/1.3 (20)	2.0/1.6 (20)	2.1/1.6 (20)	0	0	5	20	44 (16)
		C. Challenge (Day 0): WR 211,537,[d] 394 mg salt/kg, p.o.								
VII	Vehicle (Control)	0.0/0.0 (13)	0.0/0.0 (13)	0.0/0.0 (13)	0.0/0.0 (13)	35	35	40	40	0 (12)
VIII	Pheno-barbital	0.0/0.0 (19)	0.0/0.0 (19)	0.0/0.0 (19)	0.0/0.0 (19)	0	5	5	5	0 (19)
IX	SKF 525-A	0.0/0.0 (20	0.0/0.0 (20)	0.0/0.0 (20)	0.0/0.0 (20)	0	0	0	0	0 (20)

[a] Twenty mice per group.

[b] Pretreatment regimens consisted of: a daily i.p. injection of water only (control), 1% BW, or of phenobarbital sodium, 100 mg/kg, in sterile water for three days (Days -3, -2, -1) before oral challenge on Day 0; or a single i.p. injection of 50 mg/kg of SKF 525-A in 0.9% saline on Day 0, one hour before oral challenge.

[c] See Table 23, footnote c for method of scoring.

[d] All of the agents were prepared in MCT. The volume of injection was always 1% BW.

PLASMA CONCENTRATIONS OF WR 184,806 · H_3PO_4 – ^{14}C AND TOTAL METABOLITES IN MICE AFTER ORAL ADMINISTRATION OF 10 mg/kg

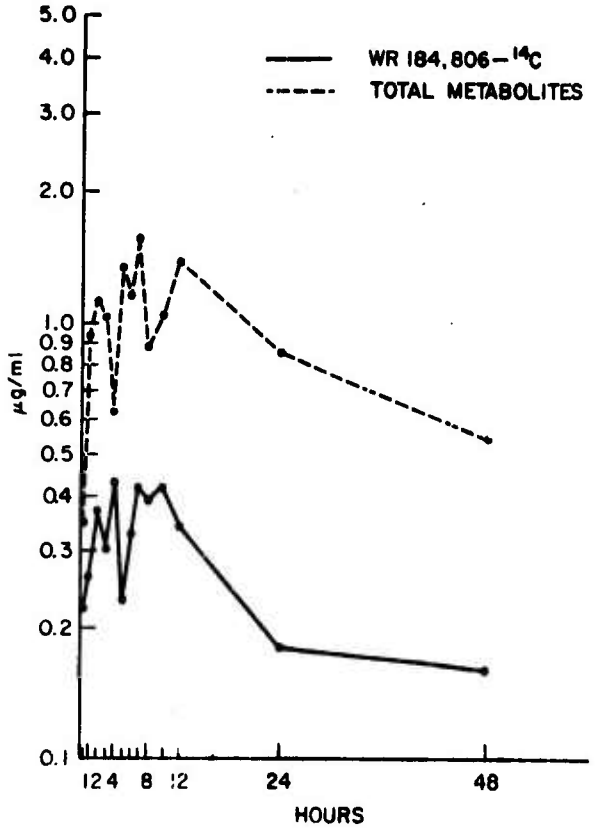


Figure 1

RED BLOOD CELL CONCENTRATIONS OF WR 184,806 · H_3PO_4 – ^{14}C AND TOTAL METABOLITES IN MICE AFTER ORAL ADMINISTRATION OF 10 mg/kg

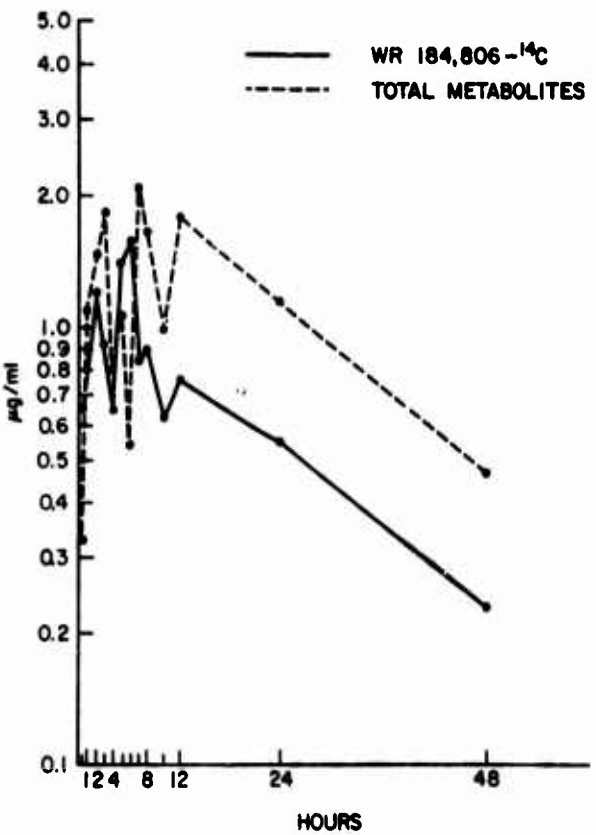


Figure 2

URINARY AND FECAL EXCRETION OF WR 180,409-^{14}C-H_3PO_4
AFTER A SINGLE ORAL DOSE (20mg/kg) TO FEMALE MICE

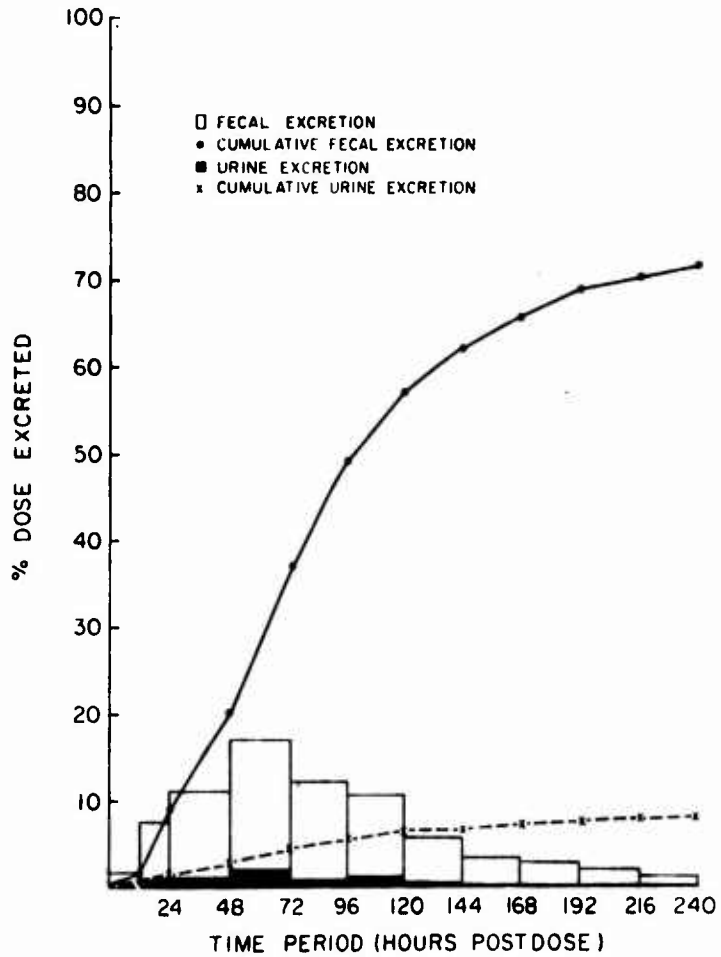


Figure 3

URINARY AND FECAL EXCRETION OF WR 180,409-^{14}C-H_3PO_4
AFTER A SINGLE ORAL DOSE (20 mg/kg) TO MALE MICE

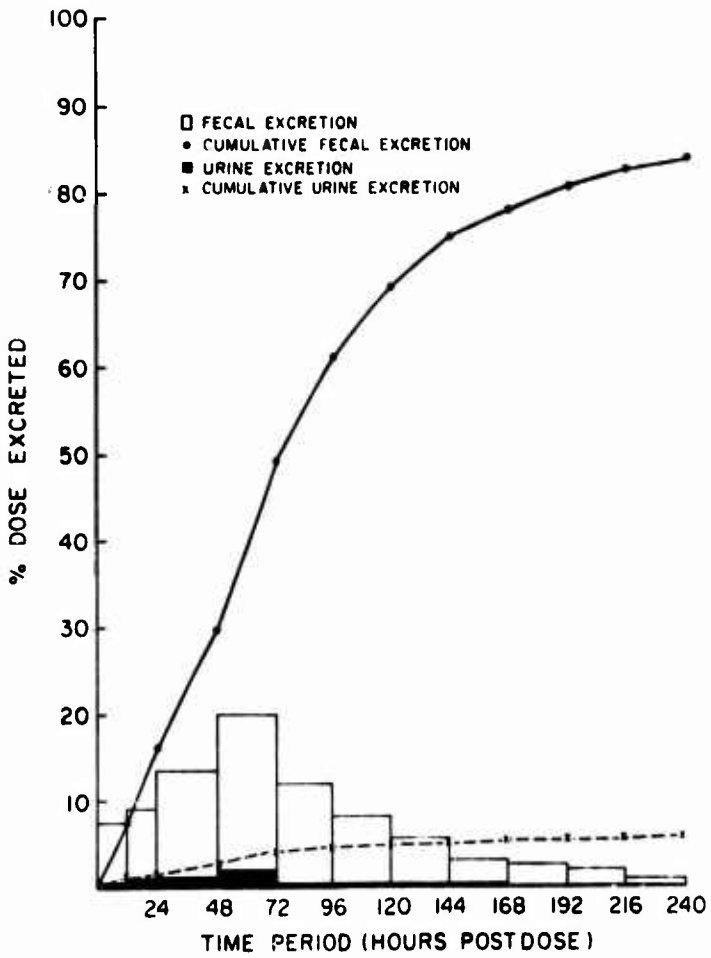


Figure 4

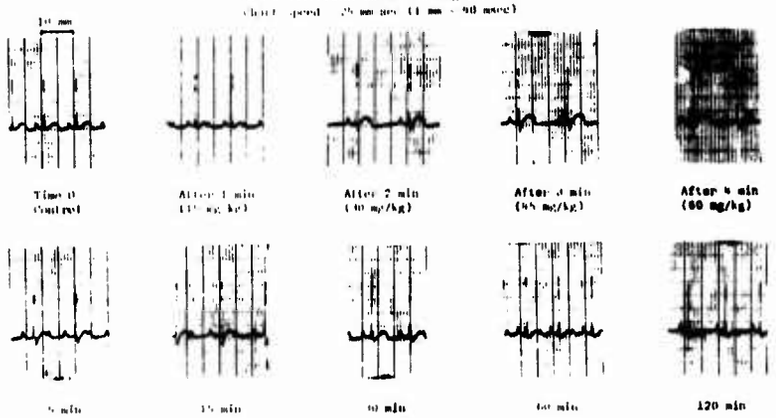
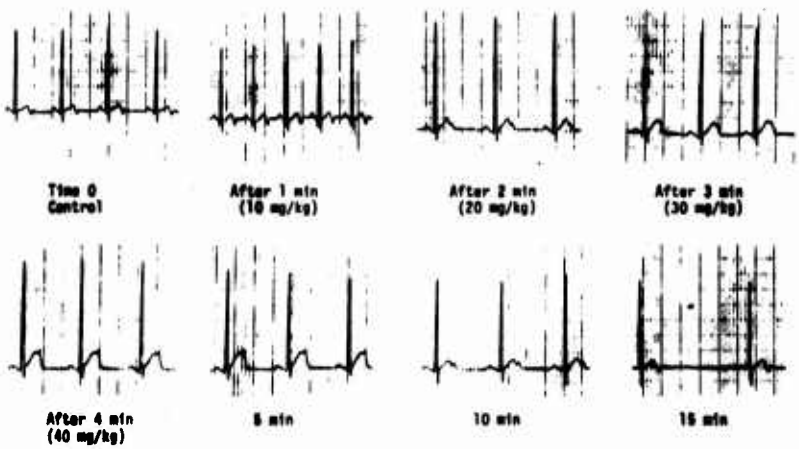

Time 0
Control
After 1 min
After 2 min
(40 mg/kg)
After 4 min
(80 mg/kg)
5 min
120 min

Figure 5

Electrocardiographic Effects Produced by Intravenous Infusion*
of WR 142,490·CH_3SO_3H in Lead CV_5 of Dog No. 70
Chart Speed - 25 mm/sec (1 mm = 40 msec)



* Drug infusion started at time 0 at a rate of 10 mg/kg/min for 4 minutes.

Figure 6

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 309 Determination of pharmacological effects of antimalarial drugs

Literature Cited.

References:

1. Lee, C. C., Crawford, C. R., Kintner, L. D., Sanyer, J. L., Hodgson, J. R., Reddig, T. W., Girvin, J. D., Seifert, W. K. and Olson, T. W.: Subacute oral toxicity of primaquine and 4-methylprimaquine in monkeys. Interim Report No. 95, Contract No. DAMD-17-74-C-4063, 18 April 1975.

2. Hayton, W. L. and Levy, G.: Effect of SKF 525-A on drug absorption in rats. Life Sciences. 10:691-697, 1971.

Publications:

1. Canfield, C. J. and Rozman, R. S.: Clinical testing of new antimalarial compounds. Bull. Wld. Hlth. Org. 50:203-212, 1974.

2. Grindel, J. M., Leany, D. M., Molek, N. A. and Rozman, R. S.: The absorption, distribution and excretion of 2,8-bis (trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl-1-^{14}C] quinoline phosphate (WR 184,806-^{14}C) in the mouse. Fed. Proc. 34:734, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6506	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	315
b. CONTRIBUTING				
c. CDK [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Blood Level Assays for Anti-malarial Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 002900 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	3	290
FISCAL YEAR CURRENT 76	3	209

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Antimalarials; (U) Pharmacokinetics; (U) Pharmacodynamics

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to study the pharmacokinetics of antimalarial drugs and to correlate these findings with pharmacodynamics in animal models in order to predict the chemotherapeutic and toxic effects of these drugs in military personnel.

24. (U) Instrumental, chemical and immunological technqiues for analysis and identification of experimental antimalarial drugs in biological materials will be developed and evaluated in animal model experiments for application to clinical specimens. These findings will be correlated with studies on the pharmacological and toxic effects of the drugs in animal models.

25. (U) 74 07 - 75 06 Several chromatographic systems have been developed using TLC, HPLC, or GLC for separation and sample preparation for several antimalarial drugs. These methods per se lack sufficient sensitivity for direct application to biological specimens; however, derivatives of the separated components are being prepared in order to apply fluorescence and other more sensitive analytical techniques for quantitative analysis. Attempts to produce antibodies to these drugs for immunoassays have been frustrated by the difficulties of preparing satisfactory hapten conjugates and because of diseases in the animal quarters. For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 315 Blood level assays for anti-malarial drugs

Investigators.
Principal: LTC (P) Douglas J. Beach, MSC; H. Kenneth Sleeman, Ph.D.; Ann R. Berman, B.S.; LTC Gale E. Demaree, MSC; Betty J. Boone, Ph.D.; MAJ John F. Mazur, MSC.
Associate: SFC Johnnie L. Harvey, Elvio A. Levri, M.S.; SP4 Cynthia D. Kindell; Nesbitt D. Brown, B.S.

The major effort under this work unit involved attempts to develop immunoassays for experimental antimalarials and the extension of toxicity studies on Dapsone. Specific studies reported for FY 75 include:

1. Synthesis of conjugated haptens of antimalarials.

2. Preparation of antisera to antimalarial haptens.

3. Preparation of labelled haptens for immunoassays.

4. Development of chromatographic methods for analysis of antimalarials in biological materials.

5. Toxicity studies on antimalarials.

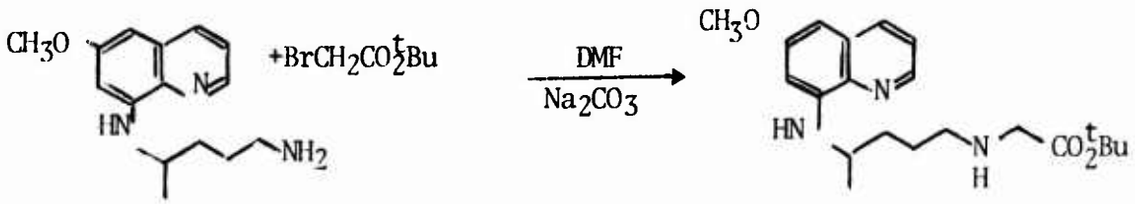
1. Synthesis of conjugated haptens of antimalarials.

In order to obtain a reasonable degree of specific antigen recognition for antibodies to be used in immunoassays, it is necessary to obtain specific, covalently bound complexes between the drug and the protein carrier. The quinoline methanol and phenanthrene methanol classes of antimalarials have presented special problems in this regard because of their very low water solubility and because of their very strong non-specific binding through hydrophobic bonds to protein.

Specific binding of drugs to protein is usually accomplished by producing a carbonyl function on the drug molecule and causing this to react with free amino groups on the protein in the presence of an anhydride or carbodiimide. Attempts to use succinic anhydride as the coupling reagent were unsuccessful in most cases because of cyclization to form an imide incapable of reacting with protein.

The succinic anhydride method proved partially successful in producing a reactive product with WR 142,490.

The problem of cyclization in the case of chloroquine and primaquine was overcome by the synthesis of 2-bromo-t-butylacetate which readily alkylates the amine functions on the drugs.



This side chain can then be hydrolyzed to form the carboxylic acid for direct conjugation with protein. This discovery of the use of 2-bromo-t-butylacetate to produce a functional group on the drug for conjugation to protein affords many advantages to other methods: (1) The reaction products to form the side chain can be monitored by GLC without derivatization. (2) The t-butyl group serves as an excellent marker for NMR identification of structure. (3) The secondary amine bond that is formed instead of ester or amide bond is much more stable and will not be hydrolyzed in other reactions. (4) The formation of a terminal ester gives excellent properties for purification prior to hydrolysis to the free carboxylic acid.

Several such derivatives have been prepared and submitted for antimalarial screening.

The standard reactions for coupling the carboxylic acid group to protein require aqueous reaction mixtures. The poor solubility of the antimalarials in water renders this approach impractical so the conditions and reagents were modified substituting dimethyl formamide for water and substituting dicyclohexyl carbodiimide for 1-ethyl-3-(3-dimethyl) aminopropyl carbodiimide.

A conjugate of WR 142,490 was formed with bovine serum albumin as judged by shifts in the UV spectra; however, the hapten number appears to be low and is undetermined at this time because of analytical problems. Nevertheless, lyophylized, dialyzed conjugate has been injected into rabbits to test the antigenic properties of the conjugate.

2. Preparation of antisera to antimalarials.

Progress on this area was seriously handicapped because an infection in the rabbit colony necessitated extermination of all immunized animals. The colony was reestablished, new lots of conjugated haptens were prepared an immunizations resumed. Preliminary

results of early bleedings indicate that the present titers are inadequate for a sensitive radioimmunoassay. All immunized animals were given booster injections of hapten conjugates. All available resources are being dedicated to this crucial aspect of the development of an RIA for experimental antimalarials.

3. Preparation of labelled antigens for immunoassays for experimental antimalarials.

Initially, two immunoassay procedures will be stressed: (1) spin immunoassay and (2) RIA. These methods involve the competitive displacement of labelled antigen by the specific antigen in the sample being analyzed.

Nitroxide free radicals have been conjugated to the experimental antimalarials for use in spin immunoassays pending production of suitable antibodies.

Similarly, antimalarials are being conjugated with tyrosine to be radioactively iodinated for use in RIA for experimental antimalarials.

4. Development of chromatographic methods for analysis of antimalarials in biological materials.

Pending the successful development of immunoassays, it is necessary to provide chemical analytical methods not only for estimating levels of drugs in biological materials, but also for validation of the immunoassays.

Because of the very low concentrations of these drugs in blood and tissues and the very strong bonds between the drugs and protein materials, it appears that some type of chromatographic separation is essential. The good spectral qualities of these drugs make detection by optical methods following separation by high pressure liquid or thin-layer chromatography very feasible. TLC systems for these drugs have not yet achieved the sensitivity required for quantifying these drugs in blood. High pressure liquid chromatography is being exploited to take advantage of its greater sensitivity.

At this time, a major problem yet to be overcome is the development of a highly efficient system to extract the drugs from tissues and fluids. The drugs bind very tightly at low concentrations so that extraction efficiencies that are constant regardless of drug concentrations have not been achieved. Thin-layer chromatography results for extraction and separation of four antimalarials are given below.

Blood (1 to 2 ml) was extracted with 40 ml of extracting solution (Table 1). The organic phase was treated with anhydrous sodium

sulfate to remove water, filtered, and evaporated to dryness. The residue was dissolved in methanol and spotted on F-254 silica gel plates. The solvent systems which were found to produce the best R_fs are shown in Table 2.

TABLE 1

Solvent Systems for Extracting Drugs from Blood

Drug	Extractant
WR 30,090	Chloroform:ethyl acetate:acetic acid (75:25:5 by volume)
WR 33,063	Chloroform:ethyl acetate:acetic acid (75:25:5 by volume)
WR 122,455	Ethyl acetate: $(NH_4)_3PO_4$ buffer, pH 11 (40:1)
WR 142,490	Ethyl acetate: $(NH_4)_3PO_4$ buffer, pH 7.4 (40:1)

TABLE 2

Developing System for Drugs on TLC Plates

Drug	Developing Solvents
WR 30,090	Chloroform:ethyl acetate:acetic acid (45:55:5 by volume)
WR 33,063	Chloroform:ethyl acetate:acetic acid (45:55:5 by volume)
WR 122,455	Chloroform:ethanol:NH_4OH (65:33:2 by volume)
WR 142.490	Chloroform:ethanol:NH_4OH (50:50:4 by volume)

The plates were developed to a height of 15 cm, dried and examined under ultraviolet light. The ultraviolet absorbing spots were eluted and measured in a spectrophotometer. Table 3 shows the R_fs, eluting solvents, and percent recovery of 1 to 20 µg/ml of drug added to plasma.

TABLE 3

Results of TLC Analysis of Drugs Added to Plasma

Drug	R_f	Eluting Solvent	% Recovery Average
WR 30,090	0.33	Chloroform:ethyl acetate:acetic acid (75:25:5)	92
WR 33,063	0.56	Chloroform:ethyl acetate:acetic acid (75:25:5)	96
WR 122,455	0.57	Ethyl acetate:ethanol:NH_4OH (90:10:5)	95
WR 142,490	0.63	Ethanol	98

Recovery of Drug from Rats. Rats were given by oral intubation, WR 30,090 suspended in methyl cellusolve (0.5%) and Tween-80 (0.1%), olive oil, and olive oil containing 10% ethanol. When doses of 1.5 mg to 50 mg were given for 3 days, no detectable drug was found in the blood. The sensitivity of the TLC method for WR 30,090 was 1 µg/ml.

In other experiments in rats, WR 142,490 suspended in methyl cellulose and Tween-80 was administered. When 25 mg/rat was given for 2 days, the blood levels were 10 µg/ml; 10 mg/rat for 2 days was not detectable. The sensitivity of the method for WR 142,490 was 2 µg/ml.

The results indicated that TLC was not sufficiently sensitive for routine blood analyses of orally administered drug. The extraction procedure, which produced 90 percent or better recovery of spiked samples, may be useful for analysis by gas chromatography, liquid chromatography and radioimmunoassays.

Gas chromatographic studies are being concentrated on formation of derivatives that will give satisfactory volatility and on detection methods using electron capture detection methods to take advantage of the halogens in these molecules.

In addition to the strong binding of these drugs to protein, it was discovered also that the drugs adsorb onto the inner walls of glass or plastic containers. Silicone treatment of the containers increasedbinding. This binding reaches equilibrium in about 24 hrs with as much as 60% of the drug being adsorbed to plastic containers and up to 30% on glass and about 50% on siliconized glass. The drug was desorbed very efficiently with alcohol rinses in 10 minutes. A greater percentage of the drugs is adsorbed at lower concentrations.

These findings point out the extreme importance of exercising caution in any aliquoting procedures and the necessity of alcohol washing of containers of drugs and specimens. Standards should be maintained in alcohol when possible.

5. Toxicity studies on antimalarials.

In previous reports we have described the effects of Dapsone administration on endocrine functions. Those results indicated that some form of pathophysiology affects the adrenals and thyroid consistent with suppression of the function of these glands.

Since ascorbate depletion of the adrenals is an index of generalized adrenal stress, the effect of Dapsone on ascorbate levels was studied. A high pressure liquid chromatographic method was developed which eliminates all problems associated with interference from other reducing substances found in biological materials.

Deproteinized tissue extracts were injected onto a Partisil SAX-10 μ strong anion exchanger column and eluted with 0.005 M, pH 3.35 KH_2PO_4 buffer at a flow rate of 0.66 ml/min maintained at 25°C. Ascorbate was detected as a well resolved peak at 254 nm with an elution time of 9.0 min.

Rats were given Dapsone orally at doses of 4, 20 and 100 mg/kg for 5 days and then sacrificed, the adrenals were removed quickly, extracted in 2.5% metaphosphoric acid and frozen (-70°C) until assayed. The results of these studies suggest that at doses of Dapsone which cause the greatest degree of histopathology of the adrenals, the levels of ascorbic are also reduced by 30%. This may indicate some generalized drug toxicity of adrenal tissues or stress. Certainly if the 30% reduction of ascorbate which was observed here can be verified, it suggests a mechanism by which Dapsone may interfere with endocrine function.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 315 Blood level assays for anti-malarial drugs

Literature Cited.

Publications:

1. Giles, R. C., Jr., Berman, A., Hildebrandt, P. K., McCaffrey, R. P.: The use of ^{51}Cr for sheep red blood cell survival studies. Proc. Soc. Exp. Biol. Med. 148: 795, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6514	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	318
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biological Studies of Insect Infection and Disease Transmission

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
65 07	CONT	DA		C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	4.5	190
	CURRENT 76	4.5	190

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Anopheles; (U) Mosquitoes; (U) Malaria; (U) Immunization; (U) Plasmodium

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Development of physiological means of interrupting malaria transmission through an understanding of factors influencing susceptibility of anophelene vectors to malaria and of the various factors affecting gametocyte infectivity in vivo and in vitro. Test systems are developed for studying the mechanisms underlying sporozoite induced immunity for the eventual prevention and control of malaria in military troops.

24. (U) Studies are conducted to determine quantitatively such parameters as the minimum number of sporozoites required for the initial immunizing dose as well as the spacing, number and strength of the subsequent boosters. Attempts to develop one or more serological assays for demonstrating the course of the immune response in the blood serum of animals immunized against the sporozoite stage. Evaluation of the effects of various environmental stresses upon the infectivity of the gametocytes. Isolation of these forms from the other blood stages on density gradients for subsequent use in culture systems.

25. (U) 74 07 - 75 06. Using an immunizing schedule which relies on an initial dose of 75,000 sporozoites followed by four boosters of 5000 sporozoites at biweekly intervals, complete protection to challenge by 1000 homologous sporozoites was conferred upon 27 C3H,HEJ and 23 BALB,c mice. Approximately 120 ml of serum from the immunized mice has been pooled and stored for precipitation of the globulin fraction. Erythrocytic stages of a murine malaria were successfully isolated on continuous density gradients in the following order beginning with the most dense: noninfected erythrocytes, trophozoites, schizonts and gametocytes. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 318 Biological studies of insect infection and disease transmission

Investigators
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Description

The two major and quite disparate objectives of this work unit have been (1) understanding the mechanisms underlying sporozoite induced immunity in Plasmodium berghei malaria and (2) separation of the erythrocytic forms of malaria parasites on density gradients for subsequent studies involving gametocyte infectivity and the use of such stages for initiating the sporogonic cycle of this parasite in vitro. Emphasis has also been placed on isolating and concentrating ookinetes of P. cynomolgi on density gradients and on refining a technique involving the mass isolation of salivary glands from infected Anopheles stephensi mosquitoes. In addition, incipient cell lines have been derived from two species of tsetse fly for proposed studies on the metabolism of the crithidial and metacyclic forms of African trypanosomes.

Progress

1. Immunization against P. berghei malaria with attenuated sporozoites

Immunization of rodents with repeated injections of X-irradiated P. berghei sporozoites results in extensive and often complete protection against a subsequent challenge of infectious sporozoites. Quantitative data regarding the minimum number of sporozoites required for the initial immunizing dose as well as the spacing, number and dosage of the subsequent boosters have been lacking as has a serological assay for demonstrating the course of the immune response in the blood serum of animals immunized against the sporozoite stage. {Investigators using the P. berghei model have relied on the circumsporozoite precipitation reaction (CSP) to demonstrate the presence of sporozoite antibodies in the blood serum of immunized animals.} But recent work at New York University and at Walter Reed has shown that there may be little, if any, relationship between CSP antibodies and protective immunity.

A standardized procedure for immunizing A/J mice with P. berghei sporozoites relies on an initial dose of 7.5×10^4 sporozoites followed

by 4 boosters of 5 x 10^3 sporozoites each at biweekly intervals. Using a similar schedule, complete protection to challenge by 1 x 10^3 sporozoites was conferred on 27 C3H/HEJ and 23 BALB/c mice. However, it was found that initial doses as low as 2 x 10^4 followed by 4 boosters with a cumulative total of 6.5 x 10^4 sporozoites was sufficient to protect the C3H/HEJ mice whereas even lower numbers (1.5 x 10^4) in the initial dose sufficed for the BALB/c mice. An additional 49 BALB/c mice are being immunized on schedules designed to test the spacing and the minimum number of sporozoites needed per booster to achieve total protection.

To determine whether the protection in mice immunized with attenuated sporozoites is associated with immune serum and immune serum globulin and whether the protection is dose dependent, approximately 120 ml of serum from immunized mice has been pooled and stored for precipitation of the globulin fraction and for subsequent separation of the 7S and 19S globulins. The fractionation work utilizing gel filtration and anion-exchange chromatography is currently in progress.

Also in progress is the immunization of congenitally athymic (Nude) mice to test whether T cell dependent or independent antibodies are involved in sporozoite induced immunity. None of these mice have as yet been challenged.

2. Mass isolation of A. stephensi salivary glands infected with malarial sporozoites for immunization studies

The antigencity of P. berghei sporozoites is quite stage specific in that parasites from the salivary glands confer total protection upon challenge by homologous sporozoites whereas the use of younger sporozoites results only in a delay of patency. Since the manual dissection of the glands is both laborious and time consuming and the yield of parasites quite limited, a method was devised to isolate the salivary glands en masse from infected anopheline mosquitoes (see 1974 Annual Progress Report). The method has recently been modified to increase the percentage of glands recovered and to reduce the amount of debris in the final preparation. The mosquitoes are anesthetized, decapitated and placed in a monolayer on a cold glass plate having a thin film of buffered saline on its surface. Feeler gauges of specific thicknesses are placed at either side of the mosquito mass and a glass or stainless steel rod rolled over the gauges to crush the bodies. Following three successive rolls over gauges with thicknesses of 330μ, 254μ and 203μ, respectively, the material is washed and filtered and the glands collected in a water-cooled aspirator. The percent recovery averages 70% and the range of infectivity varied between 24 and 45% with an average of 30%. This is comparable to the 21-23% infection rate reported for manually dissected glands.

Infectivity of the sporozoites from 5 different runs was found to be 100% in tests involving 40 mice. The prepatent periods of 4-5 days were comparable to direct mosquito transmission. In fact, infective sporozoites were recovered from mosquitoes decapitated as much as 20 hours prior to gland removal, providing the mosquitoes were held at 4°C during this interval.

Using this technique, one individual can extract salivary glands from approximately 1,200 mosquitoes in a day. A routine run involving this number requires about 2.5 hours for decapitation, 1.5 hours for the rolling and filtration procedures and from 2-3 hours for the aspiration and trituration of the glands. For comparative purposes, a standard malarial procedures manual suggests a figure of 200 per day for manual dissection.

3. Separation of erythrocytic forms of malaria parasites and an analysis of their infectivity to hosts

This effort has been directed at isolating on density gradients the blood stages of P. berghei and P. cynomolgi for the purpose of separating the parasites into discrete populations based on density differences between the various developmental stages. Isolation of viable parasitized cells at specific points in their developmental sequence will provide the necessary material for the initiation of in vitro cultures of the sporogonic cycle. In addition, the ability to isolate and concentrate gametocytes permits a detailed evaluation of gametocyte infectivity under varying conditions.

Erythrocytic stages of P. berghei were successfully isolated on continuous density gradients using either a mixture of Path-O-Cyte V: Renografin or Ficoll as the supporting medium. Infected erythrocytes were separated in the following order beginning with the most dense (Mean cellular density in parenthesis): noninfected erythrocytes (1.650+ g/cm^3); early trophozoites (1.155 g/cm^3); mature trophozoites (1.150 g/cm^3); schizonts (1.100 g/cm^3); and gametocytes (1.085 g/cm^3). Variations in this pattern were observed when more than one intraerythrocytic parasite was present. In these instances the cellular density more closely approximated that of the mature trophozoites or the schizonts. Also, no density differences were observed between macrogametocytes and microgametocytes.

Trophozoites of P. berghei were infective after isolation on density gradients as determined by the intraperitoneal injection of these cells into mice. The resulting infections demonstrated that the number of days to patency and the parasitemia levels were comparable to those of control mice infected simultaneously by direct blood passage of an equal number of nonisolated parasites.

The infectivity of isolated P. berghei gametocytes was evaluated by feeding concentrated cells to A. stephensi with a miniature vessel covered by a membrane composed of sheep mesentery. Membrane feeding permitted a quantitative assessment of the numbers of gametocytes ingested by individual mosquitoes. The results of numerous trials indicated that isolated P. berghei gametocytes do not develop to the oocyst stage in A. stephensi. Subsequent experiments in which mosquitoes ingested blood directly from parasitemic mice showed an inconsistent incidence of oocyst development even though mouse parasitemias ranged between 3 and 10 percent. When blood

was drawn from parasitemic mice and fed to mosquitoes no oocysts developed. Due to the poor ability of P. berghei gametocytes to infect mosquitoes, experiments were initiated to develop a P. cynomolgi - A. stephensi nonhuman primate model utilizing Rhesus monkeys (Macaca mulatta) as vertebrate hosts. Initial experiments compared the infectivity of gametocytes ingested by the mosquitoes feeding directly on an infected monkey with those from freshly withdrawn blood fed via a membrane feeder. Results show that fertilization was completed and that oocysts developed in A. stephensi after feeding on blood infected with P. cynomolgi under in vitro conditions and that the incidence of infection was comparable to that resulting from in vivo feeding.

Establishment of a successful in vitro feeding system permitted a detailed analysis of the effect of various physical and chemical factors upon the ability of gametocytes to infect mosquitoes. The results of a series of experiments indicated that P. cynomolgi gametocytes remained infective for at least 30 minutes at 25°C after the plasma layer was replaced with tissue culture medium M199. Gametocytes subjected to centrifugal forces up to 1000 x g for 10 minutes remained viable. However, incubation of gametocytes in the presence of 15% Ficoll for periods of 10 minutes at 25°C, or incubation with 15% Ficoll for one hour at 4°C resulted in a total loss of infectivity. Also, gametocytes maintained at 4°C for one hour with no additional treatment failed to retain their viability.

4. Attempts to isolate and concentrate P. cynomolgi ookinetes from A. stephensi mosquitoes on density gradients

Initial efforts were directed toward determining the optimum time after feeding and the optimum parasitemia for obtaining ookinetes from mosquitoes. Ookinetes were demonstrated on stained slides made from midguts of mosquitoes which fed on infected monkeys 16 to 26 hours prior to dissection. However, at times no ookinetes could be found even though there was a high parasitemia and control mosquitoes developed oocysts. The highest oocyst counts were observed when the parasitemias were above 1%; however, no correlation was observed between parasitemia, oocyst numbers and ookinete numbers.

In an attempt to free ookinetes from the blood meal, the midguts of mosquitoes which had fed on infected Rhesus monkeys were ground in a tissue grinder and incubated for varying lengths of time with the enzymes hyaluronidase and protease. The material was then centrifuged at 50 x g for 5 minutes, after which the pellet resuspended in M199 and the procedure repeated four times. The combined supernatants were centrifuged at 500 x g for 15 minutes to pack the ookinetes. The pellet was resuspended in M199 and placed on the gradient and centrifuged at 12,300 x g for 30 to 45 minutes. Both continuous and discontinuous Ficoll gradients in concentrations of from 5 to 30% were used. Only a few ookinetes were found in the material placed on the gradients and none were recovered from the gradients proper.

5. Development of incipient cell lines from two species of tsetse-fly

Varying degrees of success have been obtained in attempts to culture the insect cycle of salivarian trypanosomes in vitro. Without exception, the most promising cultures have been achieved when tsetse fly tissues were incorporated into the culture system. Although the trypanosomes may remain viable for up to 30 days in such cultures, development of the parasites is usually arrested once the midgut form has been attained after a short one or two days in vitro. Further advancement to the crithidial and metacyclic (infective) forms does not take place.

On the assumption that cell lines derived from tsetse fly tissues might provide a more suitable substrate for the parasites, more than 400 primary cultures were set up over a period of 2 1/2 years. Eggs, larvae and pupae of various ages as well as newly emerged adults served as sources of tissue for the cultures. Except for the embryos which readily dispersed into single cell suspensions, each culture consisted of lightly macerated tissue fragments from a single individual. Of all the stages tested the one which appeared to be most amenable to culture was the third larval instar in which the polypneustic tubes were grey to light black in color and still pliable. Three primary cultures, two from Glossina morsitans and one from G. austeni third stage larvae, were capable of being subcultured after intervals varying between 8 and 15 months. The doubling time of the cells in the G. morsitans cultures is approximately 48 hours whereas that of the G. austini cells approaches 72 hours. Once sufficient subcultures of the three lines are available, quantitative studies on the growth rates of both Trypanosoma brucei and T. congolense in such cell cultures will be carried out.

Conclusions and recommendations

1. The P. berghei- A. stephensi-mouse system promises to yield useful information concerning the role played by serum antibody as well as other factors in sporozoite induced immunity. In addition, to the work now in progress, efforts will be focused on the determination of antibody titer and specific immunoglobulin levels before, concurrent with, and after the immunization process as well as whether protection is associated with a specific class or subclass of immunoglobulin. Cell transfer studies using lymph nodes, spleen and thymus from immunized animals should also be undertaken to determine to what extent cell mediated immunity is involved in this protection.

2. The technique of isolating infected salivary glands en masse from A. stephensi mosquitoes insures the availability of sufficient numbers of sporozoites for immunization purposes. The time spent in isolating and collecting the glands, while much less than required with manual dissection, is still quite extensive. The possibility of automating at least a part of the procedure should be explored.

3. Since gametocytes can be isolated from other blood forms quite effectively on density gradients, further evaluation of the effects of various stresses upon the gametocytes would be useful in determining optimal conditions for in vitro maintenance. Devising a gradient system for the large scale isolation of P. cynomolgi should be undertaken concurrently with the above.

4. The possibility of isolating ookinetes on density gradients following enzymatic treatment of the midguts from engorged mosquitoes does not appear very promising unless a method is found for predicting the presence of large numbers of ookinetes at specified intervals after blood feeds. Emphasis should be placed on this particular problem before resuming the density gradient runs.

5. The G. morsitans and G. austeni cell lines should prove useful for a wide variety of studies involving the salivarian trypanosomes. They should also be available to any investigators from other institutions upon request.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 318 Biological studies of insect infection and disease transmission

Literature Cited

References: None

Publications:

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3. Schneider, I. The relative merits of various enzymatic pretreatments, culture media and supplements for the invitation and establishment of dipteran cell lines. Conf. on Invertebrate Tissue Culture, Tokyo, Japan. pp. 7-8, 1974.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6520	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9A. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	324
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Procede with Security Classification Code)

(U) Host Responses to Malaria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	4	200
	CURRENT 76	4	200

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Periodicity; (U) Cellular Immunity; (U) Lymphocyte; (U) Tolerance; (U) Serology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To study the physiopathology, immunology and serology of malaria -- a disease of prime concern to troops stationed in tropical areas, causing considerable loss of man-days due to severe morbidity and mortality.

24(U) Description of periodicity of Plasmodium falciparum in Aotus monkeys. Evaluation of cellular and humoral immunity in rodent malaria by passive-transfer protection studies with lymphocytes, macrophages and serum. Study of mechanisms of immune response to antigens of rodent malaria through induction of antigen-specific unresponsiveness (tolerance) to P. berghei.

25(U) 74 07 - 75 06 Rats which had been immunized against Plasmodium berghei by a series of injections of gamma-irradiated parasitized erythrocytes were used as cell or serum donors. Recipients of lymphoid cells from gamma-PRBC-immunized donors had lower parasitemia than did their gamma-PRBC-injected counterparts, the greatest difference in median peak parasitemias being 25 PRBC-500 RBC vs 88 PRBC-500 RBC. There was a complete suppression of circulating parasites in recipients of lymphoid cells from rats which had recovered from an active P. berghei infection. In recipients of PEC from gamma-PRBC-immunized donors there was evidence of suppression of malaria challenge. There was a partial suppression in recipients of serum from rats which had recovered from an active malaria infection. There was no evidence of an effect of immune cells on the periods of patency or prepatency of infection in recipients. For technical report see WalterReed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 324 Host responses to malaria

Investigators
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Associates: W.L. Bowie; W.R. Hildreth; CPT F.A. Hines, VC; MAJ L.K. Martin, MSC; MAJ R.A. Wells, MSC

1. Passive transfer of immunity to Plasmodium berghei in rats with cells and serum.

Mechanisms of host defense against malaria have not been described definitively, but numerous past studies have demonstrated that both humoral and cellular factors are involved. Several workers have shown that lymphocytes transferred from malaria-recovered rats confer to malaria-susceptible rats of the same inbred strain, a partial or complete protection against subsequent malaria challenge. In the present study cell and serum transfers were made from donor rats that had been immunized against P. berghei, not by the infection-recovery method, but by a series of injections of γirradiated, malaria-infected rat erythrocytes (γ-PRBC). These irradiated plasmodia are regarded as viable but incapable of replication, and an intravenous or intraperitoneal injection produces low level parasitemia without progression to disease. By the serial injection of large numbers of irradiated parasites (total of about 10^9 parasitized erythrocytes over a 2-month period) rats become refractory to subsequent challenges with non-irradiated P. berghei blood forms. By the use of such γ-PRBC-immunized rats as cell or serum donors in the present study, it was expected that a relatively "clean" and possibly more efficient cell-transfer model might result, since there should be a smaller likelihood of interference from the other consequences of infection (viz. host and/or parasite components or products which are present as a result of the various pathological changes in a fulminating infection, and which might be expected to mask some of the protective effects of lymphoid cells, macrophages or serum).

In four experiments cells from immunized adult male Fischer rats* (Charles River Laboratories) were transferred to malaria-susceptible male rats of the same strain, and the effects were evaluated by parasite counts following malaria challenge of the recipients.

In all experiments a rat-passage NYU-2 strain of Plasmodium berghei was used.

Malaria infections in all recipient groups were monitored by determining the relative number of parasitized erythrocytes (No. PRBC/500 RBC) from giemsa-stained thin smears.

Rats were immunized by a series of four or five intraperitoneal (IP) injections of homologous γ-irradiated parasitized erythrocytes (γ-PRBC) -- a total of about 8×10^8 PRBC exposed to 20,000 rad from a γ-cell irradiator. Normal control rats from the same rat pool were injected with a comparable volume of irradiated homologous normal erythrocytes (γ-RBC) over the same immunization period. Some rats from each donor group were removed and held for later challenge, in order to verify their immune status. Rats preselected as peritoneal exudate cell donors were injected IP with 20 ml sterile mineral oil 24 hours prior to cell transfer in order to produce a cellular exudate.

Cell Transfer. Three weeks following the final immunizing injection, all donor rats were ether-anesthetized and exsanguinated by heart puncture. The spleens were removed immediately and placed into cold Hanks' Balanced Salt solution with phenol red indicator (HBS). The spleens were macerated and the cells collected by a previously described technique. The anterior and posterior submandibular and cervical lymph nodes were removed and the cells collected by the same procedure as that used for the spleen cells. For peritoneal exudate cell (PEC) harvest from the predesignated donors (mineral oil recipients) exudate material was collected by aspiration and lavage of the peritoneal cavity with HBS. The mineral oil was separated from the exudate material after one hour.

Each cell suspension was centrifuged at 700 rpm for 7 minutes, the supernatant was aspirated and the suspension was washed three times in HBS with centrifugation. A count of parasitized erythrocytes was made for dosage calculation and the suspension brought to the desired volume by the addition of HBS. In each experiment trypan blue counts were made to determine cell viability, which was found to be at least 85% in all of the experiments.

Experiment 1

On the day prior to challenge with parasites, each recipient rat was injected intraperitoneally with the following approximate numbers of viable cells:

spleen cells (3×10^7)
- immune cells-6 recipients
- normal cells-3 recipients

lymph node cells (6×10^7)
- immune cells-4 recipients
- normal cells-3 recipients

PEC (2.5×10^7)
- immune cells-5 recipients
- normal cells-2 recipients

lymph node cells plus PEC (above amounts of each)
immune cells-4 recipients
normal cells-2recipients

Twenty-four hours later (day 0), all recipient rats (plus 2 challenge-controls from each donor group) were injected with 2 x 10^7 PRBC, IP.

There was evidence of varying degrees of suppression of parasitemia in the P. berghei-challenged rats which had been injected with cells from immunized donors (spleen or lymph node cells, PEC, or pooled spleen cells plus PEC) when compared to their respective control groups (Figs. 1a-1d). Parasites were detectable in all rats, and the period of patency was similar for experimental and control groups, but in all immune cell recipients the peak parasitemias were considerabley reduced, and parasitemia levels were generally lower throughout the course of infection.

Experiment 2

Donor rats. A γ-PRBC injection schedule similar to that in Experiment 1 was used in the immunization of 40 donor rats. for normal-cell donors, 40 rats of the same age were maintained. In each of these groups 20 rats were injected IP with 10 ml sterile mineral oil 24 hours prior to harvest of the peritoneal exudate.

Forty-eight hours prior to challenge, cells were collected and processed by the same technique as in Experiment 1. Sera from experimental and control groups were frozen at -20°C. The recipient rats, grouped as indicated below, were challenged with x 2 10^6 PRBC, IV.

A. Received material from immunized donors*

Recipient Group	No. Rats in Group	Received
1	6	$2x10^8$ lymphoid cells from spleen node mixture
2	4	$4x10^6$ macrophages PEC
3	6	2 ml serum
4	4	lymphoid plus PEC
5	6	lymphoid cells plus serum
6	6	PEC plus serum
7	4	lymphoid cells plus PEC plus serum

LEGENDS FOR FIGURES

Fig. 1. Median number parasitized RBC per 500 RBC in rats challenged IP with 2×10^7 PRBC one day after receiving IP from either normal (-----) or γ-PRBC-immunized (_____) rats: (a) 3×10^7 spleen cells, (b) 6×10^7 node cells, (c) 2.5×10^7 PEC, or (d) 6×10^7 node cells plus 2.5×10^7 PEC.

Fig. 2. Median number parasitized RBC per 500 RBC in rats challenged IV with 2×10^6 PRBC two days after receiving IP from either normal (-----) or γ-PRBC-immunized (_____) rats: (a) 2×10^8 lymphoid (spleen-node) cells, (b) 4×10^6 PEC, (c) 2×10^8 lymphoid cells plus 4×10^6 PEC, or (d) 2 ml serum.

Fig. 3. Median number parasitized RBC per 500 RBC in (a) rats challenged with 1×10^7 PRBC IV after receiving IP from either normal (-----), γ-PRBC-immunized (_____), or recovered-immune (.....) rats: (a) 1×10^8 spleen cells or (b) 3 ml serum.

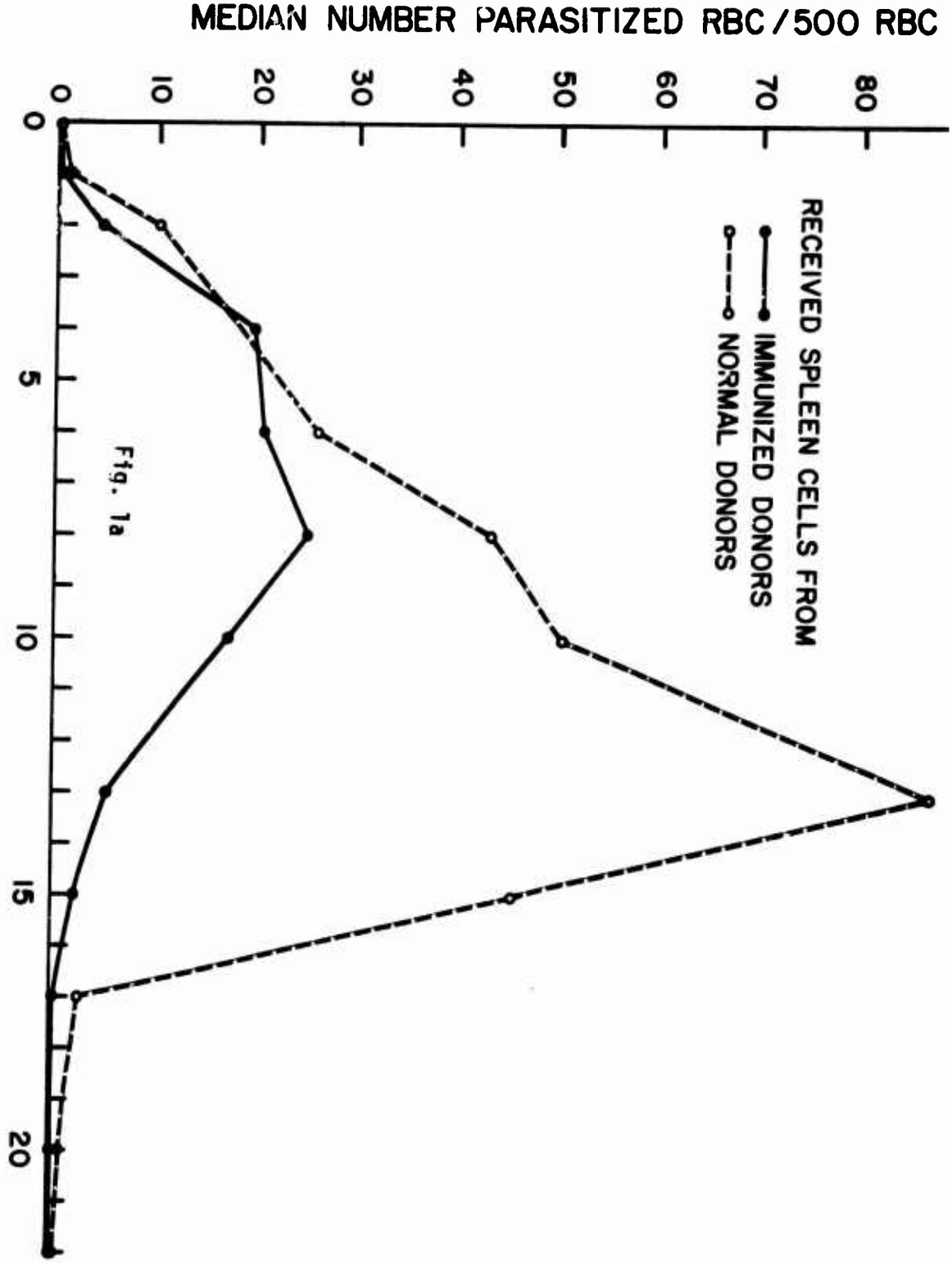
MEDIAN NUMBER PARASITIZED RBC / 500 RBC
0
10
20
30
40
50
60
70
80
0
5
10
15
20
RECEIVED SPLEEN CELLS FROM
IMMUNIZED DONORS
NORMAL DONORS

Fig. 1a

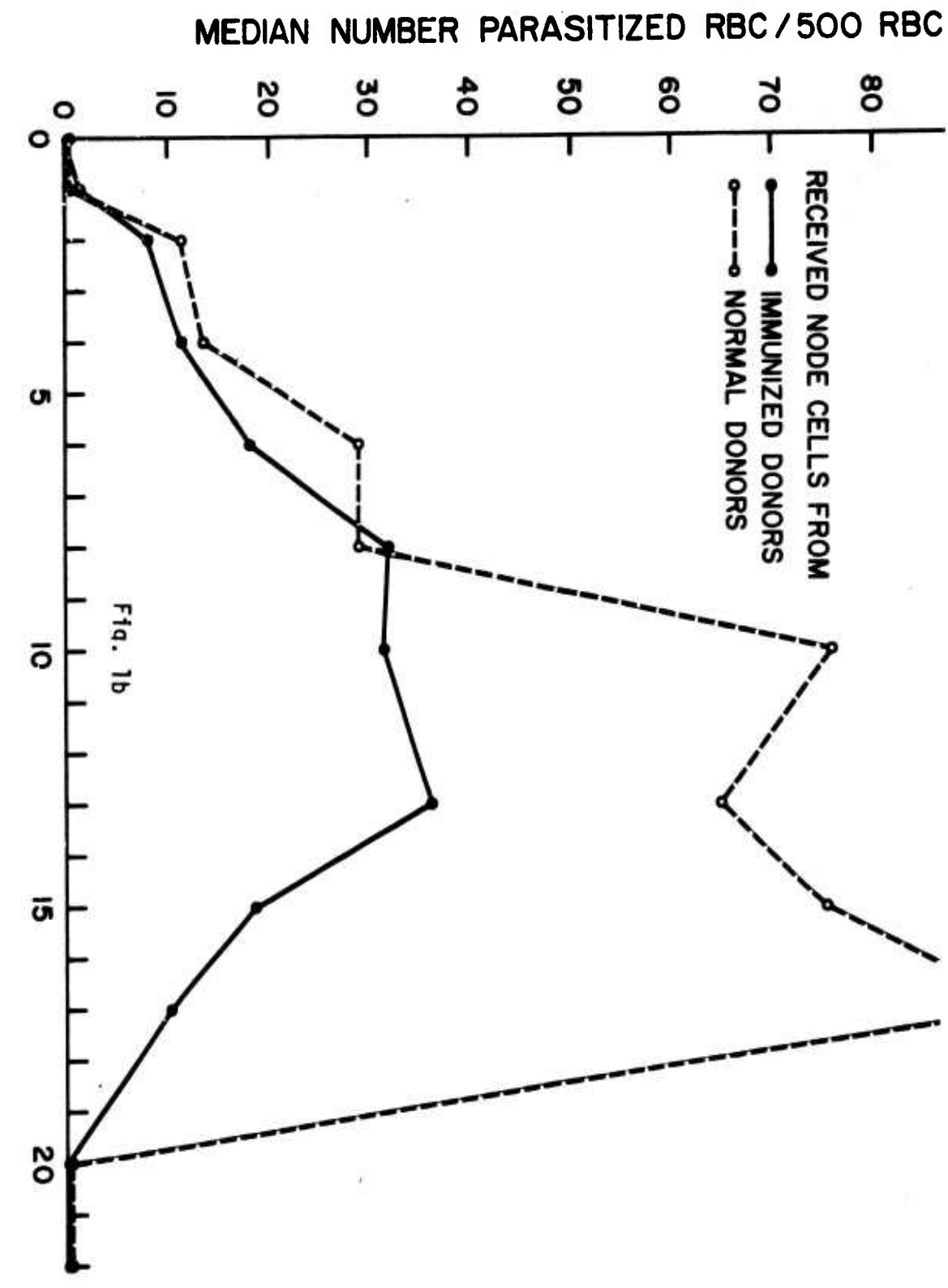
MEDIAN NUMBER PARASITIZED RBC / 500 RBC
0
10
20
30
40
50
60
70
80
0
5
10
15
20
RECEIVED NODE CELLS FROM
IMMUNIZED DONORS
NORMAL DONORS

Fig. 1b

B. Received material from nonimmunized donors*

Recipient Group	No. Rats in Group	
1	6	Each control group received material as respective groups in A, above.
2	6	
3	6	
4	6	
5	6	
6	6	
7	6	

C. Received no cells or serum; challenge-only controls (8 rats)

Percentage parasitemia levels of the malaria-challenged recipients of cells or serum are shown in Figures 2a-2d. The challenge-only control group is represented in 2a only, for the sake of simplicity. There was evidence of a slight suppression of parasitemia throughout the course of infection in recipients of immune spleen and node cells (Fig. 2a). Recipients of immune PEC, however, had parasitemias that generally approximated those of normal PEC recipients (Fig. 2b). However, in those recipients of immune lymph node-spleen cells plus PEC (Fig. 2c) there was a markedly lower parasitemia level as well as a shorter period of patency in comparison to the normal-cell recipient control group.

In this experiment there was no apparent suppression of parasitemia in recipients of serum from immunized donors (Fig. 2d). Moreover, rats which had received immune serum in addition to immune cells (groups A5, A6, A7) did not reflect any further suppression of parasitemia beyond that shown by those respective groups (A1, A2, A4) which had received immune cells without serum.

Experiment 3

Six donor rats were immunized with 5 IP injections of about 8×10^8 γ-PRBC total, and 6 rats received a similar number of γ-NRBC. A third donor group consisted of 6 rats given an active P. berghei infection and allowed to run the course of infection and recovery; the rats then were given a final test challenge of P. berghei, to which they appeared to be completely refractory. These "recovered-immune" rats served as a postive-control group. Spleen cells and serum were

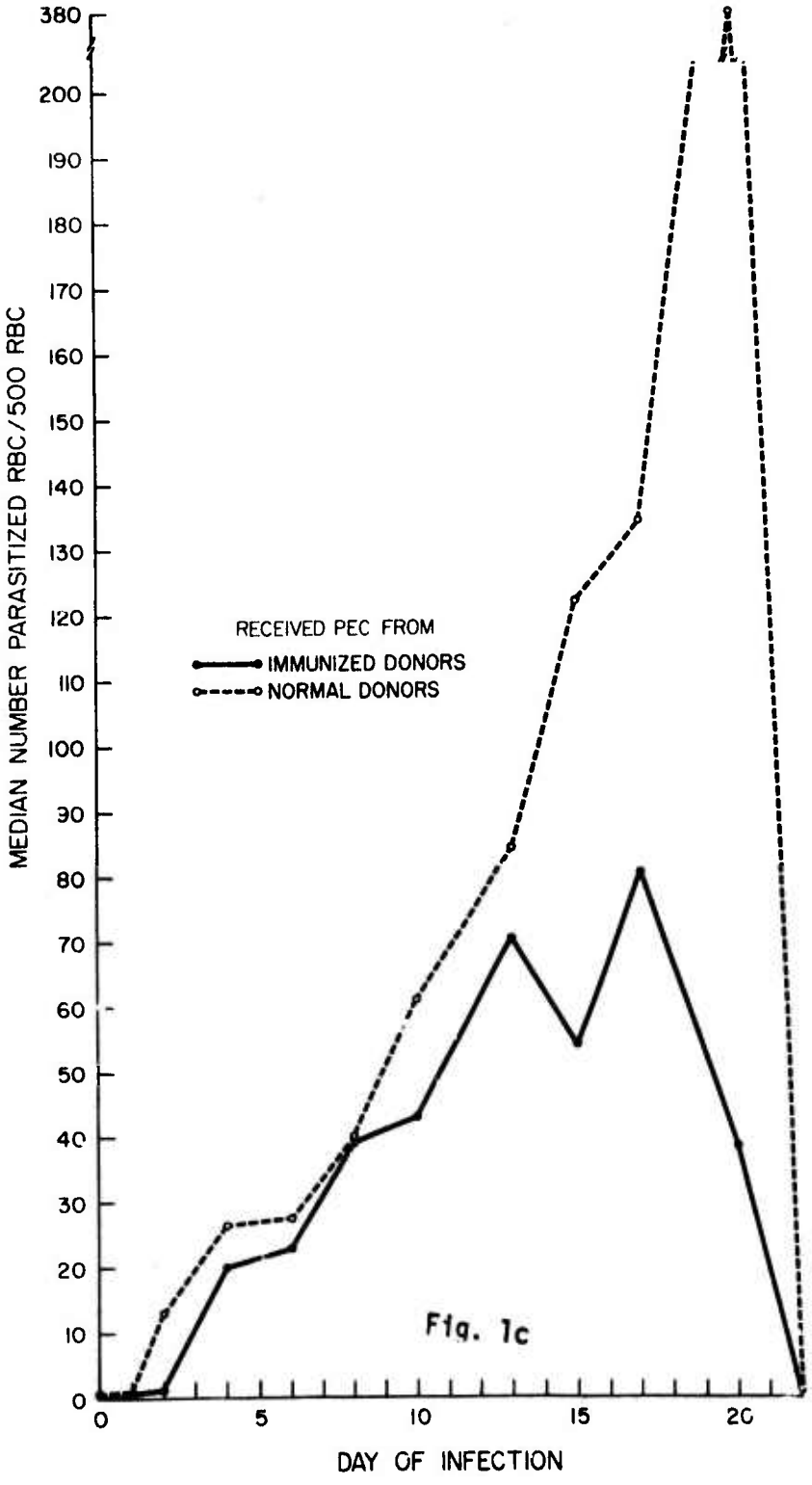
MEDIAN NUMBER PARASITIZED RBC/500 RBC
380
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
DAY OF INFECTION
RECEIVED PEC FROM
IMMUNIZED DONORS
NORMAL DONORS

Fig. 1c

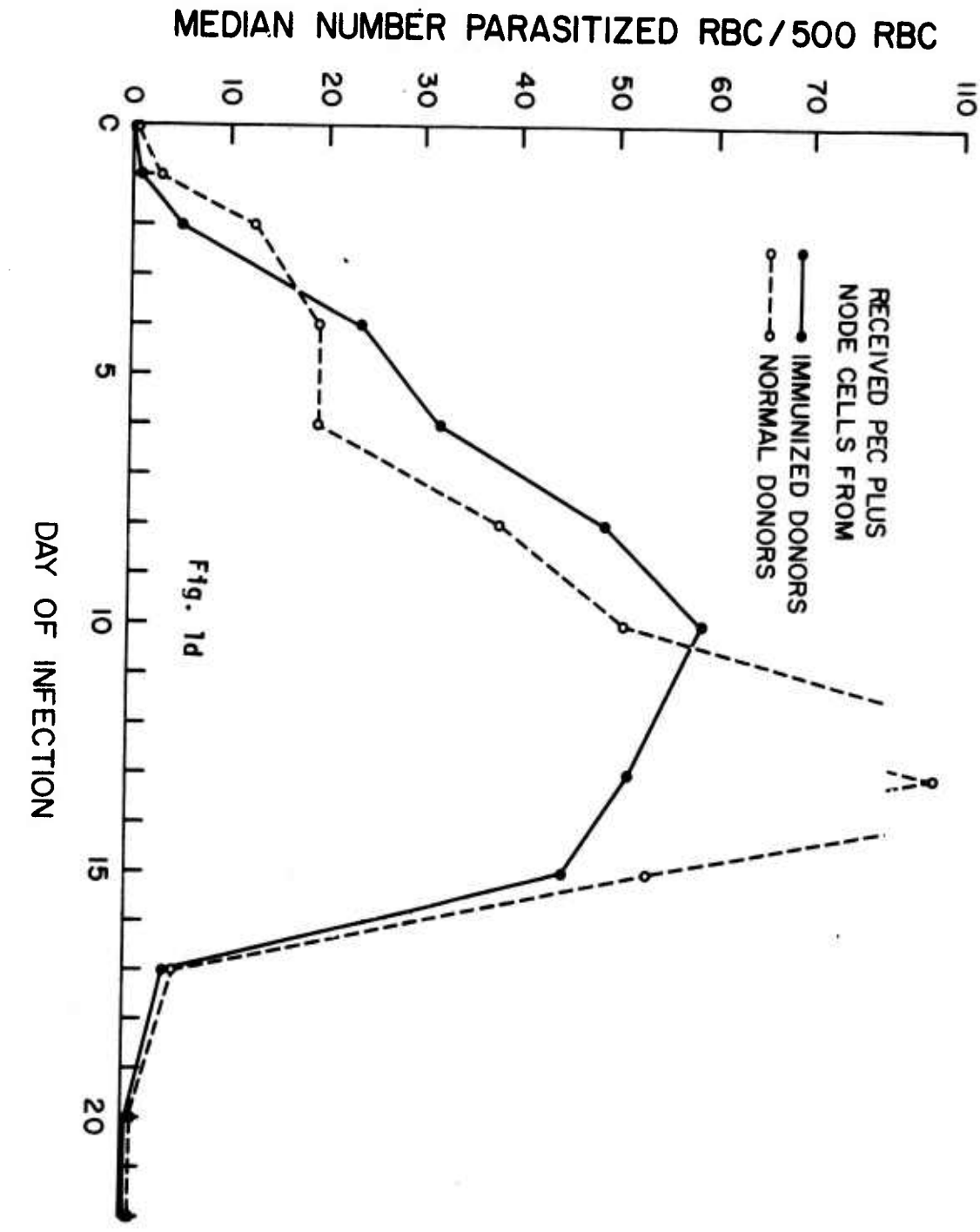
MEDIAN NUMBER PARASITIZED RBC/500 RBC
0
10
20
30
40
50
60
70
110
C
5
10
15
20
DAY OF INFECTION
RECEIVED PEC PLUS NODE CELLS FROM
IMMUNIZED DONORS
NORMAL DONORS

Fig. 1d

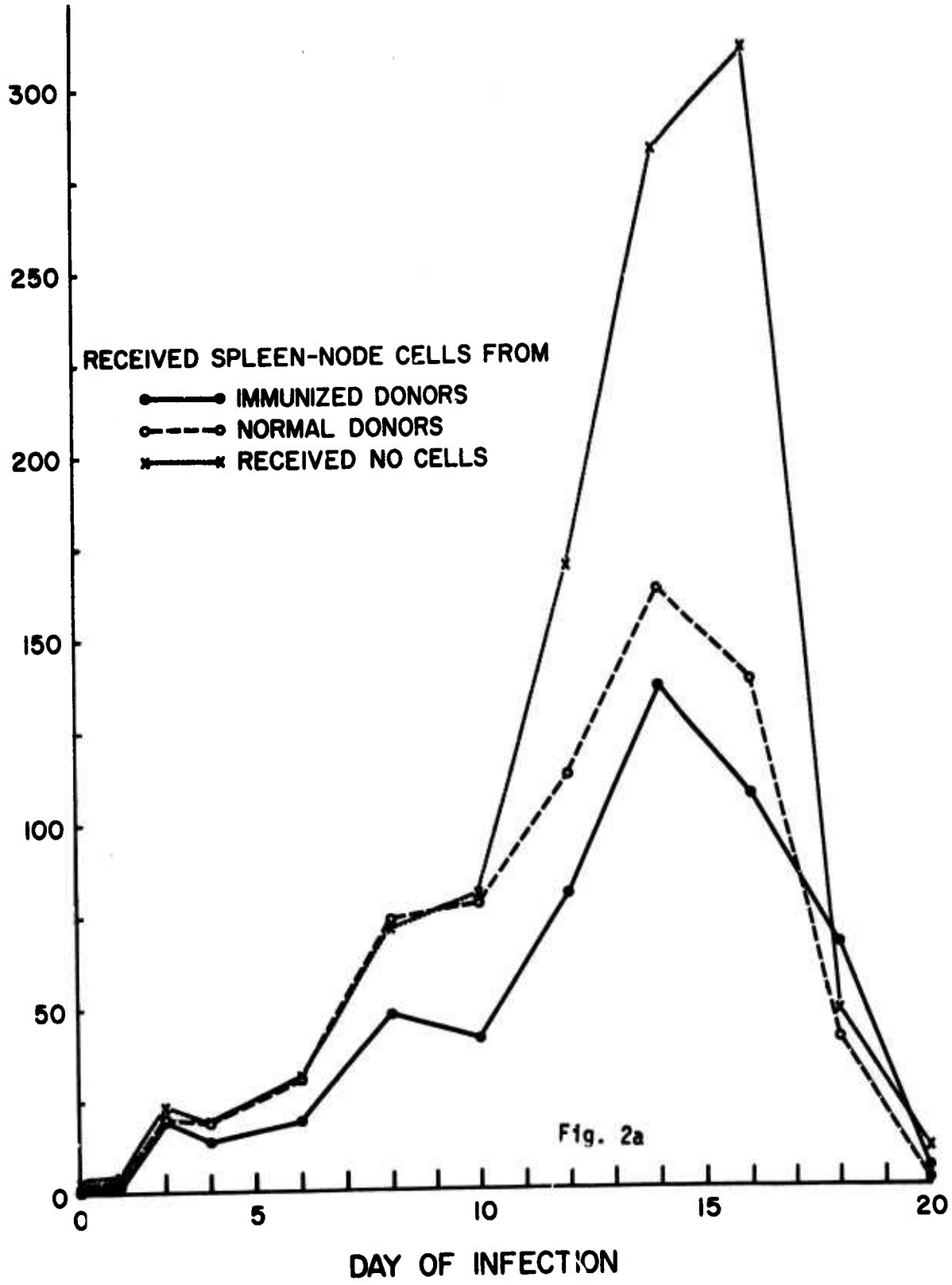
300
250
200
150
100
50
0
0
5
10
15
20
RECEIVED SPLEEN-NODE CELLS FROM
IMMUNIZED DONORS
NORMAL DONORS
RECEIVED NO CELLS
DAY OF INFECTION

Fig. 2a

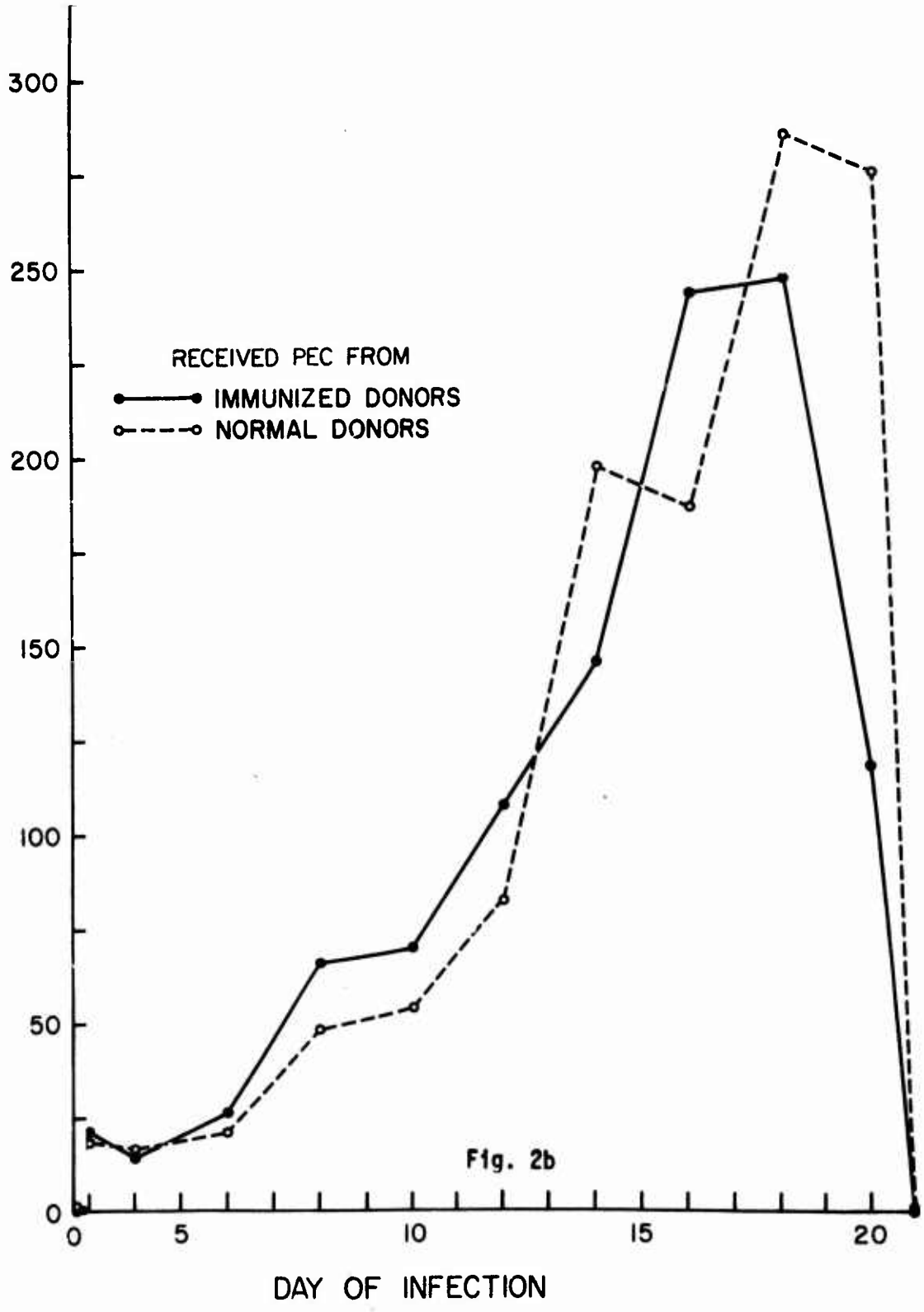
300
250
200
150
100
50
0
RECEIVED PEC FROM
IMMUNIZED DONORS
NORMAL DONORS
0
5
10
15
20
DAY OF INFECTION

Fig. 2b

collected and transferred IP as in previous experiments. No PEC transfer was made. Serum from each group of donors was frozen for use in Experiment 5. For rats in each of the three recipient groups, a $1x10^8$ spleen cell suspension was injected IP. Twelve rats received spleen cells from the γ-PRBC-immunized donors, 8 rats were given spleen cells from the normal donors, and 12 rats were given immune spleen cells from the malaria-recovered donors, all by the IP route. A challenge infection of 1×10^7 PRBC was given intravenously to each recipient rat, and the percentage parasite levels were determined as in previous experiments.

The median peak parasitemia (day 17) for rats receiving spleen cells from normal rats was more than 400 PRBC/500 RBC, whereas the median peak (day 13) for recipients of spleen cells from γ-PRBC immunizes donors was 280 PRBC/500 RBC (Fig. 3a). During the course of infection through day 13, the parasitemia levels for these two groups remained about the same; however, following day 13, parasite levels dropped more rapidly in the latter group. In the recipients of spleen cells from malaria-recovered rats parasitemia was suppressed below the 1% level throughout the experiment.

The parasitemia levels in recipients of serum from γ-NRBC donors and from γ-PRBC donors were similar throughout the infection (Fig. 3b). There was a partial suppression of parasitemia as well as a delayed patency in those rats given serum from malaria-recovered donors.

Experiment 4

Six rats were immunized by the same series of γ-PRBC injections as for those in Experiment 3, and 6 rats were given similar injections of γ-NRBC. Spleen cells and PEC were collected and transferred as described previously: 6 rats received 1×10^8 spleen cells from the γ-PRBC-immunized donors and 6 received 1×10^7 PEC from the same donors. Similarly, 6 rats were given spleen cells and 6 received PEC from the γ-NRBC donors, in numbers corresponding to the respective γ-PRBC groups. A 1×10^7 PRBC malaria challenge was given IV to each recipient rats. Serum from the immune and control donor groups was frozen and stored for use in Experiment 5.

In this part of the experiment parasitemia levels in all recipient groups were unusually low (all less than 125 PRBC/500 RBC), probably due to the greater age of the rats. Parasitemias in recipients of spleen cells from γ-PRBC donors approximated those in recipients of spleen cells from γ-NRBC donors. Similarly, recipients of PEC from γ-PRBC immunized donors had essentially the same course of infection as their γ-NRBC-injected counterparts.

Experiment 5

Serum from experiments 3 and 4 were kept at -20^0C in three pools, according to the donor category, viz., γ-PRBC immunized, γ-NRBC normal

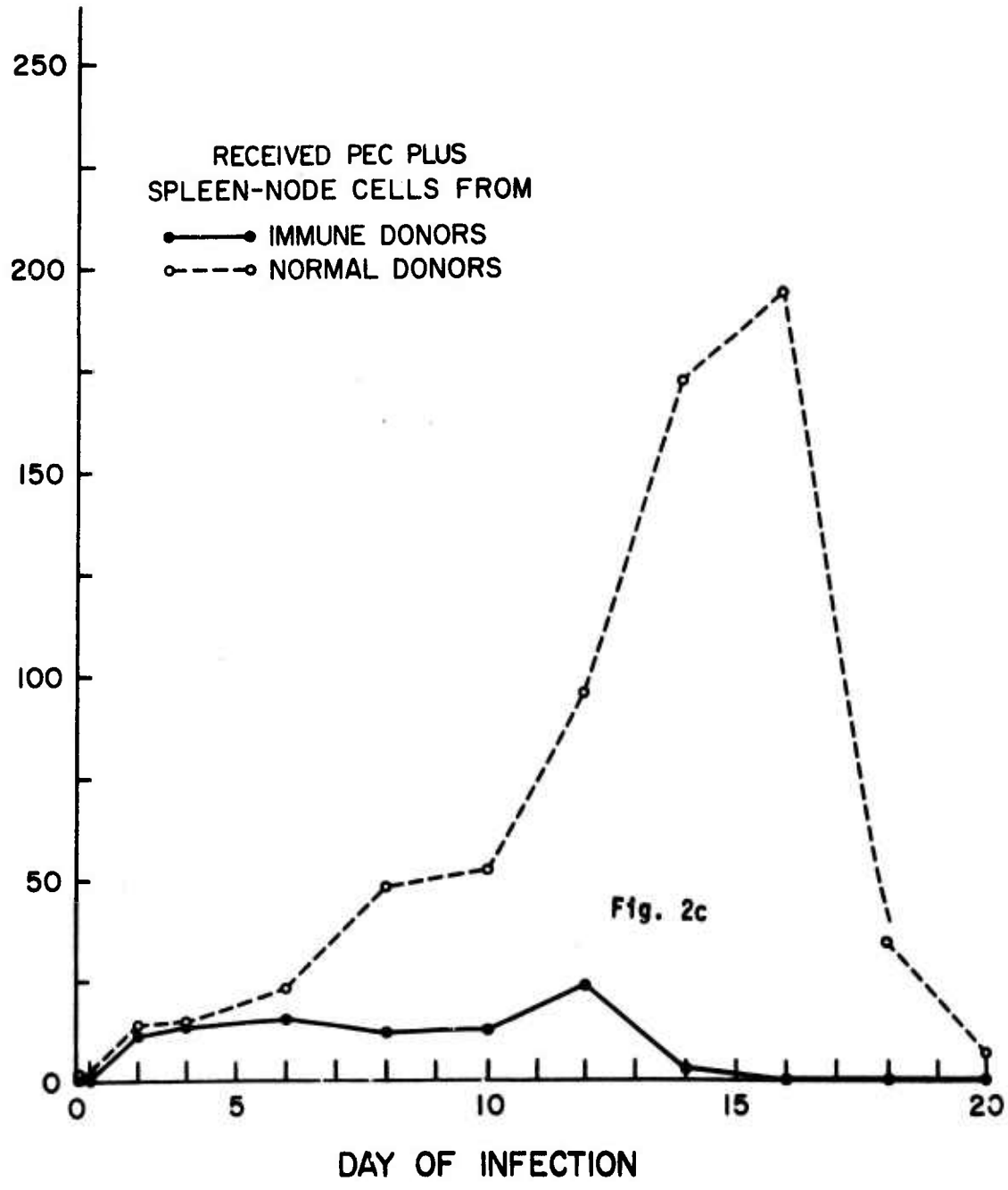
250
200
150
100
50
0
RECEIVED PEC PLUS
SPLEEN-NODE CELLS FROM
IMMUNE DONORS
NORMAL DONORS
0
5
10
15
20
DAY OF INFECTION

Fig. 2c

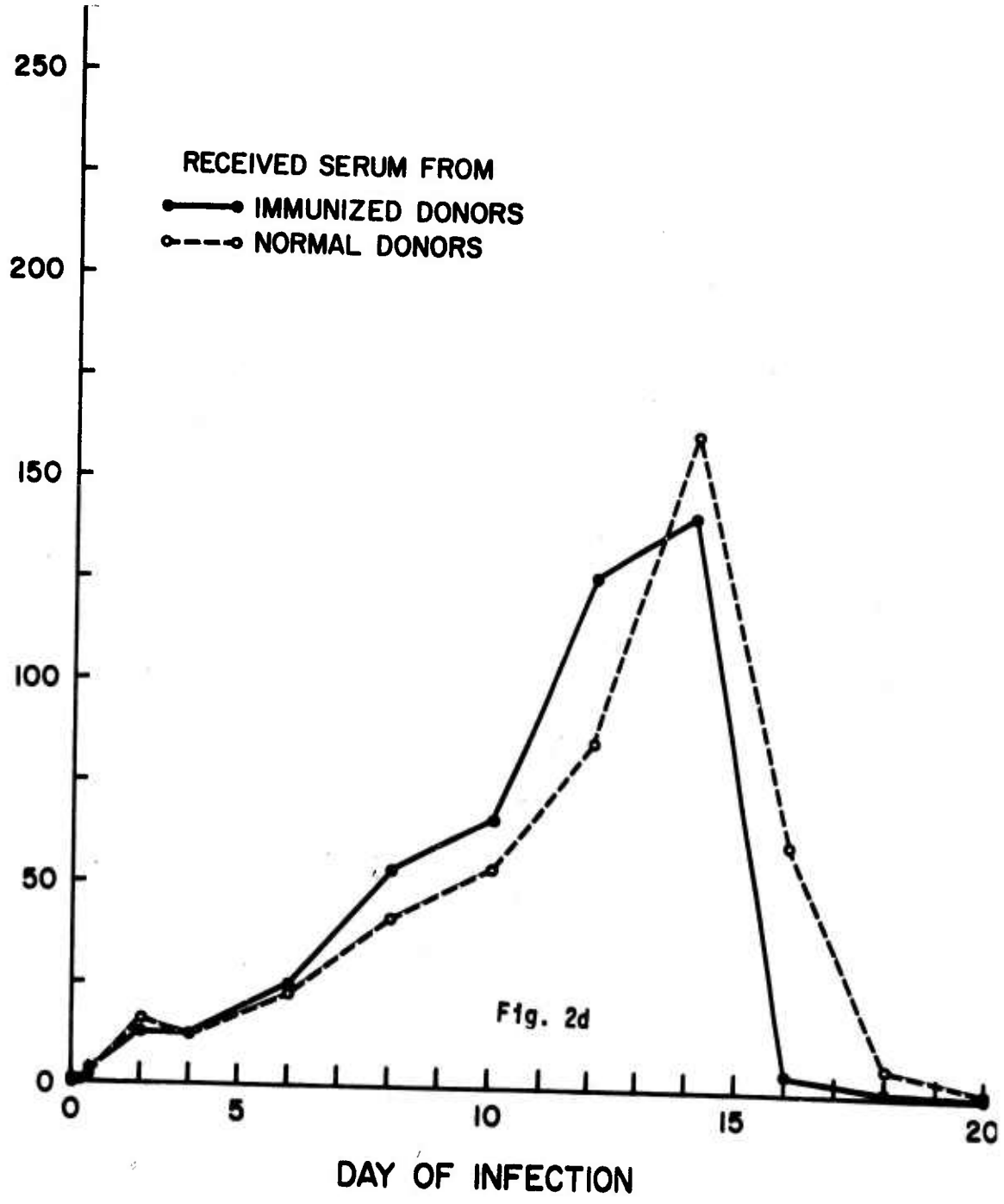
250
200
150
100
50
0
RECEIVED SERUM FROM
IMMUNIZED DONORS
NORMAL DONORS
0
5
10
15
20
DAY OF INFECTION

Fig. 2d

controls, or immunized-recovered. Serum transfer was made in two parts: in the first, a single injection of 3 ml serum was given IP to each of 17 recipients, four hours preceding a 2 x 10^7 PRBC malaria challenge IV. There were 5, 5 and 7 rats in the three respective recipient groups.

In the serum transfer part of this experiment, again, all parasitemias were relatively low. However, the relationships described previously were observed throughout the infection period, viz., a partial suppression of parasitemia with the serum from γ-PRBC immunized donors, relative to those of recipients of serum from γ-NRBC injected donors, and a more pronounced suppression in recipients of serum from recovered rats (peak parasitemias: 30 PRBC/500 RBC in recipients of normal serum; 18 PRBC/500 RBC in recipients of serum from γ-PRBC immunized donors; and 9 PRBC/500 RBC in recipients of serum from recovered donors).

During the past decade there has been in intensified effort to develop models for active immunization against malaria infections. Generally, there has been very little success except for those studies in which some form of the living malaria parasite is utilized for immunogenesis; viz., virulent infection and chemotherapy pre-infection with strains of low virulence or with parasites attenuated by incubation in serum or drugs or by irradiation.

Gamma-irradiation apparently produces metabolically active, viable parasites deprived only of their capability to replicate DNA, although the occurrence of other physiologic consequences to replicate DNA, although the occurrence of other physiologic consequences cannot be ruled out.

As has been suggested in previous reports, vaccination with irradiated parasites might be expected to elicit an acquired immunity as great as, or greater than, that produced in response to an active non-attenuated parasite infection.

The present experiments demonstrate that in a rat malaria model, the spleen and lymph node lymphocytes are stimulated by the influence γ-irradiated plasmodia. These lymphoid cells were shown to confer a partial protection to malaria challenge in passive transfer to non-immunized recipients. Yet, from such donor rats (immunized by as many as 1-2 billion irradiated parasitized cells, and resistant to parasite challenge), the lymphoid cells and serum were less effective than those from rats which had recovered from a full course of malaria. Hence, even in rodents made challenge-resistant by such a vaccination procedure, the cellular and humoral responses have not been stimulated to a degreee achieved by infection and recovery from infection with non-attenuated parasites. It is apparent from this study that if any of the pathologic changes which occur during a fulminating malaria infection produce inhibitory effects on the responsive capacity of lymphocytes, such negative effects are not sufficient to offset the greater efficacy of

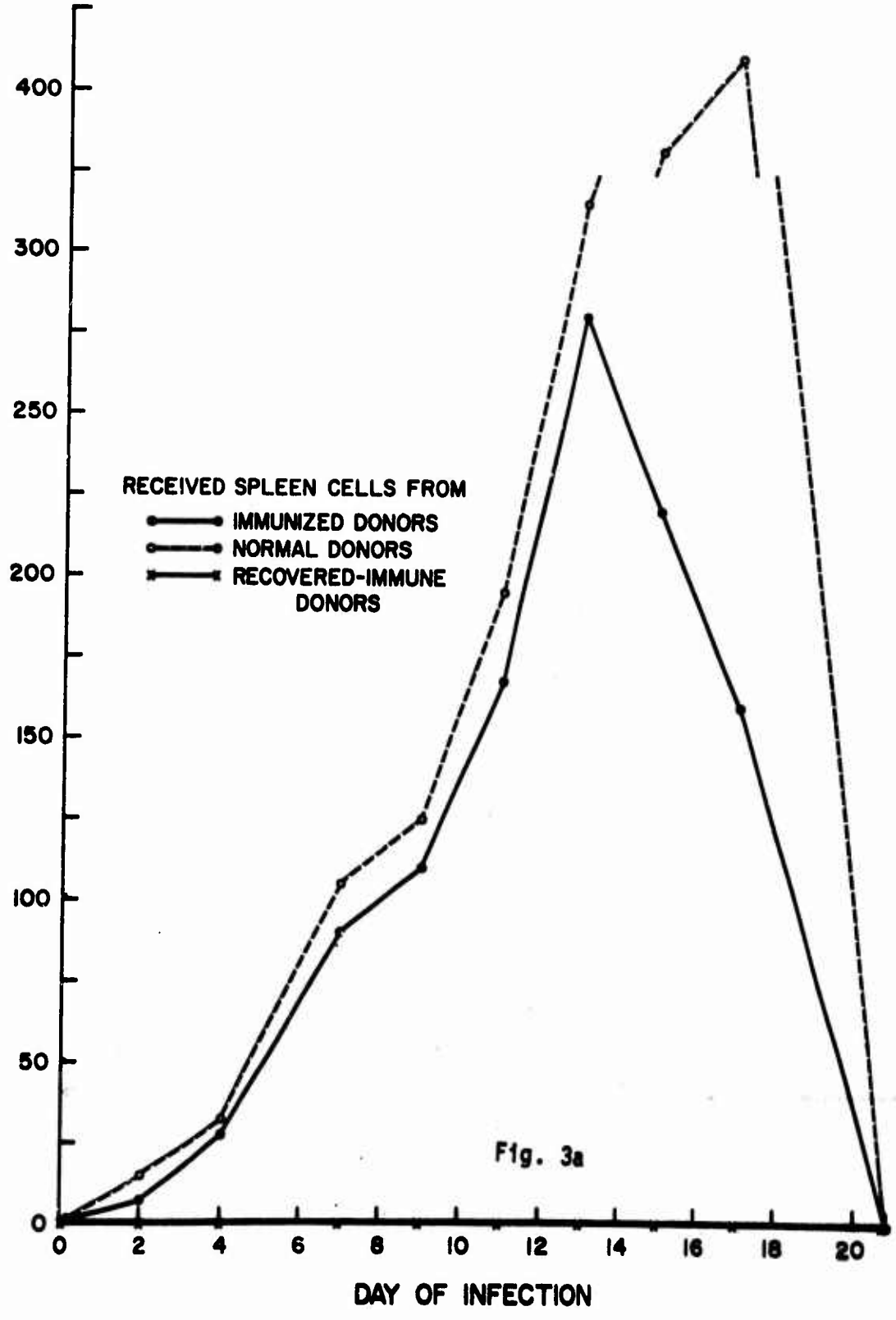
400
300
250
200
150
100
50
0
RECEIVED SPLEEN CELLS FROM
IMMUNIZED DONORS
NORMAL DONORS
RECOVERED-IMMUNE DONORS
0
2
4
6
8
10
12
14
16
18
20
DAY OF INFECTION

Fig. 3a

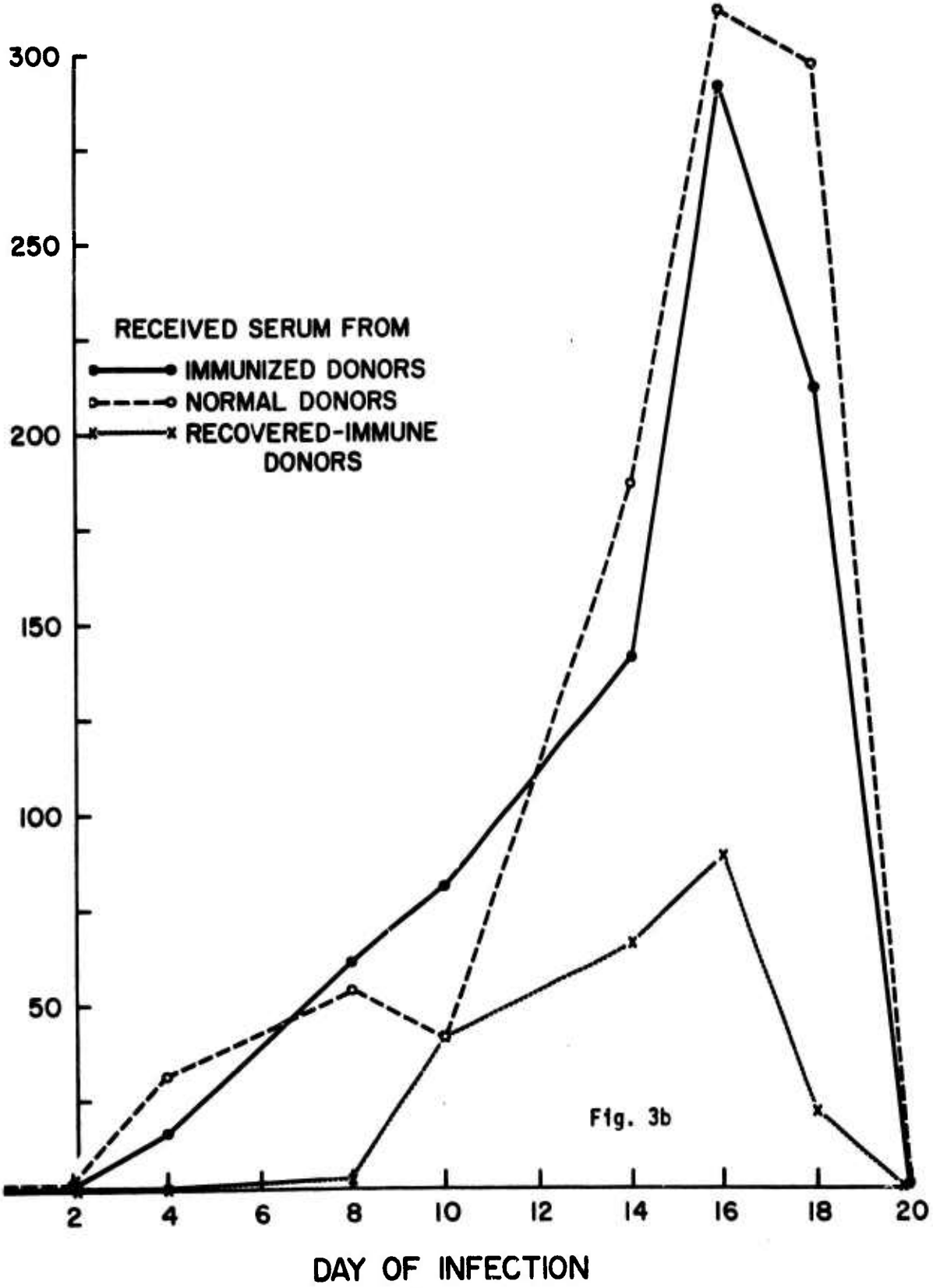
300
250
200
150
100
50
RECEIVED SERUM FROM
IMMUNIZED DONORS
NORMAL DONORS
RECOVERED-IMMUNE DONORS
2
4
6
8
10
12
14
16
18
20
DAY OF INFECTION

Fig. 3b

cells from these malaria-recovered donor animals.

The observed differences in effects from cells of recovered rat or immunized rat origin might be solely quantitative. For example, in an active malaria infection of an adult rat - even with a parasitemia of only about 1%, there is a continuous level of about 10^9 PRBC in the circulation. Consequently, a cumulative total (not constant total) of 10^9 circulating, non-dividing PRBC (γ-irradiated) as used in this study falls considerably short of the total parasite burden in the ordinary malaria infection. Although the 4-6 week γ-PRBC injection schedule equals or exceeds the period for the usual time course of P. berghei (NYU-2) in Fischer rats, the γ-PRBC injections are intermittent and thus do not provide an exact simulation of total time and continuity of host-parasite interaction that takes place in the active malaria infection.

Whether or not there is a difference in proportions of lymphocyte subpopulations (e.g., B versus T cells) stimulated by the injection of γ-PRBC, in contrast to the response caused by the presence of parasites during active infection, is under investigation. The immune response in some malaria infections has been shown to be limited to particular stages of the parasite. In the case of irradiated parasites, it is assumed that stage development is restricted at the point of DNA replication, and it is thus inferred that development of any parasite irradiated and injected, will become limited to the late trophozoite stage, regardless of the stage at which irradiation was made (in non-gametocyte-producing strains, as was used in these studies). Consequently, it is entirely possible that the primary difference between the immune response to irradiated versus non-irradiated parasites is simply a matter of proportional differences in parasite stages.

The question of antigenic variation also arises in considering the response to irradiated or to nonirradiated parasites. In an infection of nonirradiated parasites, the course of parasitemia and recovery may well involve a series of variant-specific responses as selection pressure favors the proliferation of variant strains. Such a response against multiple variants is unlikely in the animal harboring only non-reproducing irradiated parasites. Although the use of multiple injections allows some possibility of introductior of variant strains, the situation is not analogous to that of an active infection. Each of the irradiated parasite pools was derived from a single parent strain of P. berghei maintained by rapid passage in young rats. Consequently, there is relatively little selection pressure from host immune responses, in contrast to, for example, chronic infections, which often involve proliferation of variant populations.

The use of γ-PRBC immunization-inoculations in outbred rodent systems has led to the question of whether at least part of the immune response might have been directed against host erythrocyte membrane antigens. However, the finding that γ-PRBC injections lead to protec-

tion against malarial challenge in an inbred rat model as in the immune cell donors of the present study, indicates that the response is primarily against the parasite rather than heterologous host RBC antigens.

In one experiment (number 2) combined lymphoid (spleen and node) cells plus PEC appeared to produce a considerably greater malaria-suppressive effect than did either of the two types of cells alone. Such an additive effect might be expected to have occurred from either dependent or independent mechanisms (for example, anti-merozoite activity by IgG production from transferred B lymphocytes, plus an enhancement of phagocytosis from the introduction of immune PEC). Moreover, in that experiment a greater total effect apparently was produced by combined immune lymphocytes and PEC, than what might be expected to result as a simple total effect of independent mechanisms. Such an apparent echancement effect suggests that some form of cellular synergims is involved, one possibility being a lymphocyte-macrophage interaction in cell-mediated immunity as hypothesized in the Mackaness model.

However, in another experiment (number 1) there was no such additive or synergistic effect between node cells and PEC.

Criswell, et al. by use of diffusion chamber implants, demonstrated that humoral factors in chronically infected mice enhance the malaria-suppressive capacity of macrophages. The present experiments did not verify that a serum-macrophage interactions occurs in acute P. berghei infection in rats, since the addition of immune serum to immune PEC did not further suppress infections beyond the level of suppression elicited by immune PEC alone. It should be emphasized, however, that in the present experiments the cells harvested from spleen or peritoneal exudate are not regarded as pure populations, but rather, as pools which predominate in lymphocytes and macrophages, respectively.

The role of a recipient animals' own non-committed lymphocytes or non-activated macrophages, in relation to the activity of transferred malaria-immune cells, has not be ascertained. It has been reported that if recipients of cells from animals immune to Listeria or ectromelia receive whole-body irradiation prior to cell transfer, adoptive immunity is precluded. Studies are presently in progress to determine whether whole-body irradiation of recipients influences immune response to P. berghei. By the use of irradiated (or otherwise immunosuppressed) recipients a more precise measure of involvement by various donor cell populations can be measured with less influence from the recipient's own response to malaria challenge.

As in many previous reports, the present study reflects the crucial role of the cellular response in active immunity to malaria. Other studies have been initiated to further define the relative roles of lymphocyte subpopulations and to describe specific mediators of cell-mediated immunity (e.g., macrophage inhibition factor, blastogenesis, and lymphokine activity).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6525	75 06 30	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	H. Term.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	328
b. CONTRIBUTING				
XXXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Clinical Studies of Human Malaria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 11	75 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING: 74	2	195
FISCAL YEAR CURRENT: 75	2	195

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Div of Medicine
Washington DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Canfield, COL C. J.

TELEPHONE: 202-576-3268

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATOR

NAME: McCormick, G. J., Ph.D.

NAME: DA

21. GENERAL USE

Foreign intelligence not considered.

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Study pathophysiology of acute falciparum and vivax malaria; assess various modes of antimalarial therapy as to clinical responses and radical cure. Document metabolic alterations of human and animal red blood cells infected with malaria parasites; assess effect of antimalarial drugs on these alterations to develop new drugs effective against resistant falciparum malaria. Malaria is an extremely important factor in tropical military operations.

24. (U) Document clinical features of acute disease, evaluate available therapeutic agents as to clinical response and radical cure; provide surveillance for toxicity and efficacy testing of new antimalarial agents by government contractors; provide expert consultation on treatment of resistant falciparum infections and secure new strains of malaria for introduction into the volunteer test program. Measure effects of antimalarial drugs on morphologic growth and radiolabeled precursor incorporation into protein and nucleic acids during in vitro schizogony. Establish presence of metabolic pathways in the malaria parasite.

25. (U) 74 07 - 75 06 Consultations with many physicians treating patients for malaria from Vietnam continued informally. Several new drugs and combinations were introduced into clinical test centers; cooperative field trials with investigational compounds in Thailand were conducted. In vitro culture of P. knowlesi parasites has continued to screen antimalarial activity and to demonstrate drug potentiation. This in vitro cultivation system was adapted for study of P. falciparum. A difference in utilization of thymidine was observed between the pyrimethamine-resistant and -sensitive strains. This work unit is being terminated as a function of Division of Medicine. Mission will be taken over by Division of Medicinal Chemistry. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 328 Clinical studies of human malaria

Investigators.
Principal: COL Craig J. Canfield, MC
Associate: Gerald J. McCormick, Ph.D., Gloria P. Willet

Description:

The objectives of this work unit are to assess the clinical state and therapeutic response of patients to acute falciparum and vivax malaria, provide surveillance for toxicity and efficacy testing of new antimalarial agents by contractors, provide expert consultation on treatment of resistant falciparum infections, secure new strains of malaria for introduction into the volunteer test program, study metabolic pathways of the host red blood cell parasite complex and assess the effect of antimalarial drugs on these pathways in order to develop new drugs effective against resistant falciparum malaria.

Progress:

Admission to Walter Reed Army Medical Center for acute or recrudescent falciparum malaria remained at low levels during the reporting period. An undocumented number of telephone consultations were provided on problems associated with malaria infection from a variety of civilian and military treatment facilities throughout the United States.

The principal investigator of this work unit also served as principal investigator for all new antimalarial drugs undergoing evaluation in the clinical centers and in field studies in Thailand. The results of these new drug studies are reported elsewhere. The procedures and techniques used in conducting the trials were published (1). This publication included a summarization of all human work to date with U.S. Army Investigational Antimalarial Drugs.

A previously completed study of antimalarial activities in vitro of analogs of purine bases and nucleosides and of pyrimidines such as orotic acid was published (2).

The in vitro cultivation system was adapted to allow study of P. falciparum. Infected aotus monkeys have been provided by Dr. L. H. Schmidt, Southern Research Institution. A difference in utilization of thymidine was observed; the pyrimethamine-resistant strain (Vietnam-Smith) incorporated labeled thymidine into DNA, while

incorporation by the pyrimethamine-sensitive strain (Vietnam-Oak Knoll) appears to be insignificant. This finding raises the possibility that one mechanism of resistance to pyrimethamine may be the ability of pyrimethamine-resistant parasites to produce thymidylate needed for DNA synthesis by a process independent of the normal methylation of deoxyuridylate. The normal mechanism is affected by pyrimethamine's action against dihydrofolate reductase; its inhibition of production of tetrahydrofolate results in depletion of the methylene-tetrahydrofolate which is the donor of the methyl group for thymidylate synthesis and thus the parasite's subsequent production of DNA ceases in the normal pyrimethamine-sensitive case. Both strains incorporated labeled cytidine into RNA and DNA. This contrasts to the inability of P. knowlesi to incorporate pyrimidine nucleotides or precursors other than orotic acid in this in vitro system.

In the collaborative study with Dynatech Corporation in which antimalarial efficacy of implanted drug preparations is being studied, WR158122 in a modified polymer vehicle (10:90 glycolate: (+) lactate copolymer) was implanted subcutaneously in mice and immediate and weekly challenges with P. berghei were commenced. At an implanted drug dosage of 20 mg/kg, patency of parasitemia was delayed for 10 weeks. In a similar system, sulfalene at dosages of 16 to 130 mg/kg did not protect against infection but apparently alleviated its severity. Parasitemia was patent in mice as early as the first week, but some of these mice survived longer than 10 weeks. Survival time of untreated mice is approximately 10 days.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malarial Investigations

Work Unit 328 Clinical studies of human malaria

Literature Cited.

Publications:

1. Canfield, C. J. and Rozman, R. S.: Clinical testing of new antimalarial compounds. Bull. World Health Org. 50:203-212, 1974.

2. McCormick, G. J., Canfield, C. J., and Willet, G. P.: Antimalarial activity in vitro of analogs of nucleic acid precursors in the simian malaria, Plasmodium knowlesi. Antimicrobial Agents and Chemotherapy 6:16-21, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6471	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	336
b. CONTRIBUTING				
c. [illegible]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Field Studies on Drug Resistant Malaria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	5.8	130
FISCAL YEAR CURRENT 76	4.7	160

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Winter, COL P.E.

TELEPHONE: 985-2267

SOCIAL SECURITY ACCOUNT NUMBER [redacted]

ASSOCIATE INVESTIGATORS

NAME: Hall, COL A.P.

NAME: Pearlman, MAJ E. J.

21. GENERAL USE

Foreign intelligence not considered

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Drug Resistance; (U) Chemotherapy; (U)Chemoprophylaxis; (U)Human; (U)Monkey; (U)Vectors; (U)Chroloquine; (U)Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To determine the efficacy of conventional and experimental antimalarial drugs in the treatment and prophylaxis of drug resistant falciparum malaria. To define vector bionomics which influence the transmission of drug resistant malaria and to develop rationale for vector control. To evaluate the activity of candidate antimalarial drugs against simian malaria. To characterize the clinical features of malaria in Thailand, and to develop improved methods of hospital management of diseases of military importance.

24. (U) U.S. Army investigation antimalarial drugs were compared with standard drugs for treatment of drug resistant falciparum malaria in hospitalized human volunteers in SE Thailand. Chemoprophylactic studies were conducted in rural populations in central Thailand. Chemotherapeutic drugs were studied in rhesus monkeys with P. cynomolgi.

25. (U) 74 07 - 75 06 In studies of chemosuppressive agents in patients with falciparum malaria, a combination of diformyldapsone-pyrimethamine caused a four-fold reduction in the number of people with parasitemia. A similar study of sulfodoxine-pyrimethamine (Fansidar) is underway. The falciparum malaria cure rate for a single dose of Fansidar was 85%, compared to clindamycin, 50%; amodiaquine, 38%; and chloroquine, 0%. Preliminary results with WR 14290 indicate a cure rate equal to or greater than that of Fansidar. A single dose of Fansidar was not gametocidal. Intravenous quinine gave the quickest relief of symptoms. The pathogenesis and treatment of childhood malaria, malaria coma, anemia and pulmonary edema are under continous study. Of nineteen drugs tested in monkeys, WR 194965 and WR 204165 were found to have excellent antimalarial activity. A comparison of erythocytic ATP levels in Thai residents of endemic and non-endemic malarious areas failed to show a difference. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 June 75.

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Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 336 Field studies on drug resistant malaria

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1. The Suppression of Plasmodium falciparum and Plasmodium vivax Parasitemias by a Diformyldapsone-Pyrimethamine Combination

OBJECTIVE: To study the effectiveness of the combination of diformyl-dapsone (DFD) 200 mg and pyrimethamine (Py) 12.5 mg in suppressing parasitemias in an area with known chloroquine resistant falciparum malaria.

BACKGROUND: The combination of dapsone (DDS) and pyrimethamine (Py) in the chemosuppression of chloroquine resistant falciparum malaria has been previously shown to be efficacious. The longer half life of the diformyl congener of dapsone should render this sulfone in combination with pyrimethamine a better chemosuppressive agent.

DESCRIPTION: Six hundred and fifty-nine semi-immune study subjects from three villages in Prachinburi Province, Northeast Thailand were assigned to one of five drug study groups. Subjects received a weekly medication, under a double blind design, of one of the following:

a. DFD 200 mg and Py 12.5 mg
b. DFD 400 mg
c. DDS 100 mg and Py 12.5 mg
d. Py 25 mg
e. Placebo

Each study subject was visited weekly; at which time the medication was given and swallowed under supervision; a capillary blood drawn for a thick-thin malaria smear; and a history of illness since the prior visit noted. Following the drug phase of the study four additional followup visits were made.

PROGRESS: Five hundred ninety-three study subjects (90%) completed the twenty-six week course of medication. Figure 1 shows that the weekly attack rates of new falciparum infections were lower during the medication phase of the trial in the sulfone groups (DFD-Py, DDS-Py, and DFD) compared to either the pyrimethamine or placebo group. It can be further seen that parasitemias were suppressed in the DFD-Py, DDS-Py, and DFD groups in the early phase of the study. An increased number of falciparum cases was seen in weeks 7-8 in both the DFD-Py and DFD groups, while a similar increase was seen in the DDS-Py group later (week 13).

Figure 2 shows the cummulative infection rates of individual study subjects in the course of the 26 week trial and the subsequent follow-ups. The data indicate a 4.6 fold reduction in the cumulative

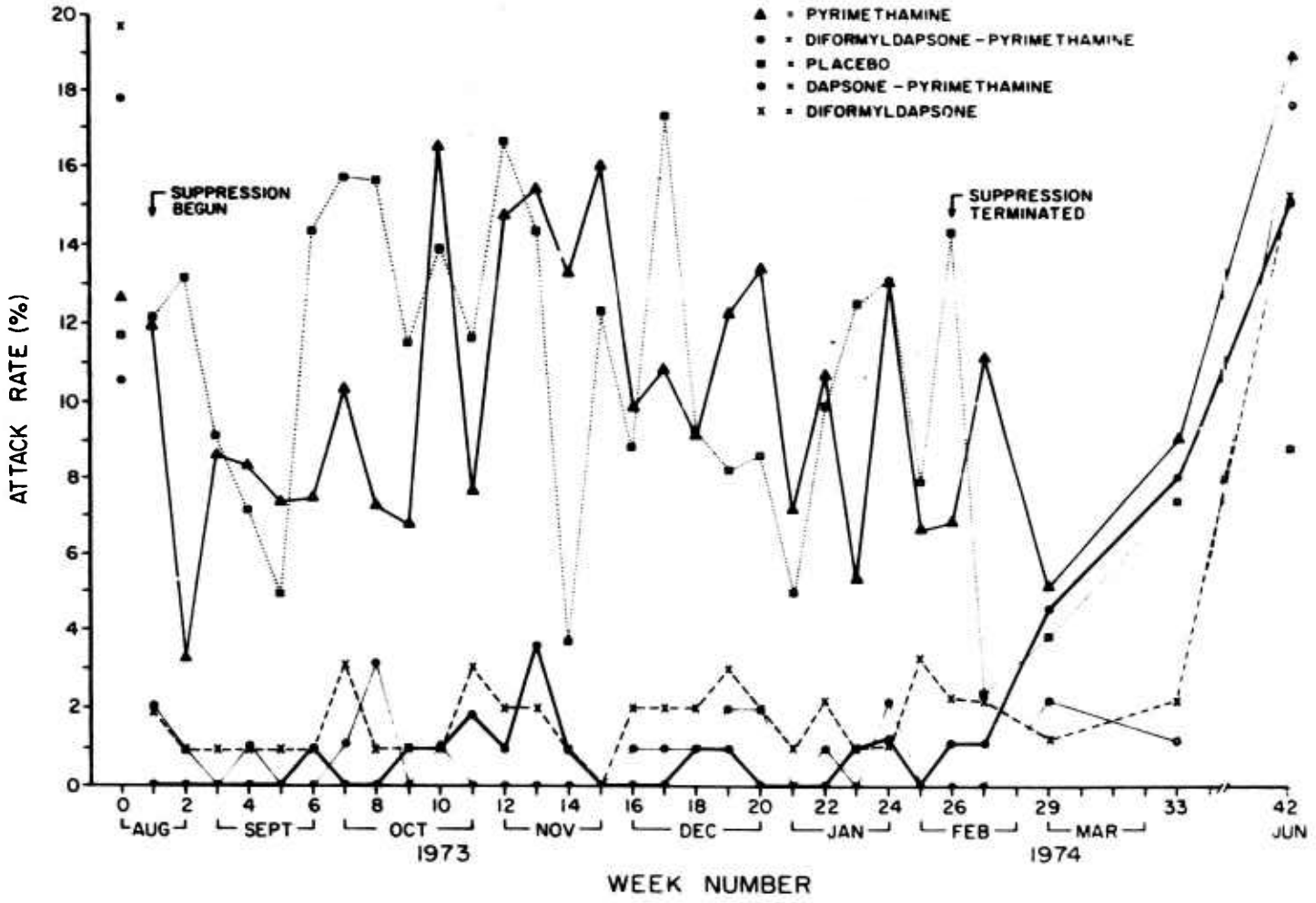

FIGURE 1. WEEKLY ATTACK RATE OF SUBJECTS INFECTED WITH P. FALCIPARUM BY STUDY GROUP

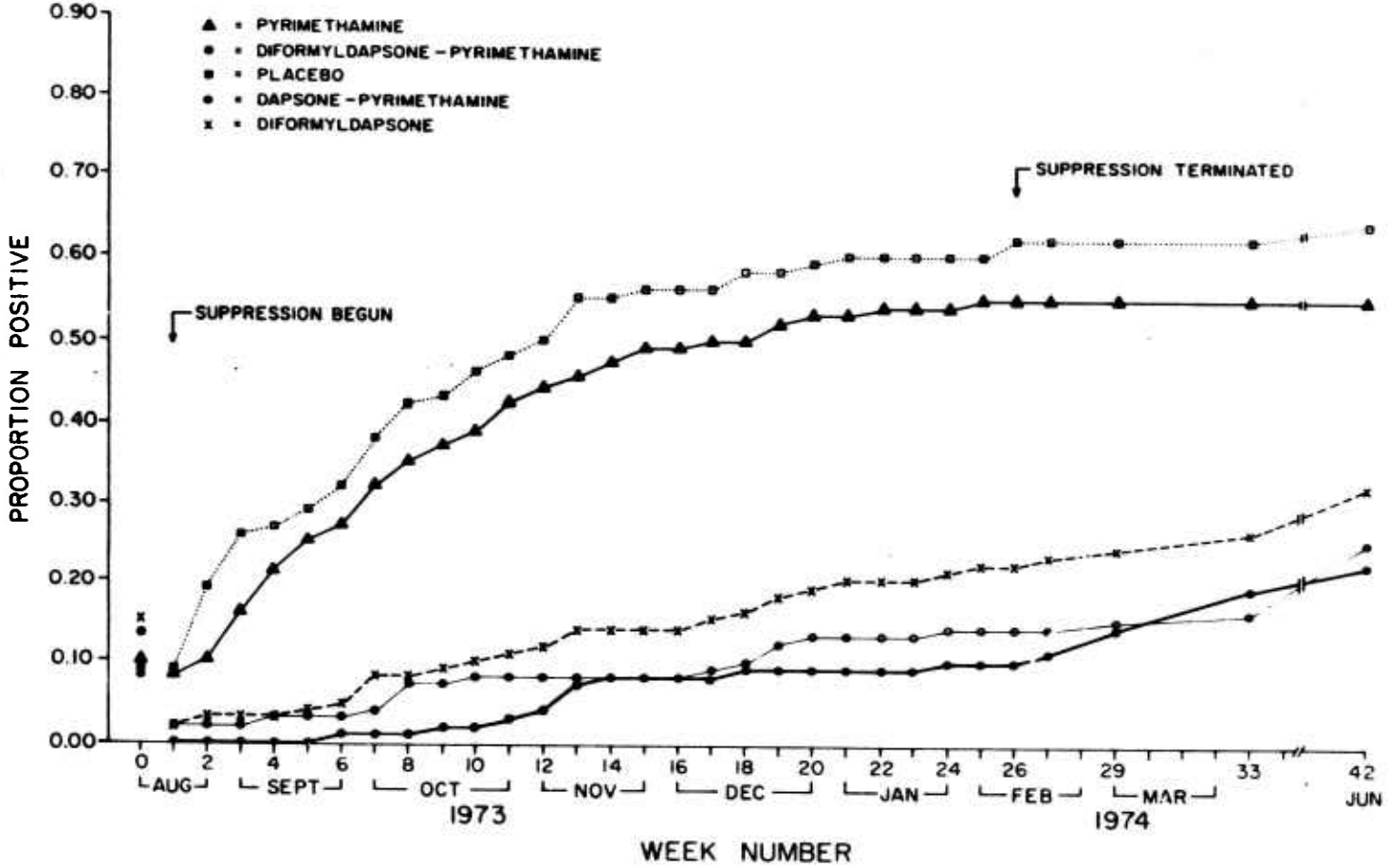

FIGURE 2 CUMULATIVE PROPORTION OF SUBJECTS INFECTED WITH P FALCIPARUM BY STUDY GROUP

parasitemic rate for P. falciparum in the DFD-Py group and a 6.4 fold reduction in the DDS-Py group when compared to the placebo group.

The results of microscopy for falciparum parasitemias are given in Table 1.

Table 1. Results of Slide Microscopy* Drug Groups

Slide results	Pyrimethamine	DFD-Py	Placebo	DDS-Py	DFD
Negative slides	2139	2697	2015	2716	2528
Falciparum(P.f.t.)positive	433	27	449	28	58
Vivax(P.v.t.)positive	124	25	148	46	111
Mixed(P.f.t.+P.v.t.)positive	22	2	14	1	6
Total	2718	2751	2626	2791	2703

*Excluded are P. falciparum gametocytemia (P.F.G.) results and smears preceeded the week before by absenteeism.

Statistical evaluation comparing the various drug regimens was undertaken. Highly significant results were obtained which showed that DFD-Py, DDS-Py, and DFD alone were effective chemosuppressive agents against P. falciparum when compared with placebo alone ($0.0005 > p$). DFD-Py, as a combination, was more effective than its component parts: DFD ($0.0005 > p$) or Py ($0.0005 > p$) in suppressing a falciparum parasitemia. Significant differences, likewise, were seen when DDS-Py was compared with DFD alone ($0.0005 > p$), and Py alone ($0.0005 > p$); however, a direct comparison between DFD-Py and DDS-Py failed to reveal any significant difference in efficacy ($0.4875 > p > 0.475$). Table 2 summarizes these statistics.

Table 2. Calculated Student-t-values for Falciparum Parasitemias (p value)

	DFD	Py	Placebo	DDS-Py
DFD-Py	3.7470* ($0.0005 > p$)	20.4216* ($0.0005 > p$)	21.0752* ($0.0005 > p$)	0.0553 ($0.4875 > p > 0.475$)
DDS-Py	3.8181° ($0.0005 > p$)	20.5830° ($0.0005 > p$)	21.2391° ($0.0005 > p$)	

*Values significant in favor of DFD-Py
°Values significant in favor of DDS-Py

While study subjects receiving the drugs: DDS-Py, DFD-Py or DFD alone were parasitemic less often (Table 3), no statistically significant differences (X^2 = 2.1613; 0.40>p>0.35) in the density of falciparum parasites was seen (Table 4).

Table 3. P. falciparum Asexual Parasitemias Experienced by Study Subjects During Chemosuppression

Group	Number subjects	Number(prop.) infected	Total(average) number of episode	Average duration (weeks) of episode
DDS-Py	123	12(0.10)	17(1.42)	1.42
DFD-Py	118	16(0.14)	19(1.19)	1.42
Diformyldapsone	117	26(0.22)	42(1.62)	1.43
Pyrimethamine	118	64(0.54)	207(3.23)	1.89
Placebo	117	73(0.62)	234(3.21)	1.98

Table 4. Densities of P. falciparum Asexual Parasitemias Experienced by Study Subjects During Chemosuppression

Group	Number (proportion) parasitemias*	
	≤20	>20
DDS-Py	16(0.94)	1(0.06)
DFD-Py	16(0.84)	3(0.16)
Diformyldapsone	39(0.93)	3(0.07)
Pyrimethamine	180(0.87)	27(0.13)
Placebo	208(0.89)	26(0.11)

*In parasites per 100 white blood cells.

With cessation of chemosuppression the following new falciparum infections were seen in the five groups: thirteen in the DFD-Py group; eleven in the DFD group; fifteen in the DDS-Py group; one in the pyrimethamine group; and two in the placebo group. Of these post-treatment falciparum parasitemias (24 of 42) occurred four months later. This would correspond to June, 1974, and the start of a new malaria transmission season.

A large number of vivax infections were seen in this study as shown in Table 5.

Table 5. P. vivax Parasitemia Experienced by Study Subjects During Chemosuppression

Group	Number of subjects	Number (prop.) infected
DDS-Py	123	17(0.14)
DFD-Py	118	16(0.14)
DFD	117	28(0.24)
Py	117	52(0.44)
Placebo	117	51(0.44)

The Py and placebo groups each had a 44% cumulative vivax infection rate while the DFD-Py group had 14%, the DDS-Py group 14%, and the DFD group 24%. Statistical evaluation (Table 4) showed that the three sulfone groups suppressed vivax parasitemias better than pyrimethamine when compared with the control group. Py alone was totally ineffective. DFD-Py was not only more effective than its component parts: DFD ($0.0005>p$) and Py ($0.0005>p$), but DFD-Py was also more efficacious than DDS-Py ($0.01>p>0.005$) in the weekly chemosuppression of vivax malaria. DDS-Py likewise was more effective than DFD alone ($0.0005>p$) and Py alone ($0.0005>p$) in the weekly suppression of P. vivax parasitemias. Table 6 summarizes this information.

Table 6. Calculated Student-t-values for Vivax Parasitemias (p value)

	DFD	Py	Placebo	DDS-Py
DFD-Py	7.7092* ($0.0005>p$)	9.2758* ($0.0005>p$)	9.2758* ($0.0005>p$)	2.2793*
DDS-Py	5.7599° ($0.0005>p$)	7.4425° ($0.0005>p$)	8.5668° ($0.0005>p$)	-

*Values significant in favor of DFD-Py
°Values significant in favor of DDS-Py

Following completion of the chemosuppressive phase of the study there was an increase seen in new cases of vivax malaria in the DFD-Py group-15; DDS-Py-16; DFD-11; Py-4; and placebo-5. The increase in the cumulative values for vivax infections are for the DFD-Py group from 0.14 to 0.29; for the DDS-Py group from 0.14 to 0.27; for the DFD group from 0.24 to 0.33; for the Py group from 0.44 to 0.48 and for the placebo group from 0.44 to 0.49.

SUMMARY: The combination DFD-Py given weekly was shown to be an effective chemosuppressive against both falciparum and vivax parasitemias, causing a four fold plus reduction in falciparum parasitemias, and an approximately three fold reduction in vivax parasitemias; however, this combination was not more efficacious than DDS-Py for the chemosuppression of falciparum malaria. DFD was only moderately effective, while there was no difference in chemosuppression between pyrimethamine and placebo.

2. The Suppression of Plasmodium falciparum and Plasmodium vivax Parasitemias by a Sulfadoxine-Pyrimethamine Combination

OBJECTIVE: To study the effectiveness of the combination of sulfadoxine (S) 500 mg and pyrimethamine (Py) 25 mg given in two dose regimens in suppressing parasitemias in an area with known chloroquine resistant falciparum malaria.

BACKGROUND: The combination of a sulfone or sulfonamide with Pyrimethamine in the chemosuppression of chloroquine resistant falciparum malaria has been previously shown to be efficacious. The longer half life of a long acting sulfonamide, such as sulfadoxine ($t_{1/2}$ = 150-200 hrs), should render this, in combination with a matched (in terms of $t_{1/2}$) dihydrofolic acid reductase, a better chemosuppressive agent.

DESCRIPTION: Seven hundred and fifty six semi-immune study subjects from four villages in Prachinburi Province, Northeast Thailand were assigned to one of five drug study groups. Subjects received, under a double blind design, one of the following medications:

a. Sulfadoxine 1000 mg - pyrimethamine 50 mg biweekly
b. Sulfadoxine 500 mg - pyrimethamine 25 mg biweekly
c. Diformyldapsone 200 mg - pyrimethamine 12.5 mg weekly
d. Diformyldapsone 400 mg - pyrimethamine 25 mg weekly
e. Placebo weekly

Each study subject was visited weekly, at which time the medication was given and swallowed under supervision, a capillary blood drawn for a thick-thin malaria smear, and a history of illness since the prior visit noted. For those subjects receiving a biweekly medication

regimen placebo tablets were given on the alternate weeks, thus study subjects received two tablets weekly.

PROGRESS: The twenty-six week course of medication phase of the study was concluded on 8 February 1975. Currently the study subjects are being monitored bimonthly for evidence of malaria transmission in the absence of chemosuppressive agents. At the termination of the medication phase, the average weekly study subject participation rate was approximately 88%. As the microscopy has not been completed, no data reduction is possible at this time.

SUMMARY: The combination of sulfadoxine and pyrimethamine has been given beweekly at two dosage levels for 26 weeks to semi-immune individuals in an area known to have chloroquine resistant falciparum malaria. The preliminary results of this study are not yet available.

3. Evaluation of the Sporonticidal Activity of Pyrimethamine-Sulfadoxine (Fansidar) Against P. falciparum in Thailand

OBJECTIVE: To determine the effect of single dose Fansidar therapy upon the subsequent development of oocysts and sporozoites of P. falciparum in vector mosquitoes and to correlate this information with plasma levels of pyrimethamine and sulfadoxine at the time of mosquito feeding. The activity of pyrimethamine alone will also be evaluated.

BACKGROUND: Fansidar, the fixed combination of pyrimethamine and sulfadoxine, has been shown to be very effective against chloroquine-resistant and chloroquine sensitive strains of P. falciparum as well as P. vivax malaria in many parts of the world. It is currently recommended in Thailand as an alternative therapeutic regimen in the guidelines of the National Malaria Eradication Project. A number of studies (1, 2) have proven its effectiveness as a schizonticide, but it has not been adequately investigated as a gametocytocide and sporonticide. Chin, et al. (1) fed A. b. balabacensis mosquitoes on patients following single-dose therapy; however, plasma levels of the constituent drugs were not determined at any time. In their series, 47% of mosquitoes fed on patients with gametocytemia showed development of the parasite up to the sporozoite stage, after feeding on days 7-9 and 13-14 following therapy.

In view of the already widespread use of Fansidar in the therapy of malaria in Southeast Asia, and its emergence as the drug of choice for chloroquine-resistant malaria in many parts of the world, the effect of this drug upon the infectivity of gametocytes needs to be conclusively determined. Epidemiologically, it is essential to

be aware of the need for the additional use of a sporonticidal drug, such as primaquine, in combination therapy.

DESCRIPTION: Patients presenting to the Malaria Eradication Service and district hospital outpatient clinics in Phrabuddhabat, Central Thailand, who are at least 15 years old and are found to have infections with P. falciparum are considered eligible for admission to the study.

Eligible patients are then randomly assigned to one of three groups: Group A: patients are treated with a single dose of Fansidar, two tablets (total 50 mg pyrimethamine, 1000 mg sulfadoxine). Group B: patients are treated with quinine, 10 grains every eight hours for six days. Group C: patients receive quinine as in Group B, plus pyrimethamine 50 mg daily for three days.

Fansidar, either alone or in combination with quinine, is the standard therapy for P. falciparum used in the outpatient clinic and on the wards in Phrabuddhabat Hospital, and in the clinic operated by the National Malaria Eradication Project.

Medications are administered by the nursing staff, under the supervision of the study physicians. At the conclusion of the 21-day study period, patients from Group B and C are given two tablets of Fansidar. Recrudescences, if they occur are re-treated with Quinine-Fansidar on an individualized basis.

Parasite counts are performed and blood is drawn for pyrimethamine and sulfadoxine levels before treatment is begun, daily in hospital and on days 5, 10, 15 and 20. Mosquito feeds are performed on days 0, 5, 10, 15 and 20 using colonized A. b. balabacensis from the SEATO Medical Research Laboratory Phrabuddhabat insectary. Patients are asked to return to the SEATO Medical Research Laboratory insectary for follow-up. If necessary, they are followed at home.

Ten percent of the mosquitoes fed on the patients are dissected ten days after feeding, and all mosquitoes are dissected on day 15, regardless of the results of the 10-day dissection.

Plasma pyrimethamine and sulfadoxine levels are performed at the SEATO Medical Research Laboratory Biochemistry Laboratory.

Numbers of mosquitoes developing oocysts and sporozoites will be evaluated as a function of plasma levels of drug at the time of feeding. Patients treated with quinine, which is known to have no effect on gametocytogeny or the development of mosquito forms, will provide the control population.

PROGRESS: Data collection is incomplete, and biochemical analyses are still pending, but it is becoming apparent that gametocytes from patients with detectable pyrimethamine and sulfa are infective to mosquitoes in some cases. To date, four of eight patients treated with the pyrimethamine-sulfadoxine combination developed gametocytes infective to the vector mosquitoes. All patients had detectable serum levels of pyrimethamine and sulfadoxine at the time of mosquito feeding.

4. Hospital Survey of Malaria in Trad Province, Southeast Thailand

OBJECTIVE: To conduct a comprehensive outpatient and inpatient survey of the clinical picture of falciparum and vivax malaria in a highly endemic area and to relate these findings to the results of therapy.

BACKGROUND: Certain parts of Trad Province are highly endemic for malaria and this disease accounts for 50% of all admissions to the Trad Hospital. SEATO studies were initially performed at the Hospital by Colwell and his team in 1970. The Rieckmann in-vitro test showed that the falciparum malaria was highly resistant to chloroquine. Extensive experience was also gained with the combination of quinine and tetracycline in the treatment of falciparum malaria. These studies terminated in 1971. In January 1973 an outpatient clinic was established at the Trad Hospital and maintained for an 18 month period.

DESCRIPTION: The SEATO outpatient clinic at Trad Hospital was open daily from 11 January 1973 to 21 July 1974. The patients were self referred or referred by the Hospital staff. The subjects varied from healthy people requesting a blood checkup to patients in deep coma admitted to the ward and then examined. On each patient the following details were recorded: the SEATO OPD number, date, time, age, sex, name, asexual count, gametocyte count, history of malaria and the fact whether the patient had been born locally (local) or migrated from another part of Thailand (migrant). On patients with a positive malaria slide, a clinical history was taken, the temperature recorded and examination performed for splenomegaly. Selected patients positive for malaria were admitted to the ward and treated with one of the therapeutic regimens being evaluated. Further data was systematically collected. The data was entered on punch card transcripts and computer analysis will be performed. Complete data on an individual patient comprises the following: age, sex, parasite count in clinic, temperature in clinic, presence or absence of splenomegaly (in clinic or ward), migrant or local status, prior history, initial parasite count in hospital, maximum parasite count in hospital, parasite clearance

Table 1. SEATO Clinic, Trad Hospital 1973-1974.
Number of Patients with Falciparum and Vivax Malaria

Month	Patients Screened	Falciparum Malaria	Vivax Malaria
1973			
January*	167	75	11
February	318	102	14
March	618	278	44
April	743	344	38
May	642	252	43
June	585	221	46
July	537	225	37
August	603	208	51
September	436	103	31
October	292	71	13
November	590	359	41
December	483	268	25
Total	6014	2506	394
1974			
January	404	187	34
February	487	239	43
March	800	378	80
April	1071	511	112
May	1111	481	122
June**	950	385	102
July	404	137	42
1973-1974 Total 11241	11241	4824	929

* 11-31 January 1973
** 1-21 July 1974

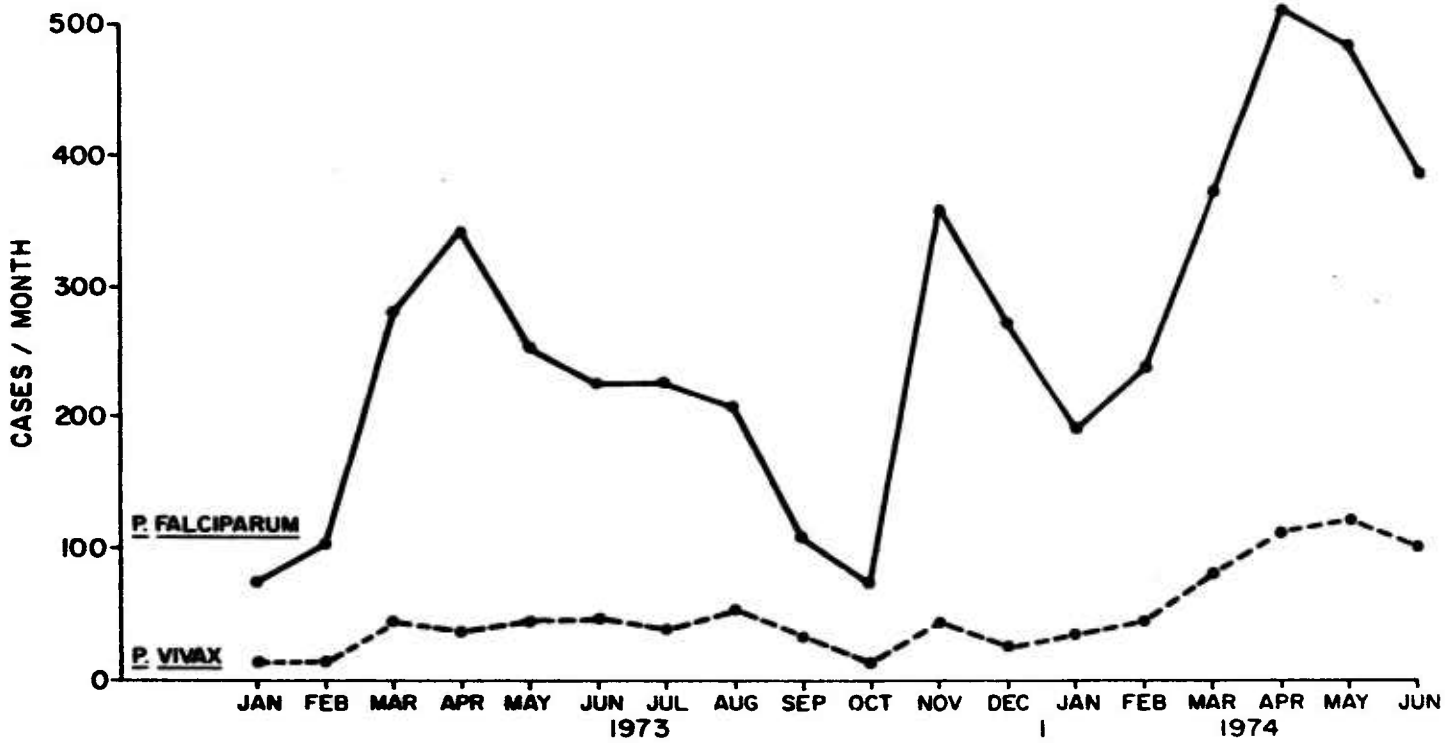

Figure 1. SEATO Malaria Clinic, Trad Hospital, 1973-1974. Number of patients with falciparum and vivax malaria.

time (in hours), initial temperature in hospital, temperature clearance time (in hours), lowest hematocrit, weight, serum bilirubin, serum creatinine, type of therapy and therapeutic result.

PROGRESS: During the study, 11,241 patients were screened for malaria in the clinic by means of a blood film. Falciparum malaria was diagnosed in 4,824 people and vivax malaria in 929 (Table 1). In 1973 the first peak for falciparum malaria (Figure 1) occurred in April (344 cases) and a second peak in November (359 cases). The incidence of vivax malaria showed an irregular fluctuation. The prevalence of malaria was much greater in 1974 and falciparum malaria peaked again in April (511 cases). Vivax malaria peaked in May 1974 (122 cases). About 950 patients with falciparum malaria were admitted to the ward and studied by the SEATO Lab.

Details of the therapeutic regimens used and results obtained are detailed elsewhere in this report. Computer analysis of all the data has not yet been completed. Preliminary analysis of the data has clearly shown that the average severity of the disease (as shown by the parasite count) was greater in the patients who had migrated from other parts of Thailand, in comparison with those born locally. Also the average level of parasitemia was lower in women and in old people. The largest group of patients with falciparum malaria had a parasite count between 10,000 and 100,000 per cu. mm. There was a positive correlation between the parasite count, the degree of anemia and the degree of jaundice. Clinically the average severity of the disease is worse during the seasonal peaks of incidence. It will be interesting to see whether the computer analysis supports this impression.

SUMMARY: Between January 1973 and July 1974, the SEATO Laboratory maintained a malaria outpatient clinic in a highly endemic area of Southeast Thailand. The peak of malaria incidence was higher in 1974 than 1973. This confirms that malaria is still a serious problem in that area. Four thousand eight hundred patients were diagnosed in the clinic as having falciparum malaria and about 950 of these patients were admitted for therapeutic studies. A computer analysis of the outpatient and inpatient data is being prepared.

5. Amodiaquine Resistant Falciparum Malaria in Thailand

OBJECTIVE: To determine the comparative efficacy of amodiaquine and chloroquine against falciparum malaria in Thailand.

BACKGROUND: Interest in 4-aminoquinolines other than chloroquine was reawakened by Schmidt (quoted by Rieckmann, 3) who found in owl monkeys that two chloroquine-resistant strains of P. falciparum

were more susceptible to amodiaquine than to chloroquine. Rieckmann obtained similar findings both in vitro and in vivo although radical cures were not achieved in volunteers. Fitch demonstrated that owl monkey erythrocytes infected with chloroquine resistant P. falciparum had a deficiency of chloroquine-^{14}C uptake, but not deficiency of amodiaquine-^{14}C uptake (4). Therefore, we compared the therapeutic efficacy of the two drugs in a chloroquine-resistant endemic area. Preliminary results of this study have been tabulated (5).

DESCRIPTION: The study was performed at Trad Hospital in Southeast Thailand in 1973 and 1974. Details of the research methods have recently been given (5). The area is forested and malaria is endemic throughout the year. Chloroquine given either orally or parenterally (but not amodiaquine) is used frequently to treat patients with the clinical diagnosis of malaria. Chemoprophylaxis is not practiced in the community. All patients were fully informed on the nature of the drug trial and consent was granted voluntarily. They all had mild or moderate falciparum malaria and an asexual count greater than 1,000 per cu. mm. Alternate patients were assigned to chloroquine or amodiaquine. Chloroquine is 7-chloro-4 (4'-diethylamino-1'-methyl butylamino) quinoline. The dosage form used was not enteric coated ("Nivaquine" tablets by May and Baker which contained 150 mg. of chloroquine base). Amodiaquine is 7-chloro-4 (3'-diethylamino methyl-4'-hydroxyanilino) quinoline, and the dosage form was a non-enteric coated tablet (Camoquine by Parke-Davis) which contained 200 mg. of the base. A 1.5 g. course of both drugs was administered during three days; 600 mg. on the first day was followed by 300 mg. six hours later and 300 mg. on each of the succeeding two days.

Because only a minority of patients were cured by 1.5 g. of amodiaquine given during three days, a 2.0 g. course during four days was studied in an additional group of patients. Most patients received 400 mg. (two tablets) initially followed by 400 mg. six hours later on day 0, then 400 mg. on the mornings of days 1, 2 and 3. All medications were administered by the study physicians.

Direct quantitative parasite counts (6) were performed before treatment, twice daily in hospital for at least three days and on days 14, 21 and 28 from the beginning of therapy. Daily hematocrits and leukocyte counts were done. Fifty milliliters of urine were obtained daily and frozen. The specimens were later analyzed for amino-quinoline content. Ten milliliters of urine (ph 8.3) were extracted with 25 ml. of a 4:1 solvent of chloroform and isopropanol. Ten grams of anhydrous sodium sulfate were then added to the extract and the solutions filtered. The dried filtrates were

dissolved in a few drops of methanol and then spotted on TLC plates precoated with silica gel. The plates were developed with a mixture of ethyl acetate, methanol and ammonium hydroxide (85:10:5) and then sprayed with acidified iodoplatinate. Control urines containing added chloroquine or amodiaquine were processed similarly and used as standards. The R_f for chloroquine was 0.85 and for amodiaquine was 0.96.

CHLOROQUINE 1.5 g. In hospital, chloroquine cleared parasitemia in only three of the 13 patients (Table 1) despite the mildness of many of the infections (average parasite count only 13,920). One of these men developed a recrudescence in the follow-up period; the other two could not be traced. In many patients chloroquine had little or no effect on parasitemia - a potentially dangerous situation. Therefore, treatment with chloroquine was stopped after 13 patients and the final four patients received amodiaquine. The following case history illustrates the severity of chloroquine resistance in the patients studied.

Patient No. 7: This patient had a headache, myalgia and cough on admission. There was a fever of 39.5°C. Therapy with chloroquine 600 mg. was given. On the next morning another 600 mg. of chloroquine was administered. His parasitemia was 8,220 which increased to 18,270 in the evening. Another 300 mg. of chloroquine was given at 1600 hours. The fever increased to 40.4°C and the patient became toxic and vomited. An intravenous infusion of quinine was given and the patient had greatly improved by the next morning. A ten dose course of quinine was administered, the fever and parasitemia cleared and the patient was asymptomatic when discharged; however, a recrudescence occurred on the 26th day.

Comment: Chloroquine 1.5 g. was followed by an RIII response and a three day course of quinine was followed by an RI response.

AMODIAQUINE 1.5 g. The average total dose for the group was 30 mg/kg compared with 28 mg/kg for the chloroquine group. The standard 1.5 g. course of amodiaquine was significantly more successful ($p<0.01$) in clearing parasitemia (15 out of 17 patients) than chloroquine (Table 2). The mean parasite clearance time was 77 hours which, in relation to the mean parasite count of only 18,000 per cu. mm., can be considered prolonged. The mean fever clearance time was 47 hours which is unusually low for an anti-malarial drug in our test system.

Four patients did not attend follow-up examinations and radical cure was achieved in 38 percent (5/13) of the remainder. This is not a successful result because most of the patients had clinically mild disease.

AMODIAQUINE 2.0 g. The average total dose for the group was 42 mg/kg. Twenty-four patients with mild to moderate disease were treated. The parasitemia was cleared in 22 men (Table 3). The mean parasite clearance time was 77 hours. The mean fever clearance time was 36 hours which was much shorter than for any other regimen that we have tested, and probably indicates that amodiaquine does not cause a drug fever. The overall cure rate of 38 percent (8/21) was the same as was obtained with the 1.5 g. course of amodiaquine in the group of patients with a lower mean parasite count (Table 4). One patient had a clear-cut RIII response and another an RII response and the case histories are described below. In eleven patients the parasitemia cleared in hospital but a recrudescence occurred after discharge.

Patient No. 34: This patient was a 34 year old gem-miner who had migrated to the highly endemic area one month previously. He was probably non-immune. He had experienced headache and myalgia for nine days and was thirsty. His parasite count was 121,125 per cu. mm. His fever was 38.5°C on the day of admission and he received 600 mg. amodiaquine followed by 200 mg. four hours later. Despite another 400 mg. amodiaquine at 0600 hours on day 1 his parasite count increased to 207,100 and his fever increased to 39.2°C with severe clinical toxicity. One dose of intravenous quinine therapy was given and then a single dose of pyrimethamine with sulfadoxine (Fansidar). His fever and parasitemia cleared but the patient did not attend follow-up.

Patient No. 37: This patient was a 38 year old farmer who had always lived in the endemic area. Therefore, using conventional terminology, he could be considered semi-immune. He had a headache for two days and had received one injection on the day before admission. His parasite count was 97,395 per cu. mm. and fever 39.4°C. On the day of admission 600 mg. of amodiaquine was administered followed by 200 mg. four hours later. Five hundred milliliters dextrose-saline was infused intravenously. On day 1 his headache persisted, as did a fever of 38-39°C; the parasitemia decreased to 4,845 per cu. mm. and another 400 mg. of amodiaquine was administered. On day 2, despite two more doses of amodiaquine (to complete the 2.0 g. course), the fever increased to 39.6°C and his parasite count rose to 36,480. A single dose of pyrimethamine with sulfadoxine was given and the temperature rapidly fell to 37.1°C. A radical cure was achieved.

The patient's falciparum malaria was resistant at the RII level to a 2.0 g. course of amodiaquine given in five doses. The infection was then radically cured by a single dose of sulfadoxine 1.5 g. with pyrimethamine 75 mg.

URINES: Urine specimens were obtained before treatment from 45 patients. Chromatography of these specimens showed spots corresponding to one or both of the 4-aminoquinolines in 39. The data do indicate that a high proportion of the patients had chloroquine therapy before admission.

All 45 patients had evidence of 4-aminoquinolines in post-treatment urine specimens. Chromatographic differentiation of the two drugs was not completely accurate.

TOXICITY: Symptoms (for example, nausea, abdominal discomfort and dizziness) were frequent during chloroquine therapy and were at least partially attributed to an unsatisfactory response to treatment.

Abdominal tightness, dizziness and other symptoms were fairly common on amodiaquine therapy (although not more so than with chloroquine) and in one patient the toxicity was alarming. Patient number 45 was aged 15 and weighed only 29 Kg. He received a 2.0 g. course during four days. Four hours after the last dose he complained of difficulty breathing and of palpitations. His pulse was normal but he appeared distressed and slightly cyanosed and had a prominent third heart sound. At this time his parasitemia was only 48 per cu. mm.; therefore, amodiaquine toxicity was the probable diagnosis. The patient improved within a few hours.

DISCUSSION: Falciparum malaria in Thailand responds poorly to chloroquine whether given as treatment (7, 8) or for suppressive prophylaxis (9). The logical deduction is that chloroquine should not be used for falciparum malaria in Thailand. In practice, chloroquine is frequently prescribed both orally and parenterally, especially in remote areas, presumably because of low cost and easy supply. Whether this is desirable is a fundamental question. We do feel that due consideration should be given to banning the use of chloroquine in countries where falciparum malaria shows severe resistance as in Thailand (where the cure rate in our study was 0 percent).

We found that vivax malaria comprises 14 percent of all cases of malaria in Southeast Thailand (5). However, species identification is not usually available in the local laboratories. Thus, detection of vivax malaria is not normally achieved. Chloroquine, as a 1.5 g. course over three days, is the appropriate treatment for the suppression of the clinical attack of vivax malaria. But without microscopic diagnosis, the patients are unlikely to receive a full course of chloroquine. In hospital the patients with vivax malaria receive the same therapy (which does not normally include chloroquine) as those with falciparum malaria. Therefore, if chloroquine were

no longer available, the patients with vivax malaria (a benign disease) would not be treated differently in hospital and the patients with falciparum malaria (a serious disease), would not be receiving this ineffective drug before admission to hospital.

Another fact that has received insufficient attention is that both parenteral chloroquine (10, 11) and oral chloroquine (12, 13) can be fatal. Cardiac arrest and convulsions are two of the most serious side-effects. High prolonged dosage can cause blindness and the indications for chloroquine therapy have been drastically reduced to malarias other than chloroquine resistant P. falciparum and extraintestinal amebiasis (14). Children are especially sensitive to the 4-aminoquinoline compounds. Amodiaquine can cause agranulocytosis (15) as well as toxicity similar to chloroquine. Fortunately in the treatment of malaria, 4-aminoquinolines by the oral route are relatively safe especially in adults.

We found (Table 4) that amodiaquine (38 percent cure rate) was more effective (Chi square = 4.16, $p<0.05$) than chloroquine (0 percent) in the treatment of chloroquine-resistant falciparum malaria. The predominant response was RI rather than RII. However, the cure rate of 38 percent with multi-dose amodiaquine is not very impressive when compared with the 85 percent cure rate we obtained with a single dose of pyrimethamine with sulfadoxine (16). The cure rates are significantly different (Chi square = 14.8, $p<0.01$). In the treatment of falciparum malaria in Thailand, quinine (5) or pyrimethamine with sulfadoxine (16, 17) are more effective than amodiaquine. Therefore, there is no clear-cut indication for amodiaquine in the treatment of falciparum malaria in Thailand. Amodiaquine might have an occasional role in patients who show hypersensitivity to quinine. But amodiaquine alone would not be satisfactory, since so many recrudescences occur. Following the initial course of amodiaquine, alternate therapy, such as a single dose of pyrimethamine with sulfadoxine, would be needed to prevent a recrudescence.

The fever clearance time was unusually low for amodiaquine compared with other antimalarial drugs which suggests that amodiaquine does not cause a drug fever.

The difference in cure rates between amodiaquine and chloroquine may be partly explained by the fact that chloroquine is widely used in Thailand whereas amodiaquine is not used at all. The falciparum parasites are probably resistant to 4-aminoquinolines in general but have not acquired a specific resistance to amodiaquine. Amodiaquine resistant falciparum malaria has also been detected in the Philippines where amodiaquine is used (18).

Table 1. Falciparum Malaria in Thailand
Therapy with 1.5 g Chloroquine Over Three Days

Patient Number	Asexual Count P. falciparum (per cu.mm.)	Parasite Clearance Time (hours)	Initial Fever (°C)	Fever** Clearance Time (hours)	Result***	Comment
1	49572	-	38.5	-	RII	
2	27391	-	37.2	-	RII	
3	21021	-	38.8	52	RII	
4	20637	-	40.2	-	RII	
5	18200	69	36.8	-	-	
6	13160	-	40.0	-	RIII	
7	12820*	-	39.5	-	RIII	
8	6552	-	39.0	51	RII	
9	3240	-	37.7	-	RII	
10	2710	41	39.0	63	RI	
11	2340	-	37.5	-	RII	
12	1820	68	37.7	-	-	
13	1500	-	38.8	74	RII	
Mean	13920	N/A	38.5	N/A		Cure Rate=0% (0/11)

*Median count

**Fever clearance time not computed if initial fever 38.0°C

***If no symbol, final result could not be determined. RIII, no marked reduction of asexual parasitemia; RII, marked reduction of asexual parasitemia, but no clearance; RI, clearance of asexual parasitemia, followed by recrudescence; S, clearance of asexual parasitemia, without recrudescence (radical cure). World Health Organization (1967) Tech. Rep. Ser., No.375, p.42.

Table 2. Falciparum Malaria in Thailand
Therapy with 1.5 g. Amodiaquine over Three Days

Patient Number	Asexual Count *P. falciparum* (per cu.mm.)	Fever Clearance Time (hours)	Initial Fever (° C)	Fever Clearance Time (hours)	Result	Comment
14	54000	70	40.3	86	S	
15	35900	-	37.9	-	RII	
16	34830	60	39.5	46	S	
17	21060	75	38.0	43	-	
18	20000	69	38.0	56	RI	*P. vivax* Day 45
19	18428	70	37.3	-	RI	
20	18425	-	38.6	55	RII	
21	18200	100	38.2	20	S	
22	14256*	93	39.5	19	-	
23	13190	117	39.0	79	RI	
24	12376	76	37.7	-	RI	
25	9100	48	37.6	-	S	*P. vivax* Day 44
26	9100	65	39.7	13	-	
27	8730	90	37.5	-	RI	
28	7917	47	37.5	-	S	
29	7735	105	39.4	55	RI	
30	4320	66	37.7	-	-	
Mean	18092	77	38.4	47		Cure Rate = 38% (5/13 followed-up cases)

* Median count

Table 3. Falciparum Malaria in Thailand
Therapy with 2.0 g Amodiaquine Over Four Days

Patient Number	Asexual Count P. falciparum (per cu.mm.)	Parasite Clearance Time (hours)	Initial Fever (°C)	Parasite Clearance Time (hours)	Result	Comment
31	168720	-	39.8	64	S	
32	152000	85	39.8	60	RI	
33	149670	-	37.9	-	RI	
34	121125	-	38.5	-	RIII	
35	102980	88	37.4	-	RI**	
36	102600	-	38.4	40	S	
37	97395	-	39.4	-	RII**	
38	91960	86	39.7	61	RI	
39	65930	59	39.2	22	-	
40	63175	-	40.3	56	RI	
41	51680	-	39.3	6	RI	
42	48070*	116	38.2	43	RI	
43	45410	-	39.3	20	S	
44	42180	-	39.1	45	RI	
45	21140	75	39.8	43	-	
46	21090	87	38.6	14	RI	
47	16562	115	39.4	-	RI	
48	9696	88	37.3	-	RI	
49	7392	94	37.7	-	S	
50	5265	43	37.2	-	S	
51	4050	63	39.6	14	-	
52	3680	41	37.7	-	S	
53	2916	67	40.0	42	S	
54	2700	41	38.9	32	S	
Mean	58,228	77	38.9	36	Radical Cure Rate=38% (8/21)	

* Median count
** Cured by single dose pyrimethamine with sulfadoxine

Table 4. Comparison of Cure Rates

Drug	Mean Parasite Count (per cu.mm.)	RIII	RII	RI	S	Total	Cure Rate *
Chloroquine 1.5 g	14000	2	8	1	0	11	0%
Amodiaquine 1.5 g	18000	0	2	6	5	13	38%
Amodiaquine 2.0 g	58000	1	1	11	8	21	38%

* Difference between cure rates for amodiaquine and chloroquine is significant ($\chi^2 = 4.23$, $p < 0.05$)

SUMMARY: Amodiaquine cured 38 percent (13/34) of patients with falciparum malaria in Southeast Thailand. Chloroquine cured 0 percent (0/13). The cure rates with amodiaquine were the same whether a 1.5 g. or 2.0 g. course was used. Most patients were resistant to amodiaquine at the RI level and to chloroquine at the RII level. In hospital amodiaquine cleared parasitemia more frequently than did chloroquine. With the 2.0 g. course of amodiaquine, the parasite clearance time was 77 hours; the fever clearance time of 36 hours was low and suggests that amodiaquine does not cause a drug fever.

Because of resistance, chloroquine should not be used for falciparum malaria in Thailand. Routine use of amodiaquine is not indicated because more effective drugs are available.

6. Falciparum Malaria Semi-Resistant to Clindamycin

BACKGROUND: The antimalarial activity of a group of chlorinated lincomycin analogues was first demonstrated in mice infected with P. berghei (22, 25) and in monkeys infected with P. cynomolgi (25, 27). Schizontocidal as well as causal prophylactic and radical curative activity was observed. Chloroquine-resistant P. falciparum infections in owl monkeys were also cured by these compounds (26). Both in animals and in man infected with malaria, clindamycin acted slowly; however, three day courses of quinine and clindamycin given in combination or sequentially proved effective against chloroquine-resistant falciparum malaria in volunteers (23). We tested clindamycin alone and in combination with quinine in Thais naturally infected with chloroquine-resistant falciparum malaria.

DESCRIPTION: The study was performed at the Trad Provincial Hospital in Southeast Thailand. Details of the research methods have been described (5). We operated a daily malaria clinic at the hospital and suitable outpatients volunteered for the inpatient studies. Informed consent was obtained in all cases. Male patients with an asexual parasite count over 1,000 per cu. mm. were included. To avoid the problem of immunity, patients with clinically mild infections were rarely studied.

Quantitative parasite counts were made at least twice daily in hospital on blood specimens obtained by finger-prick taken at 0700 and 1400 hours and at follow-up examinations on days 14, 21 and 28. Determination of the hematocrit (packed cell volume) and leukocyte count was made on admission and whenever clinically indicated. Sera were collected on admission and the concentrations of bilirubin and creatinine determined.

Throughout the study, the drugs were administered by one of the study physicians during medication-ward rounds usually made at 0600, 1400 and 2100 hours. The patients were observed by the physician as they swallowed the drug with water, then examined and kept under observation for a few minutes. The clindamycin was dispensed as 150 mg capsules (Cleocin, Upjohn) and the usual dose was 450 mg every 8 hours for three days (total dose 4050 mg). The quinine was administered as sugar-coated tablets of quinine sulfate, USP each containing 270 mg base. The usual dose was 540 mg every 8 hours for three days (total dose, 4860 mg).

Follow-up examinations on days 14, 21 and 28 were made either in the clinic or at home.

In the evaluation of the final therapeutic result in each patient, the WHO (30) classification was used (Table 1). A radical cure was diagnosed if the parasitemia was cleared and had not reappeared before day 29. Parasite clearance times were calculated in hours. Fever clearance times were computed in hours if the initial fever was at least 38.0°C. Clearance was diagnosed when the temperature decreased to 37.2°C or less and remained at this level for at least one more reading. If there was still fever or parasitemia on discharge at least 100 hours after admission, the elapsed time was arbitrarily counted as the clearance time.

CLINDAMYCIN. Eleven patients were treated with 450 mg every 8 hours for three days. One patient (Case No. 4) was a 12 year old boy weighing 28 kg. He received 300 mg every 8 hours. In five patients not responding to clindamycin, the drug was stopped and more effective therapy given.

The initial clinical response was fairly rapid in some of the patients (Table 1) but the mean parasite clearance time was slow (88 hours) as was the fever clearance time (68 hours).

In five patients the parasitemia was cleared and did not reappear on follow-up examination on days 14, 21 and 28. These patients were adjudged to be radically cured. The average initial parasite count in these five patients (33269 per cu. mm.) was less than that (73511 per cu. mm.) in the five patients who were not cured (the difference was not statistically significant). In two other patients (Cases 2 and 6) an initial clinical response occurred but follow-up was not achieved. Thus the initial clinical response was satisfactory in seven of the 12 patients.

In five patients the initial infection was not controlled by clindamycin and because of a worsening clinical and parasitemic situation,

alternate therapy had to be given. Two of these patients were cured by sequential therapy with quinine and Fansidar (pyrimethamine and sulfadoxine). Two other patients were cured by a single dose of Fansidar given alone. The case-history in one of these patients is described below. A clinical response to Fansidar occurred in the fifth patient but follow-up was not obtained. Thus the overall cure rate for clindamycin was 50% (5/10).

No clear-cut toxicity due to clindamycin occurred in these patients; however, Case No. 2 had persistent dizziness and weakness during therapy and tinnitus occurred for two days afterwards.

Case No. 12: The patient was a 43 year old farmer born locally. The main symptom was a headache for four days. He had received two intramuscular infections (content unknown) on the day before admission. He was in distress with a fever of 40.0°C, although his parasitemia was only 8265 per cu. mm. Clindamycin 450 mg was administered at 1600 and 2100 on day 0 and thereafter every eight hours. The fever abated briefly on the morning of day 1 but then returned to 40.0°C. The parasitemia decreased to 665 on day 1 but then rose to 9880 on day 2. On the evening of day 2, because of the increase in parasitemia, the high fever (40.0°C) and persistent severe symptoms, drug failure (RII type) was diagnosed. Seven doses of clindamycin had been given. The patient then received a single dose of Fansidar (sulfadoxine 1.5 g with pyrimethamine 75 mg). The parasitemia then cleared in 48 hours and the fever in 60 hours. Blood films were negative on days 13, 21 and 30 and a radical cure was diagnosed.

QUININE WITH CLINDAMYCIN (FULL DOSAGE). Six patients were begun on treatment with quinine 540 mg base every 8 hours and clindamycin 450 mg every 8 hours given at the same time for three days (Table 2). The dosage of clindamycin was reduced (usually to 300 mg) in most patients because of intolerance.

In five of the six patients, the quinine-clindamycin combination appeared to cause toxicity. In Case No. 13; his symptoms worsened during therapy but improved when the clindamycin was stopped. In Case No. 15; despite a fall in parasite count from 55,419 to 40, he developed severe anorexia after the sixth dose of clindamycin. Cases No. 16, 17 and 18 had a similar clinical picture; they developed severe retching one to four hours after the second to fourth dose of quinine and clindamycin. They all improved after the clindamycin was stopped despite the continuation of the quinine at full dosage. After the course of quinine was finished and the patients had improved, clindamycin was resumed without causing any side-effects when given alone.

Four patients were cured and two others did not complete follow-up. The mean parasite clearance time was prolonged (83 hours) and the mean fever clearance time was 67 hours.

QUININE WITH CLINDAMYCIN (HALF DOSAGE). Eight patients received combination therapy with half dose quinine (270 mg) and approximately half-dose clindamycin (150 mg) every 8 hours (Table 3). The mean parasite clearance time was prolonged (95 hours) and the mean fever clearance time was 68 hours. Complete follow-up was achieved in five patients of whom three were cured (60%). A clinical response occurred in two other patients but follow-up was not achieved. The eighth patient (Case No. 22) developed toxicity after four doses of therapy and was then treated with quinine followed by Fansidar. Five of the eight patients developed unacceptable toxicity which consisted mainly of upper gastrointestinal symptoms. Details of three of these cases are given.

Case No. 19. This patient received four full doses of quinine (three intravenously) without toxicity. He then received quinine with clindamycin at half dosage. After two doses the patient developed nausea, tightness in the chest and severe retching which persisted for eight hours. The clindamycin was stopped. The symptoms improved although the quinine therapy was continued. After the nine dose course of quinine had been completed, the clindamycin was resumed until nine doses had been given. The patient had persistent weakness during this time. The parasitemia and fever cleared but follow-up until day 28 was not obtained.

Case No. 20. This patient received five full doses of quinine at eight hour intervals uneventfully. Then the semi-dose combination was given for two doses. The patient felt generally worse and a persistent fever developed. The clindamycin was stopped and also the quinine after two more doses. Sixteen hours later when he felt better the clindamycin was resumed for five doses during which time the patient felt listless. A radical cure was achieved.

Case No. 22. The patient was virtually asymptomatic on admission, having mild headache and backache only. After two doses of quinine 540 mg and clindamycin 300 mg at 1000 and 2100 hours on day 0, he developed nausea and vomiting, headache, dizziness and prostration. On day 1 he received quinine 270 mg and clindamycin 150 mg at 0600 and 1400. The patient remained very toxic with weakness and dizziness although the parasite count had fallen from 60,000 to 650 per cu. mm. The clindamycin was stopped but the quinine was continued and the dose increased to 540 mg; the patient improved and remained well. After 12 doses of quinine, a single dose of Fansidar was given. The patient was cured.

QUININE ALONE: Three patients were treated with a three day course of quinine alone. They developed recrudescences on days 14, 22 and 25, respectively (Table 4).

TETRACYCLINE ALONE: Four patients received tetracycline 250 mg every 6 hours for three days (Table 5). Two patients had an RIII response and one patient had an RII response. A slow clearance of parasitemia occurred in the fourth patient but follow-up was not obtained.

DISCUSSION: Falciparum malaria in Thailand is difficult to eradicate in many patients. In recent studies the cure rate with chloroquine (23) or with pyrimethamine (16) was 0%. This data indicates that clindamycin is partially effective against chloroquine-resistant falciparum malaria in patients with clinically moderate disease. In some men the clinical response to clindamycin was rapid but in others it was slow or ephemeral. Our cure rate of 50% with multi-dose clindamycin compares with the 85% cure rate we obtained with a single dose of pyrimethamine with sulfadoxine (16). The average parasite clearance time for clindamycin (88 hours) was significantly longer ($p<0.05$) than that for pyrimethamine with sulfadoxine (71 hours). Wagner et al. (29) found that clindamycin has a short half-life of only 2.4 hours and consequently, frequent doses must be given which is a disadvantage in the treatment of malaria. On the other hand sulfadoxine has a half-life of about 200 hours as determined by Brooks et al. (19). In our test system clindamycin was obviously a more powerful antimalarial than tetracycline (Table 5).

Clindamycin alone was not toxic in our patients; however, several recent reports have shown that lincomycin or clindamycin (a chlorinated lincomycin analogue) can cause ulcerative colitis or even pseudomembranous colitis (20, 24, 28). The diarrhoea usually begins after 4 to 9 days of therapy. Colitis was not detected in our patients. Clindamycin is probably the most potent antimalarial among the antibiotics. However, because of its partial efficacy and potential toxicity, clindamycin alone has a limited role as an antimalarial.

Quinine and clindamycin in combination at full or half dosage apparently potentiated toxicity in our patients. Retching and frank vomiting were frequently observed, although Miller et al. (23) did not encounter gastrointestinal intolerance. Other patients had less specific symptoms and did not look well. When the clindamycin was stopped but the quinine continued, the patients improved. Likewise when the course of quinine had been completed, clindamycin alone did not cause serious side-effects. The

Table 1. Falciparum Malaria Therapy with Clindamycin Every 8 Hours for 3 Days

Patient Number	Asexual Count *P. falciparum* (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result	Comment
1	162260	-**	39.8	-	RIII	Cured by Quinine and Fansidar
2	93670	99	39.9	55	-	
3	82080	87	40.4	63	S	
4	77900	-	37.9	-	RII	Cured by Quinine and Fansidar
5	72770	-	40.5	-	RII	
6	51300	-	40.2	54	-	
7	46360*	-	40.0	-	RII	Cured by Fansidar
8	33060	97	40.0	102+	S	
9	20140	88	40.7	63	S	
10	18144	92	39.9	76	S	
11	12920	67	40.0	66	S	
12	8265	-	40.0	-	RIII	Cured by Fansidar
MEAN	56572	88	40.0	68	Radical Cure Rate=50% (5/10)	

* Approximately the median count.

** If no symbol, final result could not be determined. RIII, no marked reduction of asexual parasitemia; RII, marked reduction of asexual parasitemia, but no clearance; RI, clearance of asexual parasitemia, followed by recrudescence; S, clearance of asexual parasitemia, without recrudescence (radical cure).

Table 2. Falciparum Malaria Therapy with Quinine and Clindamycin Both Given Every 8 Hours for 3 Days at Full Dosage

Patient Number	Asexual Count *P. falciparum* (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result	Comment
13	146692	117	38.3	82	S	Drug Toxicity
14	101556	93	39.6	60	S	
15	55419	64	40.0	39	S	Anorexia
16	33943*	85	40.6	60	-	Vomiting
17	8645	69	40.0	69	-	Vomiting
18	5642	69	40.0	92	S	Vomiting
MEAN	58,650	83	39.8	67	Radical Cure Rate =100% (4/4)	

* Approximately the median count

Table 3. Falciparum Malaria Therapy with Quinine and Clindamycin Both Given Every 8 Hours for 3 Days at Half Dosage.

Patient Number	Asexual Count *P. falciparum* (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result	Comment
19	358,830	119	37.5	99	-	Vomiting
20	236,600	116	39.1	115+	S	Drug Fever
21	214,760	115	38.5	42	RI	
22	61,880	-	38.0	-	-	Toxicity
23	54,432 *	67	39.9	18	S	
24	13,312	92	38.9	32	-	Vomiting
25	4,914	96	40.2	112+	S	Abdominal Pain
26	2,730	59	40.9	58	RI	
MEAN	118,432	95	39.1	68	Radical Cure Rate =60% (3/5)	

* Approximately the median count

Table 4. Falciparum Malaria Therapy with Quinine Every 8 Hours for 3 Days.

Patient Number	Asexual Count *P. falciparum* (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result
27	87,804	84	37.9	-	RI
28	21,708	69	39.8	-	RI
29	18,270	68	40.5	84	RI
MEAN	42,594	74	39.4	-	

Table 5. Falciparum Malaria Therapeutic Results in Patients Treated with Clindamycin or Tetracycline*

Regimen	Duration Therapy	Average Parasitemia	RIII	RII	RI	S
Clindamycin	3 Days	53389	1	4	0	5
Tetracycline**	3 Days	20647	2	1	-	-

* Clinically clindamycin was more effective than tetracycline.

** One patient responded in hospital but follow-up was not achieved.

therapeutic results with full dose quinine and clindamycin therapy were excellent (4/4 cures). Perhaps quinine and clindamycin potentiate both antimalarial efficacy and toxicity.

Sequential administration of quinine and clindamycin was not toxic and could be useful in patients who have relapsed following more conventional therapy (e.g. quinine followed by Fansidar).

By studying high count rather than low count cases, we produced a more severe test of antimalarial efficacy in any regimen studied i.e. the degree of "drug pressure" was increased. If patients with counts only over 50,000 per cu. mm. and uncomplicated disease are selected for drug trials, comparative studies can be completed using fewer subjects. We have adopted this system without risk to the patients.

SUMMARY: Clindamycin, a semi-synthetic antibiotic of the lincomycin family, at a dose of 450 mg every 8 hours for three days in adults, cured 50% (5/10) of patients moderately ill with chloroquine-resistant falciparum malaria. Combination therapy with full dose quinine and clindamycin for three days was curative in 100% (four patients) and with half-dosage in 60% (3/5). However both combinations caused upper gastrointestinal toxicity and appeared to potentiate both toxicity and possibly antimalarial efficacy. Colitis due to clindamycin was not observed. Sequential therapy was not toxic and could be useful in patients who have recrudesced following more conventional therapy.

7. Single-Dose Therapy of Falciparum Malaria Using Pyrimethamine in Combination with Diformyldapsone or Sulfadoxine

OBJECTIVE: To compare the efficacy of diformyldapsone and sulfadoxine used with pyrimethamine in the treatment of falciparum malaria.

BACKGROUND: Weekly administration of pyrimethamine and diformyldapsone (DFD) has proved to be an effective chemosuppressant against drug-resistant P. falciparum malaria in both induced infection in volunteers (31) and in field populations (32).

In a trial of DFD alone as therapy for falciparum malaria, Clyde, et al found that single dose treatment was slowly effective in clearing 15 of 23 episodes of asexual parasitemia in volunteers; however, recrudescence occurred in all but one patient (33).

The combination of pyrimethamine with sulfadoxine (Fansidar) has been reported to be highly effective both for treatment (34) and prophylaxis (35) of drug-resistant falciparum malaria.

Pyrimethamine-DFD, like pyrimethamine-sulfadoxine, is a single-dose preparation, and the therapeutic potency of the two combinations was compared.

DESCRIPTION: The patients to be studied were selected from males presenting at the Trad Provincial Hospital in Southeast Thailand with an asexual parasite count of P. falciparum greater than 1000 per cu. mm. The patients also had to agree to be hospitalized and followed during the study period, and be willing to signify consent after being informed of the nature and potential hazards of the study. Since pyrimethamine with sulfadoxine is known to be therapeutically effective, 45 patients were selected who had clinically moderate disease. Their average age was 22.7 years and average weight 47.3 kg. Since the therapeutic potency of pyrimethamine with DFD was not known, most of the patients selected had clinically mild disease. Thirty-three patients received pyrimethamine with DFD. Their average age was 23.6 years and average weight 51.5 kg.

Direct quantitative parasite counts (6) were performed on admission and thereafter twice daily in hospital and 14, 21 and 28 days after admission. Hematocrit, white cell count, and urinalysis were performed on admission, and whenever subsequently indicated. Patients were taken home by a member of the study team, and follow-up examinations were made either at the clinic or at the patients' homes. Other details of the study procedure are described elsewhere (5).

All medications were administered by one of the study physicians, and patients were seen on clinical rounds made three times daily. The drugs used were combination tablets of pyrimethamine 25 mg and sulfadoxine 500 mg (Fansidar, Hoffmann-LaRoche) or pyrimethamine 12.5 mg and diformyldapsone 200 mg (supplied by the Walter Reed Army Institute of Research). Both were supplied as uncoated white tablets. The dosage of pyrimethamine-sulfadoxine used in adults was pyrimethamine 75 mg and sulfadoxine 1500 mg (3 tablets). This is the maximum dose recommended by the manufacturer. Seven boys weighing between 20 and 36 kg received two tablets. One boy weighing 18 kg received one tablet. Two dosages of the pyrimethamine-DFD combination were tested: pyrimethamine 25 mg, DFD 400 mg (2 tablets) and pyrimethamine 50 mg, DFD 800 mg (4 tablets).

Therapeutic responses were classified according to WHO criteria (30). Parasite clearance times and fever clearance times were determined for each patient. Fever was considered to be "cleared" if it remained at or below 37.2°C for at least 12 hours. Patients in whom asexual parasitemia was cleared by treatment and had not reappeared for 28 days following therapy were considered radically cured.

PROGRESS: PYRIMETHAMINE 75 MG, SULFADOXINE 1500 MG.

Forty-five patients were treated with the combination pyrimethamine-sulfadoxine (Table 1). Patients in this group had a high average parasitemia (60,000 per cu. mm.) and were moderately or severely ill. The mean parasite clearance time was 73 hours and the mean fever clearance time was 63 hours.

Thirty-nine of the forty-five men were followed for the 28 day observation period and radical cure was attained in 33 (85%). All patients with parasite counts below 30,000 per cu. mm. were cured. A typical successful response to pyrimethamine-sulfadoxine is described below.

Patient No. 5. This 18 year old farmer was admitted with a three day history of fever, insomnia, and backache. He gave a history of malaria one year previously. On physical examination, he was found to have a temperature of 39.2°C, but no other positive findings. The asexual parasite density of P. falciparum was 115,900 per cu. mm. on admission. He remained febrile for 51 hours following treatment and complained of continuing backache, but his parasitemia steadily decreased. There were rare ring forms 48 hours after therapy, but smears were negative thereafter. He remained well throughout the follow-up period.

Two patients exhibited recrudescence of asexual parasitemia and malaria symptoms before the end of the 28 days and were considered RI treatment failures. Six patients required intravenous quinine after the initiation of the pyrimethamine-sulfadoxine therapy because of rising parasitemia and worsening clinical state. However, to bring the infection under control, four patients required only one dose of quinine; one received two doses and another five doses. Some of the patients may indeed have eventually responded to the single dose of Fansidar, but in the judgement of the attending physicians, withholding the faster acting drug of known effectiveness was not warranted. Four patients were diagnosed as RIII treatment failures. In two patients (Numbers 1 and 4), it was later considered that the quinine therapy may have been given prematurely, so no result was recorded.

Patient No. 11. was an example of an RIII response. An 18 year old farmer was admitted with a two day history of headache, muscle pain, fever, and thirst. Admission temperature was 40.4°C. His spleen was not palpable and there were no abnormalities on physical examination. The asexual parasite count on admission was 79,800 per cu. mm. and he was treated with three tablets of Fansidar. By the afternoon of admission, he was in severe distress with headache, abdominal pain, restlessness, and his fever was still 40°C. The

Table 1. Falciparum Malaria in Thailand. Single Dose Therapy with Pyrimethamine 75 mg and Sulfadoxine 1.5 g. (Smaller Dose in Children)

Patient Number	Initial Asexual Count P. falciparum (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result
1	314500	-*	39.4	-*	-***
2	286900	-	39.3	-	RIII
3	258400	-	41.0	-	RIII
4	179600	-	38.5	-	-
5	115900	65	39.2	51	S
6	111000	88	37.0	-	S
7	98000	66	39.9	-	S
8	93300	118	40.4	69	RI
9	92200	-	40.0	67	S
10	90300	76	38.1	68	S
11	79800	-	40.4	-	RIII
12	71300	88	40.3	87	S
13	65300	113	40.2	88	S
14	65000	-	39.7	-	RIII
15	63100	68	39.8	55	S
16	61800	69	40.0	61	S
17	58900	-	39.3	66	S
18	58700	-	40.1	63	S
19	58700	-	40.1	66	S
20	56200	-	39.9	64	S
21	50400	70	40.9	74	S
22	44200**	-	39.4	66	S
23	36100**	-	39.8	42	S
24	30400	-	39.6	-	RI
25	27200	76	40.1	68	S
26	26100	62	39.4	37	S
27	24500	-	39.5	64	S
28	23400	66	39.0	41	S
29	21300	75	40.4	83	S
30	19400	63	40.3	62	S
31	17300	61	38.2	84	S
32	17200	-	37.3	-	S
33	16200	-	40.2	66	-
34	13400	-	39.4	-	S
35	10900	117	39.1	37	-
36	10100	93	40.0	109	S
37	9400	39	39.4	-	S
38	8600	-	40.3	-	-
39	6800	-	39.6	35	S
40	6400	70	39.9	57	S
41	4900	-	39.0	42	-
42	4800	45	38.7	44	S
43	3000	52	39.2	-	S
44	2300	-	37.3	-	S
45	1700	38	37.0	-	S
Mean	60300	73	39.5	63	Cure Rate = 85% (33/39)

* If period of observation not adequate, no result given.

** Median count.

*** If no symbol, final result could not be determined.
RIII, no marked reduction of asexual parasitemia;
RII, marked reduction of asexual parasitemia, but no clearance;
RI, clearance of asexual parasitemia, followed by recrudescence;
S, clearance of asexual parasitemia, without recrudescence (radical cure).
World Health Organization (1967) Tech. Rep. Ser. No. 375, p. 42.

parasite count seven hours after treatment had risen to 366,000 and it was decided to infuse 500 mg quinine. A second infusion of 500 mg quinine was given the following morning when the parasite count was 236,000. The patient did not require additional therapy, and attained a radical cure of his malaria. Despite the fact that the elapsed time between treatment with the drug under evaluation and the initiation of quinine therapy was only eight hours, it was felt that this patient represented a failure of the pyrimethamine-sulfadoxine combination in view of his worsening clinical condition and progressive rise in parasitemia.

Toxicity: Two patients developed rashes after the pyrimethamine-sulfadoxine combination which were considered to be related to the administration of the drug. In one patient the rash consisted of giant urticaria, appearing 34 hours after dosing, which resolved after treatment with antihistamines and dexamethasone. Another patient developed a pruritic erythematous rash four days after therapy, which disappeared spontaneously without additional treatment. Neither patient had mucous membrane lesions, and aside from multi-vitamins, neither had received additional medication. Radical cure was achieved in both patients.

PYRIMETHAMINE 50 MG, DFD 800 MG.

Thirty patients received a single four tablet dose of the combination. Fever clearance time (mean 59 hours) and parasite clearance time (mean 60 hours) were short. However this group of patients had a low mean initial parasite count (17,000 per cu.mm.), and were, usually, clinically mild cases. Twenty-three patients were followed throughout the 28 day period (Table 2); only ten were cured. Of the failures, two had RIII responses and two exhibited RII responses. Nine had recrudescences of parasitemia before the end of the 28 day observation period, and were considered RI failures. The over-all cure rate for pyrimethamine-DFD at this dosage was 43%. There was no evidence of hematologic or other toxicity.

The case-history of a patient with an RII response is given below.

Patient No. 50. A 42 year old tailor presented with a four day history of fever, headache, anorexia and vomiting. A cough had been present for six days. He gave a history of malaria 10 years previously. Examination showed a temperature of 40.0°C and rhonchi in the chest. The asexual density of P. falciparum was 40,700 per cu. mm. The four tablet dose was administered at 1100 hours. The patient felt better in the evening. By 1400 hours the following day, the parasite density had decreased to 400 per cu. mm. and

the patient had a temperature of 38.0°C. He developed fever and chills at 2000 hours, his temperature rose to 39.6°C and his parasite count increased to 10,800 per cu. mm. An RII response was diagnosed and 500 mg quinine was administered intravenously in 500 ml saline over four hours. Altogether nine doses (eight oral) of quinine were given, followed by a single dose of pyrimethamine-sulfadoxine. The patient made a rapid recovery.

PYRIMETHAMINE 25 MG WITH DFD 400 MG, AND PYRIMETHAMINE ALONE.

Early in the study, three patients were treated with the pyrimethamine-DFD combination at this lower dosage (two tablets). Of the three, one responded promptly (S), and two were RII failures. Because of the apparently unacceptable therapeutic action of the combination at this dosage, no further patients were studied.

Three patients with mild illness and low parasitemias were treated with 50 mg pyrimethamine daily for three days. In all three, only a temporary reduction in parasitemia resulted (RII), and symptoms persisted, supporting an earlier impression of resistance of the local strain of P. falciparum to this drug.

DISCUSSION: Pyrimethamine 50 mg with DFD 800 mg is only partially effective as an antimalarial, since only 43% of mildly ill patients were cured. This cure rate is similar to that for a pyrimethamine-dapsone (DDS) combination, which was 19% effective (Chi square = 2.0, $p > 0.05$) (17). DFD has a metabolic half-life of 30 hours, (36) compared with 21 hours for DDS (37). Pyrimethamine, which has a half-life of 96 hours (38), used with either DFD or DDS leads to an unbalanced synergistic combination. Obviously at certain times only an effective dose of pyrimethamine is present in the blood. Since pyrimethamine resistance was demonstrated in this study, the partial therapeutic efficacy of pyrimethamine with DFD may be explicable on this basis. Conversely, pyrimethamine-sulfadoxine is a balanced combination since the half-life of sulfadoxine is 200 hours, (19), and of pyrimethamine about 96 hours.

In this study, pyrimethamine with sulfadoxine cured 85% of patients with an average parasite count of 60,000 per cu. mm., when administered in an adult dose of three tablets (pyrimethamine 75 mg, sulfadoxine 1500 mg.). Thus the combination (Table 3) was twice as effective as pyrimethamine-DFD ($p < 0.01$) in patients whose average parasite count was three times greater ($p < 0.001$).

In Northeast Thailand, a two tablet dose (pyrimethamine 50 mg, sulfadoxine 1000 mg.) cured 82% of patients with an average count of 28,000 per cu. mm. (17).

Table 2. Falciparum Malaria in Thailand.
Single Dose Therapy with Pyrimethamine 50 mg and DFD 800 mg

Patient Number	Initial Asexual Count *P. falciparum* (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result
46	95600	93*	39.0	84*	RI*
47	63700	-*	39.2	-	-
48	46100	43	39.2	42	-
49	41000	-	38.4	-	RII
50	40700	-	40.0	-	RII
51	30300	69	39.1	69	S
52	20200	44	39.0	50	S
53	18400	117	37.8	-	RI
54	13000	52	40.5	-	-
55	13000	62	38.3	12	-
56	12400	45	37.3	-	S
57	12300	70	40.4	93	RI
58	12200	67	36.9	-	S
59	11700	84	39.8	71	S
60	11200**	-	40.5	-	RIII
61	10000**	-	40.4	-	RIII
62	7300	66	37.7	-	-
63	7200	60	39.3	59	RI
64	7200	44	37.1	-	S
65	6900	43	37.8	-	S
66	5700	40	37.0	-	S
67	5400	92	39.2	67	RI
68	4900	72	40.3	40	RI
69	2600	41	38.6	160+	RI
70	1800	61	39.6	12	-
71	1600	45	38.7	37	RI
72	1400	61	38.8	36	S
73	1200	14	39.8	-	S
74	1100	-	40.0	-	-
75	1000	61	38.7	60	RI
Mean	16,900	60	38.9	59	Cure Rate =43% (10/23)

* If period of observation not adequate, no result given.

** Median count.

Table 3. Comparison of Cure Rates*

Drug	Average Initial Parasite Count (per cu.mm.)	RIII	RII	RI	S	Cure Rate (%)***
Pyrimethamine + Sulfadoxine	60,300**	4	0	2	33	85%
Pyrimethamine + Dfd	16,900**	2	2	9	10	43%

* Patients who did not complete follow-up examinations were not included in this table.

** The average initial parasite counts were significantly different ($t=8.4$, $p < 0.001$)

*** The difference in cure rates was statistically significant ($\chi^2=9.67$, $p < 0.01$)

8. Falciparum Malaria Cured by Quinine Followed by Fansidar (Sulfadoxine with Pyrimethamine)

OBJECTIVE: To determine the efficacy of a short course of quinine (about three days) followed by a single dose of sulfadoxine with pyrimethamine against falciparum malaria in Thailand.

BACKGROUND : The treatment of chloroquine-resistant falciparum malaria has hitherto been confusing. No clearcut regimen has yet emerged (40). The World Health Organization (41) mentions 16 different regimens involving 12 drugs administered over 1 to 14 days. Based upon the results of this study we recommend one regimen involving three drugs given in about three days.

In Southeast Thailand a six day course of quinine cured 85% of patients with falciparum malaria (5); a single dose of Fansidar (sulfadoxine with pyrimethamine) was also 85% curative (16). Therefore we tested a short course of quinine (average three days) followed by Fansidar in about 400 patients.

DESCRIPTION: The Trad Provincial Hospital is in Southeast Thailand 400 Km from Bangkok. Malaria is endemic in the area throughout the year. Volunteers were selected for the study if their asexual parasitemia of *P. falciparum* was at least 1,000 per cu. mm. Initially only adult males were studied. When quinine-Fansidar was shown to be the most effective therapy, it was used routinely on adults and children. Patients with coincident vivax malaria were treated but not included in this study.

Quantitative parasite counts (6) were performed at least twice daily in hospital on finger-prick specimens taken at 0800 and 1400 hours and at follow-up examinations on days 14, 21 and 28. Hematocrit levels (packed cell volume) and leukocyte counts were made on admission and whenever clinically indicated.

The drugs were administered by the study physicians during ward rounds usually conducted at 0600, 1400 and 2200 hours. Intravenous quinine was administered as the dihydrochloride salt. The standard dose in adults was 490 mg quinine base in 500 ml normal saline infused over a four hour interval. Oral quinine was prescribed as tablets of quinine sulfate each containing 270 mg base. The routine formulation was a sugar-coated tablet, but plain tablets were occasionally used especially in small children. The standard dose in adults was 540 mg (two tablets) every 8 hours. Each plain tablet of Fansidar (Hoffman-La Roche) contained 25 mg pyrimethamine and 500 mg sulfadoxine. The standard dose in adults was three tablets given with or eight hours after the last dose of quinine.

Quinine concentrations on random sera were determined by the benzene extraction method of Brodie and Udenfriend (42). This is an accurate technique (43).

Parasite and fever clearance times were determined in hours. The patients' temperature charts were retained for analysis. Patients were considered radically cured (s response) if the parasitemia was cleared by treatment and had not reappeared before day 29 (the day of admission being called day 0). The WHO (30) classification was used (Table 2).

PROGRESS: Four hundred and fourteen patients were admitted to the study. Many patients were very seriously ill on admission and of these 13 died (3% of 414) and did not receive Fansidar. Nine patients escaped from hospital after clinical improvement but before receiving Fansidar. Therefore 392 patients received quinine-Fansidar therapy. The average age of the group was 23.9 years (range 1-73) and average weight 45.4 Kg (Range 5-80). Only 14 patients were female since we usually confined our studies to male patients.

Most patients were fairly seriously ill on admission. The average parasite count (90,676 per cu. mm.) was much higher than in our other therapeutic studies (5) and the average parasite clearance time of 77.3 hours indicates a satisfactory response (Table 1). Clearance of parasitemia was slow in a few patients, especially those with high parasite counts (e.g. over 200,000 per cu. mm.) and was possibly due to partial resistance to quinine. A typical patient was No. 391 with a parasite count of 252,720 per cu. mm. He received 18 doses (six days) of quinine (Table 1) before the Fansidar. His parasite clearance time was prolonged at 147 hours. Despite the slow clinical response, his blood was free of parasites on days 14, 21 and 28 and so he was accredited with a radical cure.

The average fever clearance time was 61.5 hours. In a few patients a persistent fever was probably caused by the quinine. Since the average course of quinine was short; quinine fever was not so frequent a problem as with longer courses in previous studies (44). In some patients, who received only a few doses of quinine, a rising temperature on day 1 or day 2 was probably caused by the Fansidar, since the fever patterns were similar to those found in patients receiving Fansidar alone (16). Many patients were discharged when they felt better but before the fever and parasitemia had cleared.

Eighty-two percent (322/392) of the patients received at least one dose (average 1.5 doses, range 1-7) of quinine as a continuous intravenous infusion. The standard dose of quinine base was 10 mg per Kg but in small children a lower dose was often used to prevent toxicity. The adult intravenous dose (490 mg quinine base in the dihydrochloride salt) was usually given in 500 ml normal saline.

The optimum infusion time for a rapid response and avoidance of toxicity was four hours. Half strength solutions (0.5 mg quinine base per ml) were administered, especially in children, if quinine toxicity was suspected. In comatose adults optimum therapy was not more than 1,000 mg quinine base (two doses) per 24 hour interval given in 1000-1500 ml fluid intravenously. Quinine metabolism is impaired in severe falciparum malaria and the half-life of the drug is prolonged. Thus lower or less frequent doses are needed to prevent overdosage. In several of our patients toxic levels of serum quinine (concentrations over 10 mg/L) occurred despite subnormal doses of the drug. Nevertheless quinine is the only drug that brings severe falciparum infections under satisfactory control in South-east Thailand.

Follow-up examinations were completed in 314 patients, of whom 302 (96%) were radically cured (Table 2). The quinine-Fansidar regimen was more effective than a six day course of quinine, or Fansidar alone, in our test system.

Initially all patients received nine doses of quinine. The questions arose whether a shorter course of quinine would be equally effective and whether, in severe cases, a longer course of quinine would be more effective. Therefore, additional patients received from 1 to 18 doses of quinine before the single dose of Fansidar. To our surprise the cure-rate was about 96% throughout the range (Table 1). However the cure-rate of 98% (43/44) with 10 to 18 doses was impressive since the patients were mostly seriously ill and their average parasitemia was high (166,000 per cu. mm.).

If the course of quinine was very short (less than four doses), then the initial clinical improvement was not always maintained and a temporary resurgence of fever and other symptoms often occurred on about the second day. If at least four doses of quinine were given, optimal clinical improvement usually resulted. Table 1 shows that the shortest fever clearance time occurred with the four dose course. Longer courses of quinine appeared to be indicated in patients with high initial parasite counts or evidence of chronic disease (e.g. large spleens).

Quinine caused typical mild side-effects in most patients (e.g. nausea and tinnitus). If blurred vision or other serious symptoms occurred, the dose of quinine was reduced. Serious side-effects were more frequent in children than in adults (e.g. coma, convulsions). Reduction or deletion of dosage usually resulted in a rapid decrease in toxicity.

Serious toxicity attributable to the Fansidar did not occur in any of the 392 patients who received the quinine-Fansidar regimen. However Fansidar as solo therapy often causes fever and less often urticarial rashes (16).

The cost of treatment comprises the hospital costs (if the patient requires admission) plus the cost of the drugs. The 392 patients were in hospital for an average of only 3.8 days which was brief in relation to the average clinical severity of the group, and reflects the fact that quinine usually acts rapidly.

We determined that the minimal effective regimen was four doses of quinine plus Fansidar. At the time of the study quinine cost the hospital pharmacy about U.S. $0.04 per tablet and Fansidar about $0.15 a tablet. Thus the cost of the regimen orally was at least $0.77. This was less expensive than any other effective regimen. Intravenous therapy was much more expensive because units of intravenous fluid were relatively costly.

Other relevant factors are the duration of therapy, the total number of doses and the frequency of dosing. Optimal quinine-Fansidar therapy comprises at least five doses (four quinine, one Fansidar) given over two days at eight hour intervals. These parameters are less than for any other effective regimen.

DISCUSSION: This study has established that quinine followed by Fansidar is the treatment of choice for chloroquine-resistant falciparum malaria. The regimen is theoretically sound because it comprises the rapidly acting drug quinine, followed by the long acting combination of sulfadoxine and pyrimethamine (Fansidar). The quinine brings the infection under control and the Fansidar assists in its eradication. This regimen is useful because we found that Fansidar is non-toxic when given at the end of a course of quinine; it is practical because it is completed in about three days, thus allowing the patient to be discharged promptly.

The value of an antimalarial regimen may be determined by five criteria viz., efficacy, toxicity, cost, duration of therapy and length of hospital stay. These criteria must be judged in relation to the average severity of the cases being treated, which is indicated by the clinical severity and the average parasite count. Considering all these factors, the quinine-Fansidar regimen is the best that we have tested.

The components of the quinine-Fansidar regimen are more powerful antimalarials than any alternative drugs. For chloroquine-resistant falciparum malaria, quinine is the only rapidly acting drug currently available. The components of Fansidar are longer

acting than any other similar drugs (Table 3). Fansidar is more effective in the radical cure of P. falciparum than pyrimethamine with DDS (17) or pyrimethamine with DFD (16). Single dose Fansidar is more effective than multi-dose clindamycin or tetracycline (45). It should be stressed that Fansidar alone is often toxic (e.g. fever, urticaria) and slow acting, but when given at the end of a course of quinine, it caused no serious toxicity in over 300 patients. Sequential quinine-Fansidar is also more logical than combination therapy because antimalarial activity in the blood is obviously maintained for a longer period.

Quinine has, of course, been used for malaria for several hundred years. Sulfadoxine was introduced for malaria by Laing (46). Chin (34) discovered the antimalarial potentiation of sulfadoxine and pyrimethamine. The U.S. Army found that a 14 day course of quinine with an initial dose of sulfadoxine and pyrimethamine was effective against falciparum malaria (47, 19). However the official U.S. Army regimen is still quinine for 10 days, pyrimethamine for 3 days and DDS for 29 days (48). In Laos a 7-10 day course of quinine plus an initial dose of Fansidar was found to be effective (49). However quinine with Fansidar is not mentioned in the regimens recommended by WHO (41) nor in the latest addition of a leading textbook of medicine (50).

There is evidence that quinine is more effective than chloroquine against chloroquine-sensitive falciparum malaria from Africa (51). Therefore comparative studies of quinine and chloroquine with and without terminal Fansidar for African falciparum are obviously indicated.

RECOMMENDATIONS: The following is the recommended treatment of falciparum malaria in Thailand based on all completely tested drug regimens to date:

1. At least four doses of quinine (540 mg base each dose in adults) usually given at 12 hour intervals followed by a single dose of Fansidar (sulfadoxine 1.5 g with pyrimethamine 75 mg in adults). Proportionately smaller doses are given to children.

2. Quinine dosage should not exceed 20 mg/kg daily. The first and sometimes subsequent doses of quinine should often be administered as an intravenous infusion usually in four hours. The standard dose in adults is 500 mg (10 gr) in 500 ml normal saline.

3. In order to prevent pulmonary edema, Thai adults with falciparum malaria should not receive more than 1500 ml fluid (including blood transfusion) every 24 hours. Children should receive proportionately smaller volumes.

Table 1. Quinine Therapy Followed by a Single Dose of Fansidar[R] No. Doses Quinine Related to Cure Rate. Data for Fansidar[R] Alone Included.

No. Doses Quinine	Average Parasite Count For Group	Average Parasite Clearance Time(Hrs)	Average Initial Fever (°C)	Average Fever Clearance Time(Hrs)	Treatment Failures (RI-III)	Cure (S)	Cure Rate
0*	60230	73.0	39.5	62.6	6	33	85%
1	57742	67.3	39.1	50.7	0	21	
2	65510	71.6	39.5	57.5	2	22	
3	71719	67.7	39.3	55.6	0	24	
4**	124624	72.4	39.4	43.4	0	22	
5	76385	67.4	39.5	54.5	2	22	
6	81481	73.8	38.8	62.5	1	21	
7	114285	75.3	39.4	58.0	0	23	
8	99600	78.2	39.3	70.3	1	21	
9	64067	76.6	39.2	59.5	5	83	
10	98651	81.5	38.5	69.5	0	20	
11	66949	91.8	39.6	102.0	0	7	
12	285057	88.0	39.0	74.8	1	6	
13	242345	92.5	39.2	93.5	0	3	
14	269754	108.8	38.7	91.0	0	3	
15	138340	117.0	40.3	109.5	0	2	
16	-	-	-	-	-		
17	515060	130.0	40.1	82.0	0	1	
18	252720	147.0	40.2	137.0	0	1	
Average 1-18 Doses (Total Quinine-Fansidar Group)	90676	77.3	39.2	61.5	12	302	96%

* Study reported in detail elsewhere[(4)].

** For Optimum Clinical Response at Least 4 Doses Quinine Should Be Given Before The Fansidar.

Table 2. Falciparum Malaria in Thailand 1973-1974.
Cure Rates with 4 Different Regimens.

	Average Duration Therapy	Average Parasitemia (per cu.mm.)	RIII*	RII	RI	S	Cure Rate(%)
Quinine+Fansidar	3 days	90,000	0	0	12	302	96
Quinine**	6 days	28,000	1	0	9	55	85
Fansidar***	1 dose	56,000	4	0	2	33	85
Chloroquine	3 days	15,000	2	8	1	0	0

* RIII, no marked reduction of asexual parasitemia; RII, marked reduction of asexual parasitemia, but no clearance; RI, clearance of asexual parasitemia, followed by recrudescence; S, clearance of asexual parasitemia without recrudescence (radical cure). World Health Organization (1967).

** Study reported in detail in Hall, A.P. et al.(3)

*** Dose in adults 1.5g sulfadoxine and 75 mg pyrimethamine.

Table 3. Dihydrofolate Reductase Inhibitors, Sulphonamides and Sulphones Used in Malaria - Half-life (t/2)

Drug	Half-Life (t/2 in Hours)	Reference
Sulfadoxine*	200	Brooks, M.H. et al.(19)
Pyrimethamine*	96	Smith, C.C., and Ihrig, J. (58)
Sulphalene	65	Seneca, H. (52)
Dfd	30	Sonntag, A.C, et al. (56)
Dds	21	Glazko, A.J. et al. (53)
Sulphadiazine	17	Richards, W.H.C. (54)
Sulphamethoxazole**	9	Schwartz, D.E., and Rieder, J.(55)
Trimethoprim**	9	Schwartz, D.E., and Rieder, J.(55)

* Sulfadoxine with pyrimethamine is marketed as Fansidar.

** Sulphamethoxazole with trimethoprim (co-trimoxazole) is marketed as Bactrim or Septrin.

4. Blood transfusion is rarely indicated in falciparum malaria and antimalarial therapy is usually sufficient. Blood transfusion should be considered if the hematocrit falls below 15%.

9. Comparison of Mefloquine (WR142490) and Pyrimethamine with Sulfadoxine for the Single-dose Treatment of Falciparum Malaria

OBJECTIVE: To compare the efficacy of mefloquine and Fansidar in the treatment of falciparum malaria.

BACKGROUND: Mefloquine (WR142490) is α-(2-piperidyl)-2, 8-bis (trifluoromethyl)-4-quinoline methanol hydrochloride. Mefloquine, given as a single dose, has been very effective in the treatment of induced falciparum malaria in prison volunteers in the United States (56). It is a long acting chemical analogue of WR30090. In Thailand WR30090 was as effective as quinine when administered every 8 hours for 6 days in the treatment of falciparum malaria.

Fansidar (a 20:1 combination of sulfadoxine and pyrimethamine) has been extensively studied as a single dose for the treatment of falciparum malaria. In Thailand Fansidar was 85% curative in Trad Province and 82% curative in Prachinburi Province in the SEATO studies.

DESCRIPTION: The study was begun at the Chao Phya Abhai Bhu Bejhr (Prachinburi Provincial Hospital) on 24 February 1975. Male patients who volunteered were selected for study if they were aged at least 15 years. Other criteria were an asexual parasite density of P. falciparum of at least 1,000 per cu. mm. and the ability of the patient to return for follow-up examination on days 14, 21 and 28 after therapy. Also the patients were asked to sign a written consent after being informed of the nature and potential hazards of the study. Thirty patients will be treated in each group.

Direct estimations of the parasite density were made on admission, twice daily in hospital and once at follow-up examination on days 14, 21 and 28. Determinations of the hematocrit and WBC count were made daily in hospital and at the follow-up examinations. Urinalysis was performed on admission and whenever subsequently indicated.

The medications were administered by the nursing staff in the presence of a study physician. Mefloquine was supplied as plain tablets each containing 250 mg of the drug. The dose was 1.5 g

(six tablets). Sulfadoxine-pyrimethamine was prescribed as plain tablets each containing 500 mg sulfadoxine and 25 mg pyrimethamine. The dose was three tablets.

PROGRESS: MEFLOQUINE acted more quickly against falciparum malaria than did Fansidar (Tables 1 and 2). However even with mefloquine, the patients' symptoms did not always respond as quickly as the parasite and fever clearance time would suggest.

The parasite clearance time for mefloquine was 59 hours which is shorter than for the 12 other regimens that we have tested. For example, the parasite clearance time for quinine, in a group of patients of similar clinical severity, was 69 hours. The average fever clearance time for mefloquine was 46 hours. Only amodiaquine had a shorter fever clearance time (36 hours). In most patients who received mefloquine, fever cleared rapidly, but in two it was more prolonged (Cases 3 and 5).

One patient showed clinical deterioration during the first few hours after the dose of mefloquine and an RIII failure was diagnosed. The patient responded to quinine and the case history is given below. Follow-up has been completed on nine other patients and all achieved radical cures of their infection.

Patient No. 1 was an 18 year old laborer with a history of headache and fever for five days. He had a bitter taste but was not thirsty. He was afebrile on admission (temperature 37.2°C) and the pulse rate was 90 per minute. The blood pressure was 90/50. He looked slightly anemic but was not jaundiced. His abdomen was soft and the liver and spleen were both moderately enlarged. He could stand and stagger about but he preferred to lie down. He was alert and signed the consent form. His skin was cool without perspiration. The initial parasite density (5 minute stain) was 96,000 per cu. mm. He was diagnosed as having moderately severe falciparum malaria. Because he was afebrile and hypotensive, the old term "algid malaria" was probably appropriate. The hypotension was probably due to salt depletion or possibly dehydration. Mefloquine 1.5 g orally was administered at 0945 hours and 500 ml normal saline was infused over a four hour interval. The accurate count (30 minute stain) was 171,000 per cu. mm. At 1330 hours the parasite count was 208,000 per cu. mm., but the patient had a large lunch. At 1600 hours the patient sat on the floor (as was his custom) and had a large meal. He then developed severe abdominal pain. On examination the patient was groaning and writhing in agony. An infusion of quinine (500 mg in 500 ml normal saline) was begun at 1650 hours and was infused in 3.5 hours. The serum quinine concentration increased from 0 to 13.3 mg per liter during the infusion.

The severe abdominal pain disappeared within 30 minutes of the beginning of the infusion and overall the patient greatly improved. The parasite density, however, was 407,000 per cu. mm. at 1745 hours and 468,000 at 2020 hours. A fever of 39.1°C developed during the day.

The patients' condition was satisfactory the next morning but the parasite density was still 450,000 per cu. mm. A second infusion of quinine 500 mg in 500 ml normal saline was infused over a four hour interval. At the end the patient had tinnitus indicating quinine toxicity. However at 1300 hours the parasite density had decreased to 141,000 per cu. mm. A third and smaller dose of quinine (270 mg orally) was given at 1800 hours. Thereafter the patient made a steady recovery and remained free of parasitemia on days 14, 21 and 28.

The parasitemia cleared in 99 hours and the fever in 88 hours. During the initial 24 hours the hematocrit (packed cell volume) decreased from 40 to 26 per cent. Thereafter coincident with the eradication of his disease, the hematocrit increased to 41 per cent on day 28 without any hematinic therapy.

FANSIDAR: The single dose combination of pyrimethamine with sulfadoxine (Fansidar) cleared parasitemia on average more slowly (76 hours) than did mefloquine (59 hours). Clinically Fansidar acted slowly in many patients (case histories given below). The fever clearance time (62 hours) was longer than that for mefloquine (46 hours). The differences between the clearance times are not statistically significant but probably will be when more patients have been studied. So far, 6 out of 8 patients have been cured.

Patient No. 16. This 35 year old laborer had headache and myalgia for four days. He had two shots three days before admission. There was no history of previous malaria. His temperature was 40.0°C, pulse rate 90, blood pressure 120/80 and weight 46 kg. He appeared mildly jaundiced but not anemic. He was alert but tired and could only walk with assistance. The parasite density was 100,000 on the slide stained for five minutes and 78,000 on the 30 minute slide. The dose of Fansidar was given at 1530 hours. Because his lips were dry, dehydration was diagnosed. One thousand milliliters 5% dextrose in saline were infused over a four hour interval. On day 1 the parasite density decreased to 12,000 but his fever resurged to 40.5°C on day 1 and to 41.0°C on day 2. The patient was observed carefully and he improved; however, anorexia persisted. His parasitemia cleared on the morning of day 6 (135 hours) but reappeared in the afternoon. Therefore a treatment failure was diagnosed, either RI or RII. The patient received four

doses of oral quinine and the parasitemia cleared. He was discharged. On day 28, the patient returned with a parasite count of 1000. Again he received four doses of quinine. On day 42 the patient returned with a count of 2000. Six days oral quinine therapy was prescribed as an outpatient. One week later the patient was free of parasitemia.

Patient No. 20. This 26 year old farmer had a history of headache and fever for three days. His temperature was 39.5°C and pulse rate 90 per minute. He was walking and alert. The liver and spleen were not palpable. The parasite density was 31,000 per cu. mm. Fansidar was given at 1730. The parasitemia decreased to 20 per cu. mm. by the afternoon of day 2, at which time the fever resurged to 39.9°C. On day 3 the patient felt better and the fever decreased to 37.8°C at 0600 hours. During the day the parasitemia increased from 20 (overnight) to 900 to 2200 per cu. mm. An RII response was diagnosed. One dose of oral quinine was given at 0900 hours. The parasitemia decreased to 0 by day 6; however, the patient developed a persistent headache and a parasite count of 10 per cu. mm. was again noted on day 9. A six dose course of quinine was now given and another dose of Fansidar. The patient was cured.

The therapeutic result in this patient is somewhat difficult to assess. On day 3 the parasite count increased but the fever decreased and the patient felt better. It could be argued that the quinine was given prematurely. However despite one dose of quinine, the parasitemia returned on day 9. The patient therefore had either an RII or an RI response.

Patient No. 22. This 38 year old laborer had received two shots (probably an antipyretic) two days previously. He did have a history of malaria eight years ago. His temperature was 38.6°C, pulse 104, blood pressure 90/60 and weight 55 kg. The spleen was enlarged. The parasite count was 13,000 per cu. mm. The dose of Fansidar was administered at 1115 hours. At 1300 hours the parasite count was 62,000 but the patient had improved clinically. At 1930 the parasite count had increased slightly further to 90,000. The patient felt better and the temperature had decreased from 40.2°C to 37.2°C. Because of the increase in parasitemia, quinine therapy was begun. The fever cleared in 63 hours. The patient received oral quinine every 12 hours, but the parasitemia persisted at 10-16 per cu. mm. for three days. Therefore the quinine dosing was increased to every 8 hours. The patient finally received 18 doses of quinine. It was later decided that quinine therapy had been instituted prematurely on day 0, so no therapeutic result could be recorded.

DISCUSSION: Fansidar (a 20:1 combination of sulfadoxine with pyrimethamine) has been extensively studied both for the treatment

and prevention of malaria. At Prachinburi Hospital in Northeast Thailand in 1972, a single dose of Fansidar cured 82% of a group of patients with falciparum malaria; in Southeast Thailand in 1974 the cure rate was 85%. However it is well known that Fansidar is often slow to bring an infection under control and some infections are resistant to the drug. The preliminary results of this study suggest that a lower cure rate will be found at Prachinburi Hospital in 1975.

In the initial group of patients, mefloquine cleared parasitemia and fever more quickly than did sulfadoxine with pyrimethamine. Clinically mefloquine acted more quickly and appears to be a very effective antimalarial drug; however quinine by intravenous infusion is still required for severe infections. The current recommended treatment for chloroquine resistant falciparum malaria is a course of quinine followed by a single dose of sulfadoxine with pyrimethamine. Mefloquine can be considered a superior substitute for Fansidar in this regimen. A short course (e.g. 2-6 doses) of quinine will probably usually suffice in severe cases and in mildly ill patients, the Mefloquine can be given alone.

SUMMARY: Mefloquine 1.5 g (a 4-quinoline methanol) was compared with pyrimethamine 75 mg and sulfadoxine (Fansidar) 1500 mg for the single dose treatment of falciparum malaria. The study is still in progress. So far, mefloquine has cured 90% (9/10) of patients; the average parasite clearance time has been 59 hours and the average fever clearance time 46 hours. With Fansidar the cure rate has been 75% (6/8), the parasite clearance time 76 hours and the fever clearance time 62 hours.

Table 1. Falciparum Malaria Treated with Mefloquine (WR142490)

Patient Number	Initial Asexual Count P. falciparum (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result*
1	171000	-	37.2	-	RIII
2	130000	62	38.2	28	
3	68000	67	39.0	46	S
4	28000	68	40.0	79	S
5	27000	44	38.8	31	
6	16000	43	39.8	10	S
7	15000	62	39.1	101	S
8	11000	88	39.8	38	S
9	8000	91	40.4	104	
10	7000	76	38.5	61	S
11	5000	66	39.6	32	S
12	4000	22	39.2	15	S
13	2000	19	39.4	10	S
Mean	38,000	59	39.2	46	

* In no result given, follow-up had not yet been completed.

Table 2. Falciparum Malaria Treated with Fansidar
(Pyrimethamine with Sulfadoxine)

Patient Number	Initial Asexual Count *P. falciparum* (per cu.mm.)	Parasite Clearance Time (Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)	Result
14	104000	91	40.1	110	S
15	81000	-	39.7	-	
16	78000	135	40.0	114	RI
17	56000	73	41.0	104	
18	41000	86	40.5	37	S
19	35000	73	40.0	52	
20	31000	-	39.5	-	RII
21	15000	87	39.9	61	
22	13000	-	40.2	-	See Text
23	13000	67	36.5	-	
24	7000	71	40.0	40	
25	6000	90	37.5	-	S
26	5000	69	39.0	64	
27	4000	88	38.0	104	S
28	4000	67	40.2	58	
29	4000	64	39.6	47	
30	3000	43	38.0	17	S
31	2000	42	38.5	13	
32	1000	68	39.0	43	S
Mean	26,000	76	39.3	62	

10. The Management of Coma in Falciparum Malaria

OBJECTIVE: To establish the optimum management of coma in falciparum malaria.

BACKGROUND: Cerebral malaria is common in falciparum malaria and usually occurs in patients with high parasite counts; however, occasionally, patients present in coma with low parasite counts. Coma is the most serious manifestation of cerebral malaria in adults. Epilepsy is also a grave complication especially in small children. Other forms of cerebral malaria include delirium, confusional states and stupor. Often the patients enter hospital seriously ill and stuporous and lapse into coma soon afterwards or after therapy. Often the patients appear to lapse into coma or to come out of coma following minimal therapy. In some patients, therefore, the cerebral malaria is short-lived, whereas in others coma is deep and irreversible.

Quinine is the most effective drug in therapy. Corticosteroids were first recommended in 1967 but have never been subjected to a controlled clinical trial. The anticoagulant heparin has been recommended because some investigators consider disseminated intravascular coagulation to be a common complication of severe falciparum malaria. The plasma volume expander, Dextran, has also been recommended for falciparum coma as has the osmotic diuretic, Mannitol.

DESCRIPTION: Between January 1973 and July 1974 at Trad Provincial Hospital about 40 patients with the various types of cerebral malaria were treated. Further experience has been gained at the Prachinburi Hospital since the project was initiated there in February 1975. The clinical evaluation of the patients has consisted of close observation by the study physicians. Detailed clinical notes have been maintained on specific study sheets. The rate of intravenous therapy has been monitored at regular intervals usually every 30 minutes. The state of consciousness, pulse rate, and blood pressure were also regularly monitored when indicated. Complete physical examination was regularly performed. If bladder distension occurred, a urethral catheter was passed and the bladder continuously drained into a measured bottle. The rate of urine production was recorded at frequent intervals. Laboratory investigations included a parasite count at least twice daily and a daily hematocrit. Urinalysis was performed on admission and whenever subsequently indicated. Serum was taken for biochemical analysis on admission and at regular intervals thereafter. Relevant investigations were performed a few weeks later at the SEATO laboratory in Bangkok and usually comprised a

total serum bilirubin, serum creatinine and serum glutamic oxaloacetic transaminase (SGOT). A serum alkaline phosphatase was also occasionally determined. Detailed serum quinine concentrations were determined on most patients. The technique consisted of extraction of the quinine with benzene followed by sulfuric acid and estimation of the fluorescence of the quinine in a spectrofluorophotometer.

PROGRESS: Three case histories are given to illustrate the successful management of cerebral malaria.

Case 1: A 42 year old man (a tree worker) was brought to Trad Hospital at 2000 hours on 11 May 1974. He had been in coma since 1700 hours. The history was of vomiting and anorexia for three days. He consulted a physician in Trad City at midday on the day of admission and a 500 ml unit of intravenous fluid was administered as well as one tablet orally. The patient went home and then lapsed into coma. He gave a history of malaria two years previously. His temperature was 38.5°C and pulse rate 120. The parasite count was 62,000 per cu.mm. He was in coma but reacted to pain. He was sweating profusely. The obvious question arose as to whether the patient had received quinine in the private clinic earlier in the day. For this reason he was given a half dose of quinine (250 mg) in 500 ml normal saline, infused over five hours. The next morning, 10 hours later, he was awake. A half-dose of quinine was given orally (270 mg) and eight hours later a full dose (540 mg). A full dose of Fansidar (three tablets) was administered in the evening. The patient made a satisfactory recovery and his parasitemia cleared in 83 hours.

His sera were analyzed two weeks later. Surprisingly, the serum was free of quinine on admission. On the morning after receiving the half-dose of intravenous quinine his serum quinine level was 4.2 mg per L.

Case 2: A 21 year old farmer was admitted in a stuporous condition. His parasite count was 250,000 per cu.mm. but his vital signs were satisfactory. We decided to treat him with intravenous quinine in the standard 500 mg doses in 500 ml normal saline - at 12 hour intervals. The first dose was given in two hours. The serum quinine was 9.0 mg per L at the end of the infusion (Figure 1) and had decreased to 3.6 mg per L at hour 17 when the second infusion was commenced. This was infused in three hours and raised the serum quinine to 9.3 mg per L. The third infusion was begun at hour 24 for four hours and increased the serum quinine to 10.3 mg per L. The final infusion was begun at

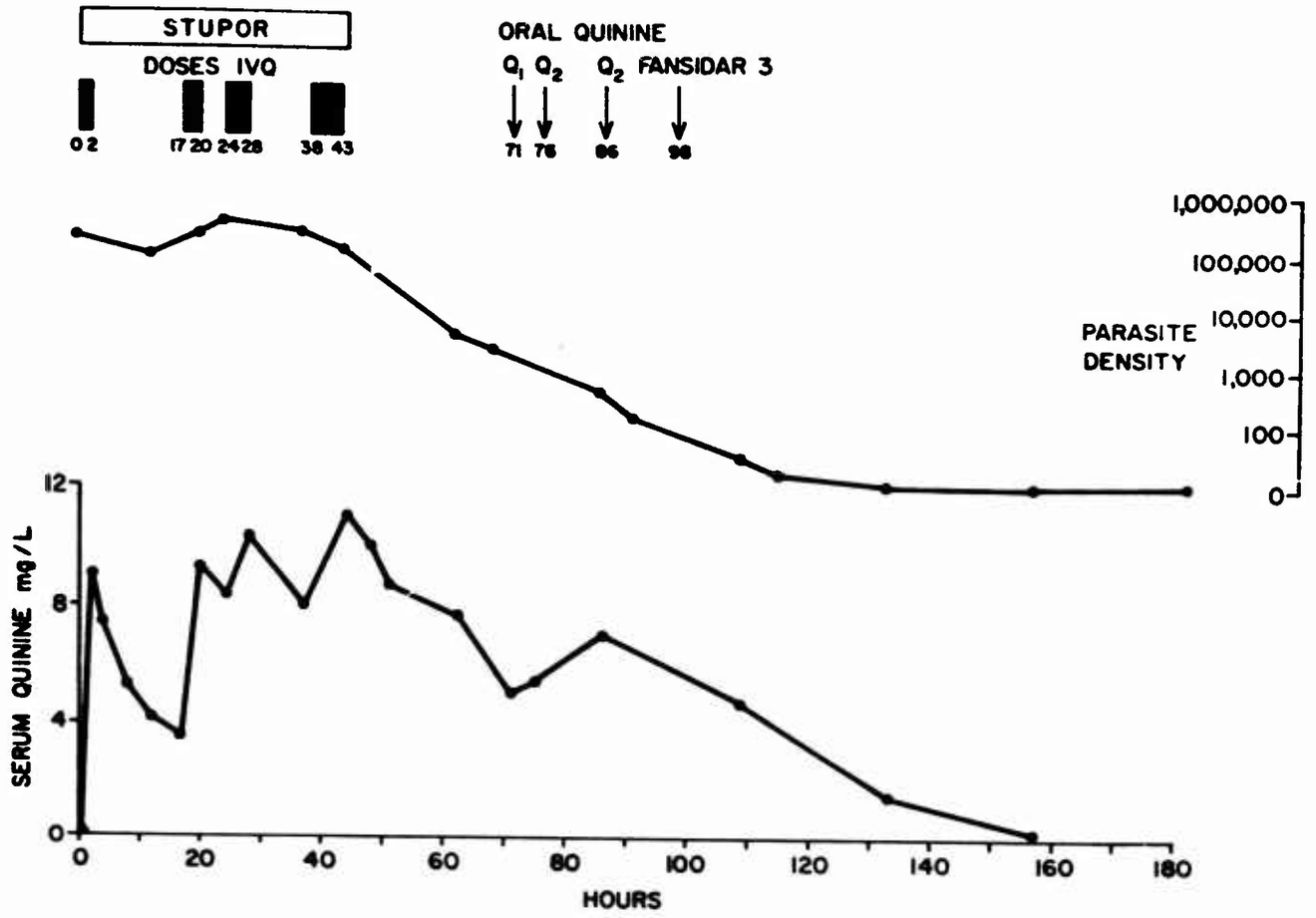

FIGURE 1 TWENTY-ONE YEAR OLD MAN WITH SEVERE FALCIPARUM MALARIA AND STUPOR. SUCCESSFUL CLINICAL RESPONSE WITH FIRST FOUR DOSES OF QUININE ADMINISTERED INTRAVENOUSLY ABOUT EVERY 12 HOURS. TOTAL DOSE QUININE 20 MILLIGRAMS PER KILOGRAM PER DAY. "IVQ" IN FIGURE DENOTES A DOSE OF INTRAVENOUS QUININE.

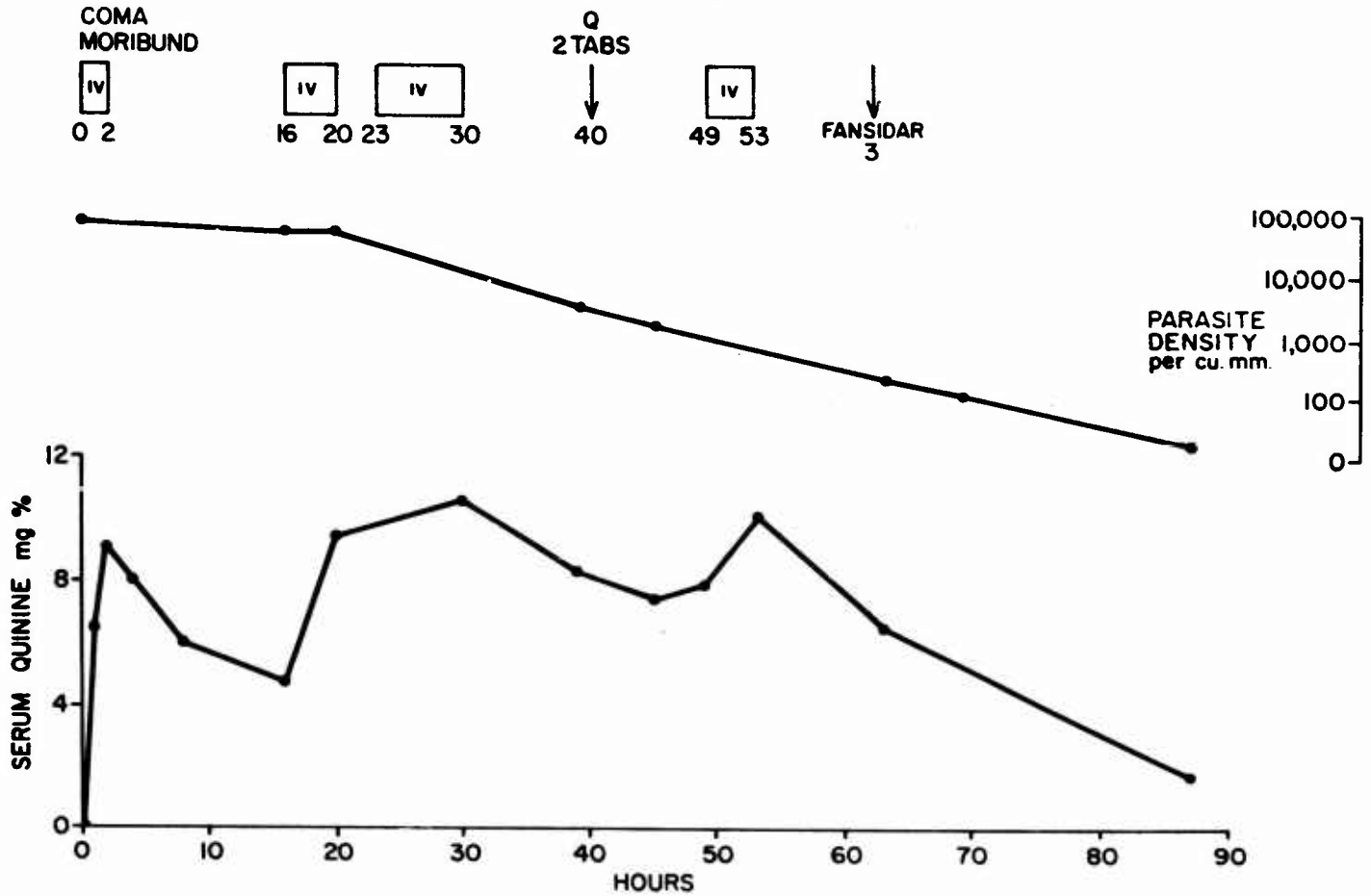

FIGURE 2. PATIENT WITH FALCIPARUM MALARIA AND DEEP COMA. SUCCESSFUL RESPONSE WITH INTRAVENOUS QUININE EVERY 12 HOURS. TOTAL DOSE QUININE 20 MILLIGRAMS PER KILOGRAM PER DAY. "IV" IN FIGURE DENOTES A DOSE OF INTRAVENOUS QUININE.

hour 38 and infused in five hours. The serum quinine then peaked at 11.0 mg per L. No further quinine was given until hour 71 when three doses of oral quinine were administered over a 15 hour interval. A dose of Fansidar (three tablets) was administered at hour 98. The patient's parasitemia remained high until hour 38 when a steady decrease begun. The patient became completely conscious about hour 43. Another notable feature of this case was that the patient had evidence of a bleeding disorder on admission. He had a large amount of recently dried blood in his nostrils and bleeding about venipuncture sites. These signs rapidly cleared with the successful management of his disease.

Case 3: This patient represented a very successful therapeutic result because he appeared moribund on admission. He was in deep coma and flaccid. His respiratory rate was increased at 40 per minute and his heart rate was 130 per minute. There was a loud systolic murmur. There was bleeding about venipuncture sites. The Thai physician gave the relatives a very serious prognosis. The initial parasite count was about 95,000 per cu. mm. The patient's progress can be seen in Figure 2. Doses of intravenous quinine were administered at 0, 16 and 23 hours following admission. The patient's consciousness returned towards the end of the third infusion. He received a dose of oral quinine on hour 20 and another dose of intravenous quinine at hour 49. A dose of Fansidar (three tablets) was administered at hour 62. During the first 50 hours the serum quinine concentration remained in the 4-12 mg per L range. The patient made an uneventful recovery.

DISCUSSION: A system for managing severe falciparum malaria has been established by our investigations. Most patients recover with quinine therapy alone but it is important to avoid overdosage. Twenty milligrams per kilogram daily is the maximum safe dose and this is administered intravenously not more frequently than every 12 hours. As discussed elsewhere, fluid imput should be restricted to prevent pulmonary edema. Corticosteroid therapy has not been discussed in this paper and its value is difficult to determine.

11. Pulmonary Edema Due to Fluid Overload in Falciparum Malaria

OBJECTIVE: To determine whether restriction of intravenous fluid intake would decrease the incidence of pulmonary edema in comatose patients with falciparum malaria.

BACKGROUND: Brooks et al. presented case-histories on five patients with falciparum malaria who developed pulmonary edema and died (57). Four of the patients had received intravenous fluids. Punyagupta et al. described 12 patients with this complication of whom nine died (58). All patients had received intravenous fluids. Both authors claim that the pulmonary edema was not due to fluid overload but to a specific complication of the disease.

This paper reports on six comatose adult patients with falciparum malaria who developed pulmonary edema which was attributed to excessive intravenous fluid therapy. Ten other comatose patients with falciparum malaria, studied later, did not develop pulmonary edema and this was attributed to optimal intravenous fluid therapy.

DESCRIPTION: The study was carried out at the Trad Provincial Hospital in Southeastern Thailand 250 miles from Bangkok. The area is endemic for chloroquine-resistant falciparum malaria (59, 5). Diagnostic services and nursing facilities were limited at the time of the study (1973-1974). Study physicians maintained detailed clinical records, closely monitored intravenous fluid therapy and, in most patients, recorded the approximate urine output. The patients were seriously ill on admission and there was no facility for weighing them in bed before treatment. Central venous pressures (CVP) could not be monitored.

Initially eight comatose patients (six adults, two children) with falciparum malaria and pulmonary edema were studied. Not all patients were under the direct care of the research physicians. Following the restriction of intravenous fluid intake as a change in therapeutic policy, 10 comatose adults with falciparum malaria were studied who did not develop pulmonary edema. The two groups were equivalent with respect to clinical severity and average parasitic densities.

Quantitative parasite counts (6) were determined at least twice daily in hospital and at follow-up examinations on days 14, 21 and 28 and often later. Hematocrits and white blood cell counts were performed regularly. Sera were collected and taken to the SEATO Laboratory in Bangkok for determination of quinine concentrations (42). There were no facilities at Trad for radiography or autopsies.

PROGRESS: In six comatose adults the onset of pulmonary edema was apparently related to intravenous fluid therapy (Table 1). These patients received an average of 1,767 ml intravenous fluid

during the first eight hours after admission and altogether 2,825 ml in the first 24 hours. The physical signs of pulmonary edema developed following intravenous therapy. In all six patients, fluid intake greatly exceeded urine output during the period that pulmonary edema developed. In three patients, coma developed or worsened after intravenous fluids.

Ten comatose adult patients studied later did not develop pulmonary edema. Their average fluid intake was 563 ml in the first eight hours and 1, 181 ml in the first 24 hours (Table 2). The differences between the eight hour inputs ($t = 4.2$, $p < 0.001$) and 24 hour inputs ($t = 4.1$, $p < 0.001$) were statistically significant. No clinical deterioration followed intravenous therapy in the second group.

The case-histories on two adults and two children are now given.

Case No. 2: This 30 year old woman was six months pregnant. She had become comatose on the day of admission. On examination the lung-fields were clear. Her temperature was 38.8°C and her asexual parasite density of P. falciparum was 94,600 per cu.mm. Since she was pregnant, quinine was not prescribed for fear of abortion. One thousand milliliters of five per cent dextrose with 200 mg chloroquine base was infused in one hour. A second similar unit was infused in the following six hours. Thus within eight hours of admission the patient had received 2,000 ml fluid. The patient developed noisy breathing. Loud moist crepitations were present throughout both lung-fields. Since her falciparum malaria was deemed resistant to chloroquine, a third liter with 1,000 mg quinine was now administered. The next morning she awoke briefly but lapsed into coma following further intravenous fluids which were administered at the rate of 1,000 ml every eight hours. The physical signs of gross pulmonary edema persisted and she died 24 hours later at which times the parasite count had decreased to 60 per cu. mm.

Case No. 6: This 18 year old man had been ill for eight days and aphasic for two days. The patient had been taken to an unlicensed practitioner on the day before admission. Four thousand milliliters of intravenous fluid were infused by the practitioner over a 12 hour interval. After 3,000 ml, the patient was able to walk to the toilet (according to his brother). Another 1,000 ml was rapidly infused and the patient went into a deep coma from which he never recovered. He was brought into the hospital moribund about 10 hours later, coughing up blood-tinged sputum. He had cyanosis and was breathing noisily. Extensive moist rales were heard. The P. falciparum

density was 103,000 per cu.mm. In hospital he received quinine 500 mg in 500 ml normal saline intravenously over a four hour interval. The diuretic, furosemide 20 mg, was administered intravenously and urine output was 800 ml during the next 12 hours. A second quinine infusion was begun 16 hours after admission. The total fluid input in hospital was 700 ml and output 1,300 ml, but the physical signs and gross pulmonary edema worsened. The patient deteriorated and died 22 hours after admission.

Case No. 17: This four year old boy weighed 10 kg. On admission at 1100 hours he was stuporous with enlarged liver and spleen. The P. falciparum density was 273,000 per cu. mm. Quinine 250 mg in 500 ml five per cent dextrose in half-normal saline was infused over five hours. His condition deteriorated and signs of pulmonary edema supervened. On the next day he was in coma and extensive bubbly rales were heard all over both lung-fields. The parasite count had decreased to 48,000 per cu.mm. Another 250 mg quinine in 500 ml was infused in eight hours. After two hours his coma had deepened and his liver and spleen had become larger. Convulsions appeared, which were only partly responsive to nembutal intramuscularly and intravenously. He developed an extension spasm of the neck. On the next day his parasite count had decreased to 10,000 per cu.mm. The physical signs of severe pulmonary edema persisted. Another 250 mg in 500 ml was infused during the day and the parasite count decreased to 2,000 per cu. mm. The boy was moribund and his father took him home to die.

Comment: In this 10 kg boy, an initial 500 ml infusion appeared to precipitate pulmonary edema.

Case No. 18: This five year old girl weighing 12 kg was admitted seriously ill but fully alert. Her asexual count of P. falciparum was 396,000 per cu. mm. Quinine 250 mg in 250 ml normal saline was infused over 90 minutes. Her lungs remained clear at the end of the infusion. On the next morning, 17 hours after admission, her condition appeared to have improved. The parisite count had decreased to 54,000 per cu.mm. Another 250 mg quinine was infused in 500 ml and she developed fits and went into coma. Extensive rales were audible throughout both lung-fields. Twenty-four hours later another infusion of 500 ml was given. She continued to deteriorate with the clinical signs of coma and gross pulmonary edema and died.

Comment: In this 12 kg girl, an initial infusion of 250 ml was uneventful but a subsequent infusion of 500 ml precipitated a fatal pulmonary edema.

OPTIMAL INTRAVENOUS HYDRATION: As described above, pulmonary edema developed in eight patients (six adults, two children) following intravenous fluid therapy. Therefore in subsequent patients fluid input was restricted.

The patients were in coma on admission or lapsed into coma shortly afterwards. None developed pulmonary edema and this was attributed to the deliberate restriction of intravenous therapy (Table 2). A positive fluid balance was not detected in any patients. The following case description is representative.

Case No. 12: This 26 year old man weighed 45 kg and was admitted in coma. His initial asexual count of P. falciparum was 10,800 per cu.mm. which cleared within 64 hours. Intravenous fluid intake over the first four days was 1,000, 1,450, and 1,000 ml, respectively. On this restricted fluid intake his urine output was about 1,000 ml daily, indicating that fluid balance was neutral. The intravenous quinine input over the first four days was 1,000, 0, 500 and 0 mg, respectively. Coma persisted 87 hours and he made an uneventful recovery. The patient's recovery was partly attributed to the moderate doses of both intravenous fluids and quinine. This treatment was successful with Cases 7, 8 and 13-16 in particular.

QUININE DOSAGE: Detailed records of quinine therapy were available on four of the six patients who developed pulmonary edema. These men received on average 1,500 mg quinine base intravenously in the first 24 hours (Table 3). The patients who did not develop pulmonary edema received on average 1,055 mg (Table 4). The difference is statistically significant ($t = 5.1$, $p < 0,001$); however, there was no evidence that quinine caused pulmonary edema. For example, Case No. 10 was admitted in deep coma and apparently moribund. He received 1,500 mg quinine intravenously in 1,500 ml daily for the first two days. A toxic concentration of serum quinine was produced (14.1 mg/L) but there was no evidence of pulmonary edema. Case No. 11 also received 1,500 mg of quinine daily intravenously and his cerebral state appeared to deteriorate after each dose of quinine, but intravenous fluids were restricted to 1,500 ml daily and pulmonary edema did not develop. Similarly Case No. 12 was overdosed with quinine since the serum quinine concentration rose to 20.6 mg/L. Pulmonary edema did not develop but the quinine appeared to precipitate aphasia and coma.

DISCUSSION: There are three lines of evidence that pulmonary edema in falciparum malaria is usually caused by therapy and not by the disease.

Firstly, fatal pulmonary edema (or acute pulmonary insufficient) is rarely mentioned in the older literature but has only recently emerged as a serious and challenging complication of acute falciparum malaria (58). If pulmonary edema were frequently an integral part of the disease then it should appear prominently throughout the literature. However, some textbooks do not mention pulmonary edema (60, 61) and, in another, only one case is mentioned (62). This is in marked contrast to the extensive descriptions of cerebral and other complications. Pulmonary edema has emerged as a clinical problem in the disease coincidentally with the extensive use of intravenous fluids.

Secondly, 80 per cent (21/26) of recent cases of pulmonary edema described in this and other studies (57, 58, 63, 64) developed after admission to hospital. At least one other patient had received intravenous fluids before admission. This suggests that some cases are due to therapy.

Thirdly, in most patients who develop pulmonary edema, there is evidence of a high fluid intake or a positive fluid balance. A positive fluid balance did not occur in our successfully managed patients. It was striking how often the urine output matched the restricted intravenous fluid intake. Either insensible loss is an overrated factor in falciparum malaria or water is produced by tissue catabolism in the disease.

Our adult Thai patients who developed pulmonary edema received an average of 1,800 ml fluid intravenously in the first eight hours after admission and altogether an average of 2,800 ml in the first 24 hours. The patients who did not develop pulmonary edema received an average of 560 ml in the first eight hours and 1,180 ml in the first 24 hours.

Similarly, in Vietnam, pulmonary edema did not develop in any of 73 patients with recrudescent falciparum malaria who received 1,500 ml of intravenous fluid daily (65). These data suggest that re-interpretation of some recent studies is indicated.

In their first paper Punyagupta et al. described two patients. In Case No. 1, the fluid input in the first four days was 17,855 ml and the output was 8,850 ml, giving a positive fluid balance of 9,000 ml, which the authors did not discuss; but during this time the patient developed pulmonary edema. Between the fifth and seventh days the fluid intake was 7,585 ml and output 11,550 ml, giving a negative balance of 3,965 ml. During this time diuretic therapy and many other drugs were given and the pulmonary edema resolved. During the seven days of observation, the average

daily fluid intake was 3,634 ml and output of urine was 2,914 ml, which is very high. It should be remembered that homeostasis can be achieved with as little as 500 ml urine daily (67).

In the second patient, pulmonary edema developed on the second hospital day during which the fluid intake was 4,750 ml and output 1,950 ml. Although the diuretic furosemide was administered to both patients, the authors attributed survival to the treatment with heparin, dexamethasone, dextran and antimalarial drugs. These two patients were included in the total of 12 described in the second report (58). They claimed that fluid overload did not occur, and perferred the term acute pulmonary insufficiency; but they gave no information on fluid balance in their other 10 cases.

Brooks et al. described fatal pulmonary edema in five patients of whom four developed the complication between the third and tenth hospital days (57). In these four patients the average daily fluid intake was 2,900 ml and output 1,800 ml, giving an average positive fluid balance of 1,100 ml daily. Their fifth patient did not receive intravenous fluid before the onset of pulmonary edema and fluid overload was presumably not a factor in that patient.

In three of our patients and probably in many others described in other reports, fluid overload appeared to cause both pulmonary edema and coma (e.g., cerebral edema).

Dehydration is either a rare or non-existent feature of falciparum malaria. Metabolic studies have demonstrated hyponatremia, an increased plasma volume but no abnormality of total body water in many patients with acute falciparum malaria (68, 69). Thus, it is surprising that having demonstrated an increased plasma volume in falciparum malaria, Brooks et al. dismissed fluid overload as being a factor in their patients with pulmonary edema (57).

Miller et al. confirmed the hyponatremia, considered that the cause was salt depletion and water retention and said "serious errors in treatment can arise from the blanket assumption that all malaria patients are dehydrated and routinely in need of intravenous water and electrolytes. In those with severe naemia and cardiac disease this could precipitate pulmonary edema" (70). This viewpoint had been expressed before (71). Tigertt (personal communication) has confirmed that many instances of pulmonary edema among U.S. troops with falciparum malaria in Vietnam were due to fluid overload. In retrospect most patients (U.S. military in Vietnam) with edema in falciparum malaria had a progressive weight gain before the onset of dyspnoea in contrast to the usual weight loss of 1.7 to 2.6 kg in the disease (72).

There is evidence, therefore, that in falciparum malaria fluid overload can be produced by intravenous fluid therapy. Extreme thirst is a frequent symptom in the disease, which raises the question whether excessive oral intake of fluids could sometimes precipitate pulmonary edema and coma.

The central venous pressure (CVP) has been normal when measured in falciparum patients with pulmonary edema (57, 58, 64). Our interpretation would be that fluid overload can precipitate pulmonary edema without increasing the CVP. Damage to the pulmonary tissues is probably common in falciparum malaria; thus, pulmonary edema will occur more readily than in healthy lungs.

Since fluid restriction is important in falciparum malaria, the indications for intravenous therapy need to be clearly defined. Inadequate oral intake is obviously an indication. As a minimum, intravenous fluids are needed to prevent oliguria (urine output less than 400 ml daily) and as a vehicle when intravenous quinine therapy is needed.

Some cases of pulmonary edema or acute pulmonary isufficiency in falciparum malaria are probably due to the disease and are not related to therapy. Deaton reported two patients in whom respiratory symptoms occurred before treatment (64). Fluid balance was negative in one patient and slightly positive in the other.

There is evidence for several pulmonary lesions in falciparum malaria (Table 5). Non-fatal bronchitis or pneumonia is often mentioned in the older literature (73, 74). Clinically diagnosed pulmonary edema, which can resolve with therapy, has been discussed in this paper. Pulmonary edema is a terminal event detected at autopsy in some patients dying of cerebral malaria or other complications of the disease (75).

Most patients with malaria are admitted to remote hospitals. We found in a remote hospital (without facilities for CVP and detailed body weights and urine outputs) that the restriction of fluid intake greatly reduced the incidence of pulmonary edema. This finding is especially applicable to other remote hospitals where fluid intake can be regulated but CVP, body weight and urine output cannot always be determined.

CONCLUSIONS: Pulmonary edema in falciparum malaria may be caused by excessive fluid intake. The present study has shown that the incidence of pulmonary edema can be greatly reduced if fluid input is restricted. One thousand five hundred milliliters of fluid

Table 1. Patients with Falciparum Malaria and Coma. Development of Pulmonary Edema Attributed to Intravenous Overhydration

Patient Number	Age (years)	Maximum Asexual Parasite Count (per cu.mm.)	Volume Infused (ml)			
			0-8 Hrs.	8-16 Hrs.	16-24 Hrs.	Total 0-24 Hrs.
1	20	317800	1450	0	1000	2450
2*	30	94600	2000	0	1000	3000
3	30	639600	1650	350	1000	3000
4	16	96500	1500	0	500	2000
5*	22	777600	1000	500	500	2000
6*	18	103600	3000	1000	500	4500
Average	23	338300	1767	308	750	2825

* Fatal cases

Table 2. Patients with Falciparum Malaria and Coma. Absence of Pulmonary Edema Attributed to Optimal Intravenous Hydration

Patient Number	Age (years)	Maximum Asexual Parasite Count (per cu.mm.)	Volume Infused (ml)			
			0-8 Hrs.	8-16 Hrs.	16-24 Hrs.	Total 0-24 Hrs.
7	35	719800	1000	0	1000	2000
8	16	6300	900	100	0	1000
9	30	307600	630	370	500	1500
10*	45	131200	500	500	500	1500
11	24	772600	500	500	500	1500
12	26	10800	500	0	500	1000
13	42	836200	500	0	500	1000
14	14	12800	350	460	0	810
15	53	2000	500	0	250	750
16	14	72300	250	0	500	750
Average	30	287200	563**	193	425	1181**

* Fatal case

** Significantly less ($p < 0.01$) than the input in the patients who developed pulmonary edema (See Table 1).

Table 3. Patients with Falciparum Malaria, Coma and Pulmonary Edema.
Intravenous Quinine Dosage First 24 Hours

Patient Number	Quinine Infused (mg)				Comment
	0-8 Hrs.	8-16 Hrs.	16-24 Hrs.	Total 0-24 Hrs.	
1	1000	0	500	1500	Mostly chloroquine therapy
2					
3	850	150	500	1500	
4	1000	0	500	1500	
5	1000	0	500	1500	
6	?	?	(500)	?	Treatment before admission
Average	963	37	500	1500	

Table 4. Patients with Falciparum Malaria and Coma but without Pulmonary Edema. Quinine Dosage First 24 Hours

Patient Number	Quinine Infused (mg)				Comment
	0-8 Hrs.	8-16 Hrs.	16-24 Hrs.	Total 0-24 Hrs.	
7	500	0	500	1000*	
8	950	50	0	1000*	
9	650	350	500	1500	
10	500	0	750	1250	Died
11	500	500	500	1500	Quinine toxicity
12	500	0	500	1000*	
13	500	0	500	1000	Quinine toxicity
14	350	450	0	800*	
15	500	0	250	750*	
16	500	0	250	750*	
Average	545	135	375	1055	

* Modest amounts of quinine and intravenous fluids were associated with an optimal clinical response in these patients.

Table 5. Pulmonary Lesions in Falciparum Malaria

Lesion	Causation	Comment
1. Pneumonitis	Disease	Non-fatal
2. Bronchitis	Disease	Non-fatal
3. Pulmonary edema	Therapy	Fatal or reversible
4. Pulmonary edema or "acute pulmonary insufficiency"	Disease	Fatal (diagnosed before death)
5. Pulmonary edema	Disease	Autopsy finding

daily (including blood transfusions) or 500 ml every eight hours is the maximum safe intake in full-sized adults. In smaller adults (e.g., many Thais), 1,000 ml is the optimum daily intake. Children should receive proportionately smaller volumes. A position fluid balance should be avoided if urine output is adequate (e.g.,about 1,000 ml urine daily in adults). It is wise to avoid intravenous therapy at night when nursing supervision may be limited.

If pulmonary edema occurs, diuretic therapy is logical; e.g., furosemide by slow intravenous injection. Quinine therapy must also be restricted to prevent toxicity and 1,000 mg daily (two doses) is usually sufficient in adults.

12. Jaundice in Falciparum Malaria

OBJECTIVE: a) To determine whether a correlation exists between the total serum bilirubin and the parasite count, and b) to determine if serum bilirubin correlates with certain other parameters in falciparum malaria.

BACKGROUND: Published reports of the prevalence of jaundice in falciparum malaria have varied from one percent (77) to 72 percent in a group of 46 fatal cases in Africa (78). Jaundice was a common feature in therapeutic malaria; e.g., in one series of 400 syphilitics inoculated with malaria, six percent developed jaundice (79). Apart from jaundice, abnormality of hepatic function in falciparum malaria is common (76). Hepatomegaly can be demonstrated during an acute attack in approximately half the cases of malaria (77); following recovery, the liver returns to its normal size within a few days.

DESCRIPTION: This study was conducted at the Trad Provincial Hospital in Southeast Thailand. Between 11 January 1973 and 21 July 1974 a malaria clinic was operated daily from 8 AM to 9 AM. Patients were self-referred or referred by the hospital doctors. Of 11,241 patients who were screened 4,824 had falciparum malaria and 929 had vivax malaria. The present survey was conducted on all adult (over age 15) patients with malaria seen in the clinic during a 46 day interval. For each patient with malaria, venous blood was used to determine parasite density (except for those with vivax malaria) by the method of Earle and Perez, and the packed cell volume (hematocrit) using a microcapillary centrifuge. On each serum the bilirubin, creatinine, SGPT, and alkaline phosphatase concentrations were measured using an Auto-Analyzer.

PROGRESS: Slight increases in total serum bilirubin (to between 1-2 mg percent) occurred in 55 percent of the patients with

falciparum and 42 per cent of the patients with vivax malaria (Table 1). However, if jaundice is defined as a total serum bilirubin over 2.0 mg per cent, then it did not occur in any of the 24 patients with vivax malaria. Clinical jaundice is rarely reported in vivax malaria. Of the 236 patients with falciparum malaria and an average parasite count of 26,000 per cu.mm., 19 per-cent had jaundice. Depth of jaundice (as defined above) correlated ($r = 0.35$, $p < 0.01$) with the parasite count (Table 2), as it occurred in only 5 per cent of the patients with a parasite density below 1,000 per cu.mm. but in 45 per cent of those with a count over 100,000 (Table 1).

The serum bilirubin correlated positively with serum alkaline phosphatase, SGPT, serum creatinine, parasite density and negatively with the packed cell volume. The serum alkaline phosphatase correlated with SGPT, serum bilirubin and parasite density. The SGPT only correlated with serum alkaline phosphatase and serum bilirubin.

In most patients recovery from jaundice was rapid and coincided with successful treatment with quinine of the falciparum malaria. For example, in one patient the total serum bilirubin was 8.0 mg per cent on admission but had decreased to 0.9 mg per cent five days later. The severity of malaria tended to be less in the older patients as shown by the negative correlation between age and parasite count ($r = -0.19$, $p < 0.05$).

There was an inverse correlation between the total serum bilirubin and the hematocrit ($r = -0.295$, $p < 0.01$); however, not all of the jaundiced patients were anemic. Anemia was more common in the seriously ill patients; as the hematocrit correlated inversely with the parasite count ($r = 0.322$, $p < 0.001$).

DISCUSSION: There have been few studies using the direct (and accurate) method for counting malaria parasites in patients with naturally acquired falciparum malaria. Thus, it is not surprising that this is the first study to show a correlation between the parasite count and the total serum bilirubin. This supports the strong clinical impression that jaundice is more common in patients seriously ill with falciparum malaria.

We found a correlation between anemia and jaundice which confirms that hemolysis is one factor in the etiology of jaundice in malaria. The serum bilirubin also correlated with the other indices of hepatic function (SGPT and alkaline phosphatase) which suggests that the jaundice is at least partly due to liver damage. Thus, the production of excess bilirubin is the result

Table 1. Total Serum Bilirubin (mg per cent) vs Parasite Count in 236 Patients with Falciparum Malaria

Falciparum Range of Parasite Count (per cu.mm.)	Patients with Bilirubin > 1.0 mg%	Patients with Bilirubin > 2.0 mg%	Mean Bilirubin (± SEM)**	Range
0-99	24% (4/17)	6% (1/17)	0.90±0.14	0.4-2.9
100-999	38% (8/21)	5% (1/21)	0.93±0.10	0.3-2.1
1000-9999	47% (31/66)	17% (11/66)	1.27±0.10	0.4-4.9
10000-99999	59% (60/101)	17% (17/101)	1.61±0.17	0.5-12.0
10000+	87% (27/31)	45% (14/31)	3.56±0.84	0.4-20.6
Average 26000	55% (130/236)	19% (44/236)		
Patients with Vivax Malaria	42% (10/24)	0% (0/24)	0.99±0.06	0.4-1.7

* Includes data on 24 patients with vivax malaria

** Standard error of the mean

Table 2. Intercorrelations of Seven Parameters in 236 Patients with Falciparum Malaria*

Parameters	Age	Parasite Count	Hematocrit	Bilirubin	SGPT	Alk Phos	Creatinine
Age	1.0	-0.19	0.157	0.008	-0.044	-0.032	0.03
Parasite Count		1.0	-0.322	0.352	0.151	0.230	0.205
Hematocrit			1.0	-0.295	-0.007	-0.169	-0.158
Bilirubin				1.0	0.351	0.274	0.226
SGPT						0.340	0.094
Alk Phos						1.0	0.052
Creatinine							1.0

* With n=236 the following values of r are associated with the listed probability levels:

r	p
≥0.296	≤0.001
≥0.225	≤0.01
≥0.18	≤0.05

of excessive hemolysis but the failure of its removal from the blood may be due to liver damage (78). Besides the SGPT and the alkaline phosphatase, the serum bilirubin also correlated with the serum creatinine, the parasite count, and the packed cell volume. Thus, the serum bilirubin seems to be the most useful index of hepatic function in falciparum malaria, and is also a useful guideline to estimate the severity of the disease.

Ross (80) reported that quinine therapy sometimes induced an increase in the serum bilirubin level. But we found that reduction of the parasite density by quinine is usually associated with a rapid reduction in the serum bilirubin concentration.

We did not find jaundice in our 24 patients with vivax malaria (nor in others that we have studied), although jaundice in vivax malaria has been reported by some workers. It is probably rare in pure infections with P. vivax; this indicates the importance of accurate species identification.

SUMMARY: Jaundice (defined as a total serum bilirubin over 2 mg per cent) occurred in 19 per cent (44/236) of a group of patients with falciparum malaria and an average parasite density of 26,000 asexual parasites per cu.mm. The serum bilirubin correlated positively with the parasite density ($p < 0.01$) and inversely with the packed cell volume ($p < 0.01$); this confirms that the jaundice is at least partly due to hemolysis. The serum bilirubin also correlated positively with the SGPT and the serum alkaline phosphatase levels, which indicates that hepatic damage also contributes to the jaundice. The prevalence of jaundice varied from 6 per cent in patients with a parasite count less than 1,000 per cu.mm. to 45 per cent in patients with a parasite count over 100,000. Jaundice was not present in any of 24 patients with vivax malaria.

13. Anemia in Malaria and the Role of Blood Transfusion

OBJECTIVE: To determine the prevalence of anemia in falciparum malaria and in vivax malaria. To determine the rate of recovery from anemia in both diseases. To determine the role of blood transfusion (if any) in the management of the anemia.

BACKGROUND: There is little information on the prevalence of anemia in malaria. Anemia is a well-known complication of falciparum malaria but is less commonly a problem in vivax malaria. Comparative studies on the rate of recovery from anemia in falciparum and vivax malaria are not available. Most standard

sources recommend blood transfusions as part of the general therapy for the hemolytic problem (82, 83). References, however, are extremely vague, and give no data on the results of blood transfusion.

DESCRIPTION: Anemia in this study is defined as a hematocrit (packed cell volume) below 35 per cent. Hematocrits have been determined on several hundred patients with falciparum malaria over the last few years. The hematocrit and other parameters were correlated in 236 unselected patients with falciparum malaria. Some data is also available on a small group of patients with vivax malaria. Daily hematocrits in hospital and weekly hematocrits during follow-up have been taken on the SEATO ward at Prachinburi Hospital since February 1975.

PROGRESS: In an unselected series of 142 patients with falciparum malaria, 63 (44 per cent) developed a hematocrit below 35 per cent during their hospital course and could therefore be considered to have developed anemia. Data on a much larger group of patients is being analyzed.

In a study of jaundice in falciparum malaria described elsewhere, two relevant correlations were discovered. The hematocrit correlated negatively with the parasite count ($r = -0.32$, $p < 0.001$); in other words, anemia is more common in patients with higher parasite counts. Also, the hematocrit correlated negatively with the total serum bilirubin ($r = -0.29$, $p < 0.01$) which indicates that anemia is more common in falciparum patients who are jaundiced.

Clinically the anemia of falciparum malaria improves steadily once the infection has been brought under control. The data in Figure 1 support this impression. Depicted are serial hematocrits on three patients who developed a worsening anemia after admission. When the malaria was successfully treated with quinine, the hematocrit steadily increased without blood transfusion or hematinics. These results are typical of most falciparum patients with anemia that we have studied.

Altogether five patients with severe anemia (defined as a hematocrit below 15 per cent) have been successfully managed without blood transfusion. One interesting patient is shown in Figure 2. This five year old boy was admitted almost in coma and with a parasite count of 41,000 per cu.mm. The patient weighed 15 Kg. He received six doses of intravenous quinine at the rate of two infusions daily administered in a total of 500 ml fluid daily. The parasitemia cleared in 64 hours but the patient remained in coma for five days. On day 6 the patient was conscious but very weak. The hematocrit had decreased from 19 to

12 per cent. A 300 ml blood transfusion was given and the hematocrit increased to 19 per cent. On the following day a second transfusion of 200 ml was given and the hematocrit increased further to 23 per cent. A dose of pyrimethamine with sulfadoxine (one tablet) was given and the patient made a steady recovery.

DISCUSSION: Our preliminary results indicate that about 45 per cent of Thai patients with falciparum malaria develop anemia (defined as a lowest recorded hematocrit below 35 per cent). In one small study of U.S. troops with falciparum malaria in Vietnam, 22 per cent had anemia by this criterion. In another group of 50 U.S. troops, 58 per cent had a hematocrit less than 38 per - cent.

Hemolysis is the principal cause of anemia in falciparum malaria but impairment of red cell production may also occur. The question arises as to what extent the anemia in falciparum malaria is due to background malnutrition or iron deficiency rather than to the disease itself. Our studies suggest that this is a minor factor. Anemia has not been a problem in the convalescence of our patients. The data in Figure 1 is typical in that it shows an increase in the hematocrit once the malaria is brought under control. Slightly low levels of the hematocrit (around 35 per cent) do persist in some patients in convalescence. We are now studying the effect of iron therapy in patients whose hematocrit have stabilized in convalescence. The probable answer is that anemia in falciparum malaria is mainly due to the disease but in some patients malnutrition, iron deficiency or heminthiasis are minor factors.

The role of blood transfusion (if any) in falciparum anemia needs to be clearly defined. Blood transfusion is recommended by some authorities (82), and indeed Manson (83) implies that transfusion should be considered if the hematocrit falls below 30 per cent. We agree with Canfield (81) who asserts that blood transfusion is rarely required. In patients that we have observed, blood transfusion often results in pulmonary edema. Another sequel of transfusion is severe hemoglobinuria which obviously is due to hemolysis of the transfused cells. In this situation the transfusion has obviously been unsuccessful since the hematocrit does not rise. There are several problems with transfusing blood in hospitals in remote malarious areas. The donor blood may contain malaria parasites. Also it may be difficult to cross-match the blood as precisely as in more sophisticated hospitals in non-endemic areas. It is probably true that red cells transfused during the acute phase of the disease are more likely to be hemolyzed than during convalescence. This concept is supported

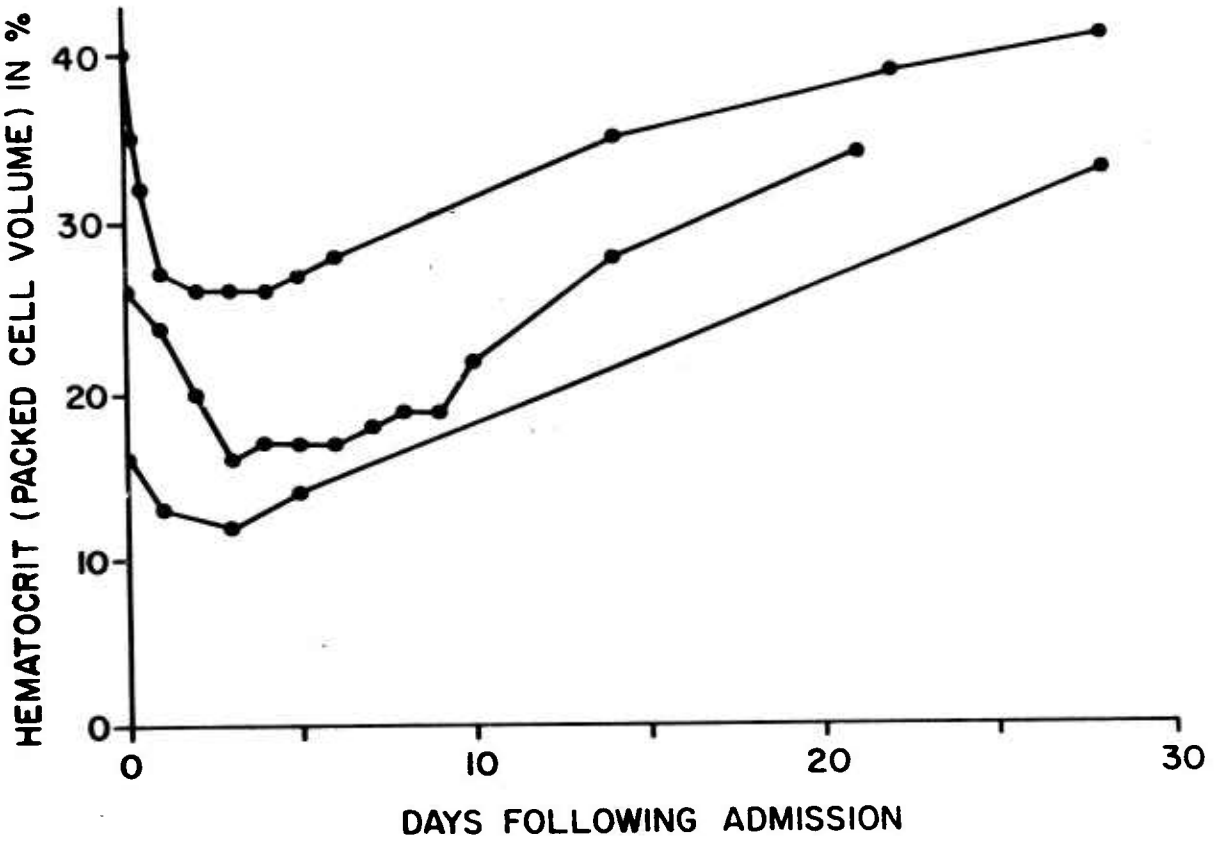

Figure I. Three patients with falciparum malaria. Recovery from anemia was associated with cure of malaria. Blood transfusion and hematinics were not used.

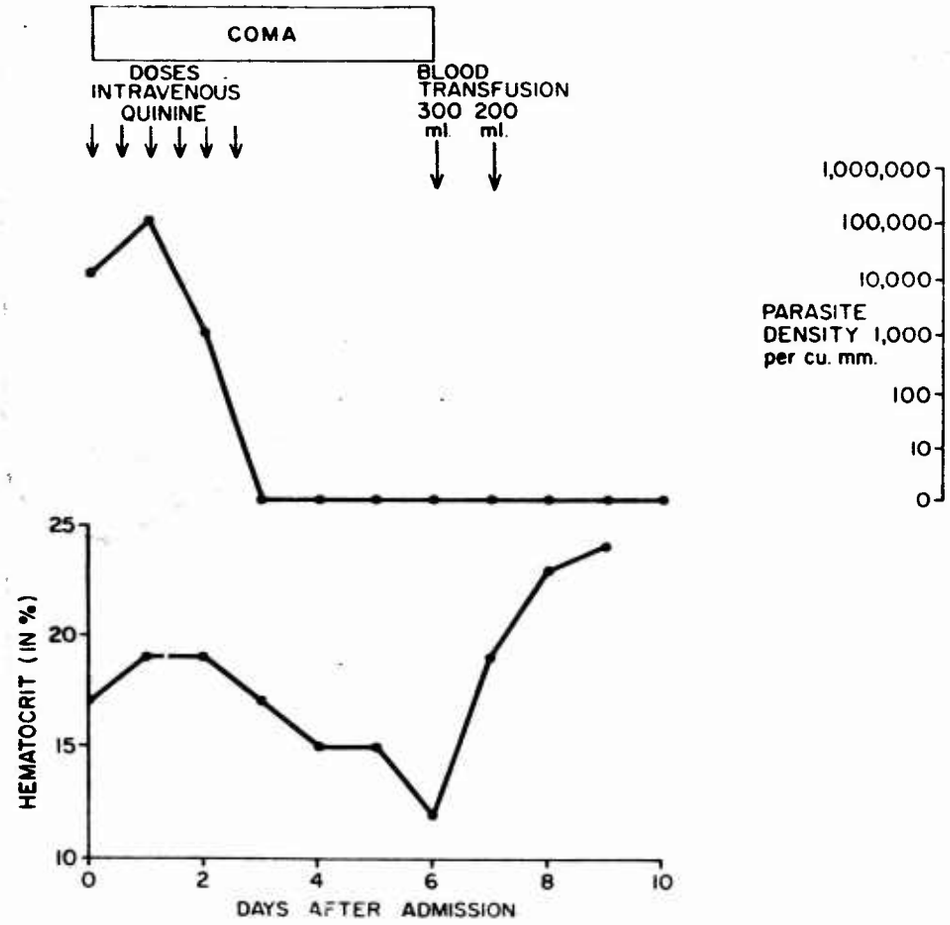

Figure 2 Boy with falciparum malaria aged 5 years. After eradication of parasitemia with quinine therapy, a satisfactory increase in hematocrit followed blood transfusion

by the patient depicted in Figure 2; after the parasitemia has been eliminated by quinine; blood transfusion caused a satisfactory increase in the hematocrit. Our, largely anecdotal, evidence suggests that blood transfusion is rarely indicated in falciparum malaria. A protocol of a controlled study of blood transfusion in falciparum anemia has been submitted to the Ministry of Health of Thailand. Patients who develop a hematocrit between 12 and 20 per cent will be randomly assigned to blood transfusion or conservative management. All patients will receive quinine therapy by intermittent intravenous infusions. Patients with hematocrits below 12 per cent will be treated according to the clinical picture and patients with a hematocrit above 20 per cent will usually be treated conservatively. The patients will be closely followed clinically and detailed relevant hematologic and biochemical tests will be performed.

14. Quinine Dosage and Serum Levels in Falciparum Malaria

BACKGROUND: The World Health Organization has emphasized the need for studying the pharmacology of antimalarial drugs in patients with falciparum malaria (41). Quinine is the only rapidly acting drug for chloroquine-resistant falciparum malaria. In healthy volunteers, quinine by intravenous infusion produced higher plasma quinine levels than did oral quinine (42).

We compared the serum concentrations of quinine following a single dose of one of the three modes of administration (intravenous infusion, intramuscular injection and tablets) in patients with falciparum malaria, since no information was available on this subject. Serum quinine levels were also determined following repeated administration of the drug at 8 or 12 hour intervals, to determine the optimum dosage intervals.

DESCRIPTION: Patients at Trad Provincial Hospital with falciparum malaria comprised the study group. Trad is in a remote area of Thailand 250 miles from Bangkok. Quantitative parasite counts on Thick blood films were made at least once daily (6).

Serum quinine levels were determined in 73 people following a single dose of the drug; sera were collected before dosing and usually 1, 2, 4 and 8 hours after the oral or intramuscular dose or after the beginning of an infusion. Twelve and 24 hour specimens were collected in a few patients. Thirteen patients were excluded from analysis because quinine was detected in the serum on admission. Sera could not be collected after 10 PM, so complete 1-8 hour data were only available on 35 patients. For technical reasons, urine could not be collected. The patients were randomly assigned to one of five dose regimens:

two intravenous (two or four hour infusion), two oral (coated or plain tablets) and one intramuscular. The patients in each group were comparable with respect to age (overall mean 23.7 years) and weight (overall mean 51.8 kg).

Intravenous infusions (IVQ) consisted of 490 mg base (2 ml) of quinine dihydrochloride (The Vitarine Company, New York) injected into an infusion bottle containing 500 ml normal saline. The bottles were infused over a two or four hour interval. All were calibrated and the rate of flow was checked frequently to maintain a constant infusion rate of 250 ml each hour.

The enteric coated tablets of quinine sulfate (Strong Cobb Arner, Cleveland) contained 270 mg quinine base. The usual dose was 540 mg. The plain uncoated tablets (Government Pharmaceutical Organization, Bangkok) contained 250 mg base. The usual dose was 500 mg.

Intramuscular quinine (IMQ) was administered as quinine dihydrochloride (same preparation as used for intravenous therapy) in a dose of 490 mg quinine base (2 ml). The injections were made deeply in the upper and outer quadrant of the buttock.

Serum quinine levels following multiple dosing of the drug were determined in 31 patients (18 of whom had been studied following the initial dose). Nineteen patients received quinine at full dosage every 8 hours and 10 patients every 12 hours. Two patients received half-dosage every 8 hours. With very few exceptions, all doses following the initial dose were oral. In multiple dose studies sera were collected at the time of dosing and at other times (e.g., 1, 2, 4, 8 and 12 hours after a dose). Serum quinine concentrations were determined by the benzene extraction method (42). Measurements were performed using an Aminco-Bowman spectrophotofluorometer (350 m u. activation; 450 mu.. fluorescence). The parenteral and oral formulations of quinine were assayed and found to contain the designated amount of quinine base ($\pm$ 2 percent).

The half-life (T/2) of quinine was determined by plotting the serum concentrations of quinine against time on semi-logarithmic paper. Data from the single-dose studies were compared using analysis of variance. Following the overall analysis of variance, individual F tests were conducted to determine the location of mean differences (84, 85).

Single Dose: Complete data (serum levels at 1, 2, 4 and 8 hours after the dose) were available on 35 patients. There were seven

patients in each of the five treatment groups. The mean serum quinine concentrations for each group are shown in Table 1. The analysis of variance indicated significant differences between the modes of administration and between the hours of sampling (Tables 2 and 3). One hour after initiation of the dose, the two hour infusion produced a significantly greater serum quinine concentration than did intramuscular quinine ($p < 0.01$) which produced a significantly greater level than plain tablets ($p < 0.01$). Four hours after initiation of the dose the four hour infusion produced a greater serum level than coated tablets ($p < 0.05$). At eight hours, coated tablets produced a higher level than the intramuscular injection ($p < 0.05$). The serum quinine concentrations following intravenous or oral administration were not significantly different at eight hours (Figure 1).

Serum quinine levels were not available at 12 hours for all 35 patients: the data were pooled (Figure 1). It is noteworthy that the serum quinine concentration 12 hours after dosing was about 6 mg per L following either intravenous or oral therapy.

Details on each of the regimens are now given.

Two Hour Infusion: Initially 10 men received a two hour infusion. Quinine was detected in the serum of three of these men on admission, two of whom developed severe cinchonism (quinine toxicity) at the end of the infusion. Seven men had no quinine in the serum on admission and the average results on these patients were used in the analysis (Table 1). One and two hours after the beginning of the infusion, greater serum quinine levels occurred than with any other regimen. Thereafter the results were similar to those obtained with the four hour infusion. In one patient the serum quinine peaked at 10.8 mg per L at two hours and decreased slowly to 5.7 mg per L at 22 hours. This data suggests a half-life (T/2) of 21 hours. The clinical response (e.g. fever, parasitemia) to the two hour infusion was excellent; however, cinchonism was more common than with any other regimen.

Four Hour Infusion: Greater serum quinine concentrations were achieved with the four hour infusion than with oral or intramuscular quinine. In most patients the clinical response was excellent.

One patient, who was jaundiced, became aphasic during the infusion and was struggling and comatose at the end, at which time the serum quinine level was 8.8 mg per L. Sixteen hours later he could speak and since no further therapy had been given, a second quinine infusion was begun. During the second

infusion he again became aphasic. The patient then received a single dose of pyrimethamine with sulfadoxine (Fansidar) and made a steady recovery. Neurotoxic hypersensitivity to quinine was diagnosed. In the other patients, the four hour infusion caused minimal side-effects.

Coated Tablets: The mean serum concentration of quinine one hour after the dose was only 0.8 mg per L. A peak serum quinine level of 7.6 mg per L occurred at four hours.

The clinical response was unsatisfactory in one patient; four hours after dosing his serum quinine was 6.1 mg per L. He complained of weakness, dizziness, cough and abdominal pain. Three episodes of diarrhoea had occurred since admission. The patient improved after 250 mg quinine was infused, although further serum quinine levels were not obtained. One patient developed urticaria on the face and chest four hours after dosing that persisted for several hours and was attributed to the quinine. Mild cinchonism occurred in several patients.

Plain Tablets: The mean serum quinine levels after plain tablets were higher than after coated tablets at one and two hours but lower at four and eight hours. With seven patients in each group the one hour data were not significantly different(Table 1). One hour data was available on several other patients not included in the group of 35, because not all 2-8 hour specimens had been obtained. In a total of 12 patients who received coated tablets, the mean one hour level was 0.7 mg per L. In 12 patients who received plain tablets, the mean level was 2.7 mg per L. The difference is significant ($t = 2.5$, $p < 0.05$).

In one patient the clinical response was unsatisfactory. Coma supervened two hours after therapy when the serum quinine level was only 0.4 mg per L. Thereafter the absorption of quinine was much improved (serum quinine level at four hours was 10.6 mg per L). The plain tablets often caused mild cinchonism but no serious toxicity was encountered.

Intramuscular Injection (IMQ): The serum concentration-time curve following intramuscular quinine was flat and did not rise appreciably above 6 mg per L.

One patient had a poor clinical response. Following the injection at 1100 hours, his parasite count increased from 12,000 to 217,000 per cu.mm. at 1700 hours. His serum quinine concentration was then only 3.1 mg per L. Intravenous quinine was then infused with a satisfactory response.

Multiple Dose Studies: Eighty percent (16/19) of the patients who received quinine at eight hour intervals developed some degree of cinchonism and in most patients this was associated with serum quinine concentrations assumed to be in the toxic range (over 10 mg per L). Only 30 percent (3/10) of the patients on 12 hourly dosing developed cinchonism. The difference is significant (chi square = 6.9, $p < 0.01$).

An example of overdosage with quinine given every 8 hours, is shown in Figure 2. Early in therapy a high fever was associated with a raised serum bilirubin and a very high concentration of serum quinine (17.1 mg per L). Improvement was associated with a decrease in fever and bilirubin.

An example of an optimum response to quinine given every 12 hours is depicted in Figure 3. Clinical improvement was associated with a decrease in serum bilirubin and in serum quinine despite constant therapy. The approximate half-life (T/2) decreased from 21 to 14 to 12 hours during therapy.

On the other hand, in two other patients, therapeutic concentrations of serum quinine were maintained with a half-dose (one tablet) given every 8 hours.

The average half-life of quinine in falciparum malaria, derived from all available data on 17 patients was 18.8 hours (range 12-31).

DISCUSSION: The serum concentrations of quinine following oral therapy were low in relation to those following intravenous therapy for the first four hours after dosing. Thereafter the serum levels were the same. These data confirm the clinical impression that the intravenous route is more effective than the oral route for at least the first dose of quinine in falciparum malaria. Plain tablets produced significantly greater serum concentrations than coated tablets one hour after dosing. If the first dose has to be oral then plain are preferable to coated tablets. There was no evidence that cinchonism was less common in the patients who received coated rather than plain tablets.

Intramuscular quinine produced a significantly higher one hour serum quinine concentration than did oral quinine, and might occasionally be preferable to oral quinine for the first dose in situations where intravenous infusions are not available. However, we have seen several patients in whom IMQ was clearly less effective than IVQ. The average serum quinine level following IMQ barely rose above 6 mg per L in distinction to the other forms of quinine. The conclusions are that, in this study, 6 to 10

mg per L was the usual therapeutic range for quinine and that IMQ did not always produce sufficiently high serum quinine levels. The bioavailability of quinine was least by by the intramuscular route, a situation analagous to that for digoxin (86) and diphenylhydantoin (87). One dose of IMQ may be satisfactory in mild cases of falciparum malaria but repeated dosing is not indicated because gluteal abscesses may be produced (88).

Intravenous infusions, either of two or four hours duration, produced serum quinine concentrations above 6 mg per L from the first hour onwards. The two hour infusions naturally produced higher levels but also more frequently caused toxicity. A two hour infusion can be considered for the initial dose but not for maintenance therapy. In two patients with quinine in the serum on admission, the two hour infusion produced very high serum quinine levels with toxicity. We have given IVQ with infusion times from 1 to 16 hours (65, 89). Lengthy infusions can cause arm discomfort and be inconvenient for the patient. The four hour infusion is the optimum form for quinine administration since therapeutic but not toxic levels of serum quinine are usually produced.

In this study the average half-life (T/2) of quinine was 19 hours in falciparum malaria which compares with the T/2 of 10 hours in healthy volunteers that can be deduced from another study (43). The T/2 of quinine is greater in volunteers with falciparum malaria than in the same volunteers without malaria (90). Thus in falciparum malaria the metabolism of quinine may be impaired presumably due to liver dysfunction since quinine is mainly metabolized in that organ (91). Conversely quinine (43) or, its optical isomer, quinidine (92), can cause liver damage. A vicious cycle may occur: if a patient with falciparum malaria has hepatitis, the half-life of quinine is prolonged; thus routine doses of quinine may result in toxic levels of serum quinine; the latter may result in greater damage to the liver; the metabolism of quinine is thus further impaired; consequently even greater levels of serum quinine are produced.

Therefore, in most patients with falciparum malaria, lower or less frequent doses of quinine should produce the same serum quinine as higher doses in healthy patients. Our data confirm this theory. In many patients, maintenance therapy with full doses of quinine every 8 hours produced toxicity and high serum quinine levels, whereas quinine every 12 hours produced a satisfactory clinical response and therapeutic levels. This is not surprising since the average serum quinine level was still about 6 mg per L 12 hours after the initial dose of oral or intravenous quinine. On the other hand, therapeutic concentrations of serum

quinine can be maintained with a half-dose (one tablet) given at every 8 hours. Dosing every 12 hours might produce greater fluctuations of serum quinine concentration than will dosing every 8 hours; however, it is not known whether fluctuation or constancy in the serum quinine level is more effective. In tropical practice, drugs are usually given three times a day (e.g. 0800, 1200 and 1700 hours) rather than every 8 hours, so administration every 12 hours is easier to accomplish.

In several patients, high serum quinine levels occurred early in therapy, but despite constant maintenance therapy, a sharp decrease occurred associated with improvement in clinical state, fever and hepatic function. Other studies have found similar results (19, 93). We attribute the decrease in serum quinine levels to improved metabolism of quinine consequent upon improvement in hepatic function.

Reduced doses of quinine (e.g. 600 mg base intravenously daily) must be administered in patients with falciparum malaria complicated by renal failure (94, 95). In those studies the impairment of quinine metabolism may have been partly due to concomitant hepatic damage.

Aphasia and coma were caused by quinine in one of our patients at a time when the serum quinine was only 8.4 mg per L. Coma in falciparum malaria is sometimes caused by quinine rather than by the disease (i.e. quinine coma). However, "It is sometimes difficult to distinguish between true quinine toxicity and complications of the diseases for which the drug is given" (91).

The WHO recommend 650 mg (540 mg base) three times daily as the standard dose of quinine (i.e. six tablets daily) (41). However, the labels on the bottles of tablets used in this study recommend three tablets daily for two days followed by two tablets daily for five days. The difference between six tablets and 2-3 tablets daily is considerable. The paradox can be explained by the fact that the WHO recommendation is based upon several studies with Americans in Vietnam whereas the manufacturer's recommendations probably take into consideration the lower average body weight of people indigenous to the malarious areas. U.S. soldiers weighing 75 kg usually tolerate about 1500 mg of quinine base per day (20 mg/kg) but the same dose in 50 kg Thais is 30 mg/kg.

Our studies indicate that 20 mg/kg/day is sufficient in the average patient with falciparum malaria. This is the dose recommended by Fletcher (88). One infusion of 10 mg/kg/day is

Table 1. Serum Quinine Concentrations (mg/L) Following Intravenous Infusion, Oral or Intramuscular Administration

Hou-	Intravenous '90 mg/2 Hrs		Intravenous 490 mg/4 Hrs		Oral 540 mg/Coated		Oral 500 mg/Plain		Intramuscular 490 mg	
	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM
0	7	0	7	0	7	0	7	0	7	0
1	7	8.7 ± 1.1	7	6.0 ± 0.6	7	0.8 ± 0.4	7	2.6 ± 0.9	7	5.5 ± 0.9
2	7	10.7 ± 1.2	7	7.7 ± 0.6	7	5.1 ± 1.4	7	5.3 ± 0.8	7	6.1 ± 0.9
4	7	9.4 ± 1.0	7	9.8 ± 0.9	7	7.6 ± 1.1	7	7.0 ± 0.6	7	6.1 ± 0.9
8	7	8.3 ± 0.9	7	7.6 ± 0.9	7	7.3 ± 1.0	7	6.8 ± 0.8	7	5.3 ± 0.8
12	6	7.2 ± 1.0	5	5.2 ± 0.7	2	7.2 ± 1.9	2	3.7 ± 2.1	2	3.4 ± 1.1

Table 2. Analysis of Variance (Hours 0 to 8)

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F	p
Hours	199.5	3	66.5	23.3	<0.001
Error	68.4	24	2.85		
Mode of Administration	351.9	4	87.9	13.3	<0.001
Hours x Mode	171.1	12	14.3	2.2	Not Significant
Error	634.9	96	6.6		

Table 3. Statistical Significance of Mean Values of Clinical Importance

Mode*	Hour**	F	p
IVQ2 v IMQ	1	5.4	< 0.01
IMQ v Oral (Plain)	1	4.5	< 0.01
Oral (Plain) v Oral (Coated)	1	1.7	Not Significant
IVQ4 v Oral (Coated)	4	2.6	< 0.05
IVQ2 v Oral (Plain)	8	1.2	Not Significant
Oral (Coated) v IMQ	8	2.1	< 0.05

*IVQ2 = Quinine infused in 2 hours
IVQ4 = Quinine infused in 4 hours
IMQ = Intramuscular quinine

**Hour refers to number of hours elapsed since treatment begun

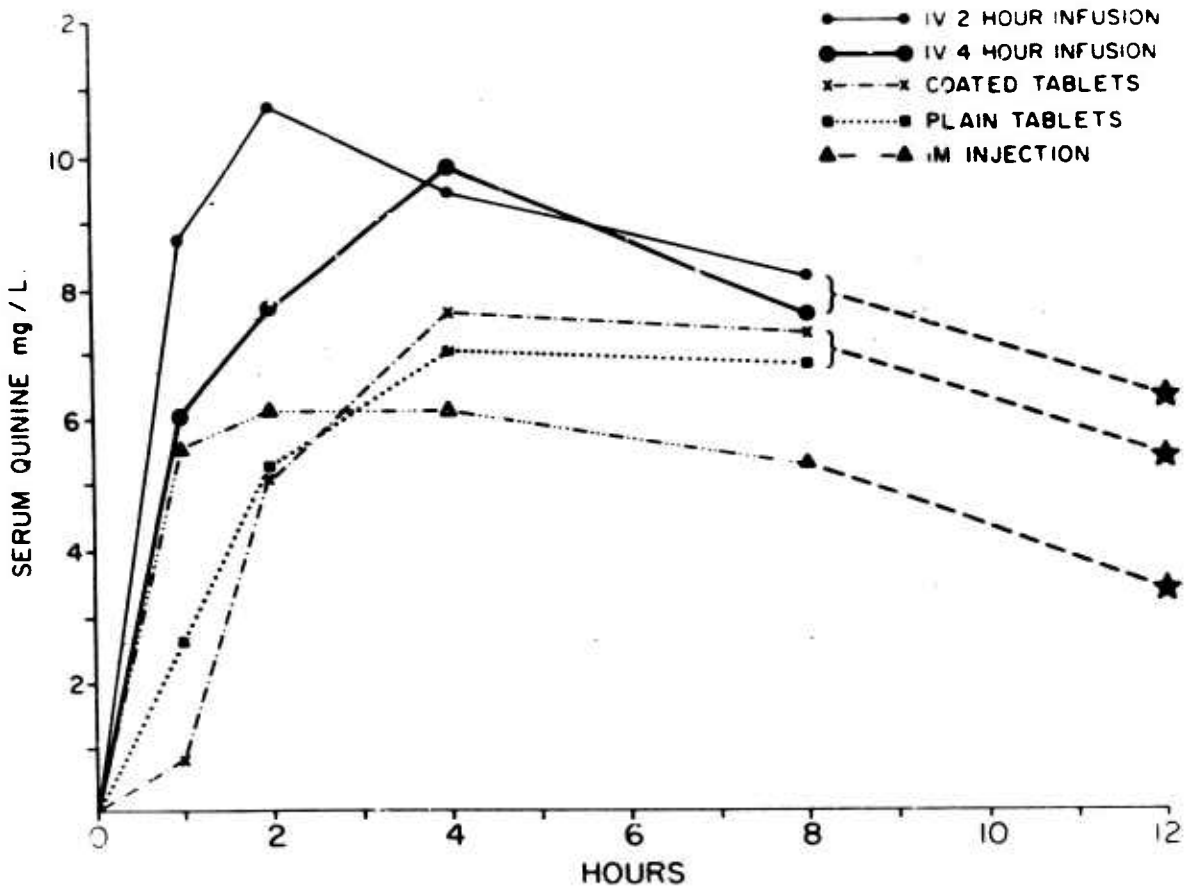

Figure 1. Mean serum quinine concentrations following a single dose of quinine using 5 different modes of administration. There were 7 patients in each group. Complete data are not available at 12 hours.

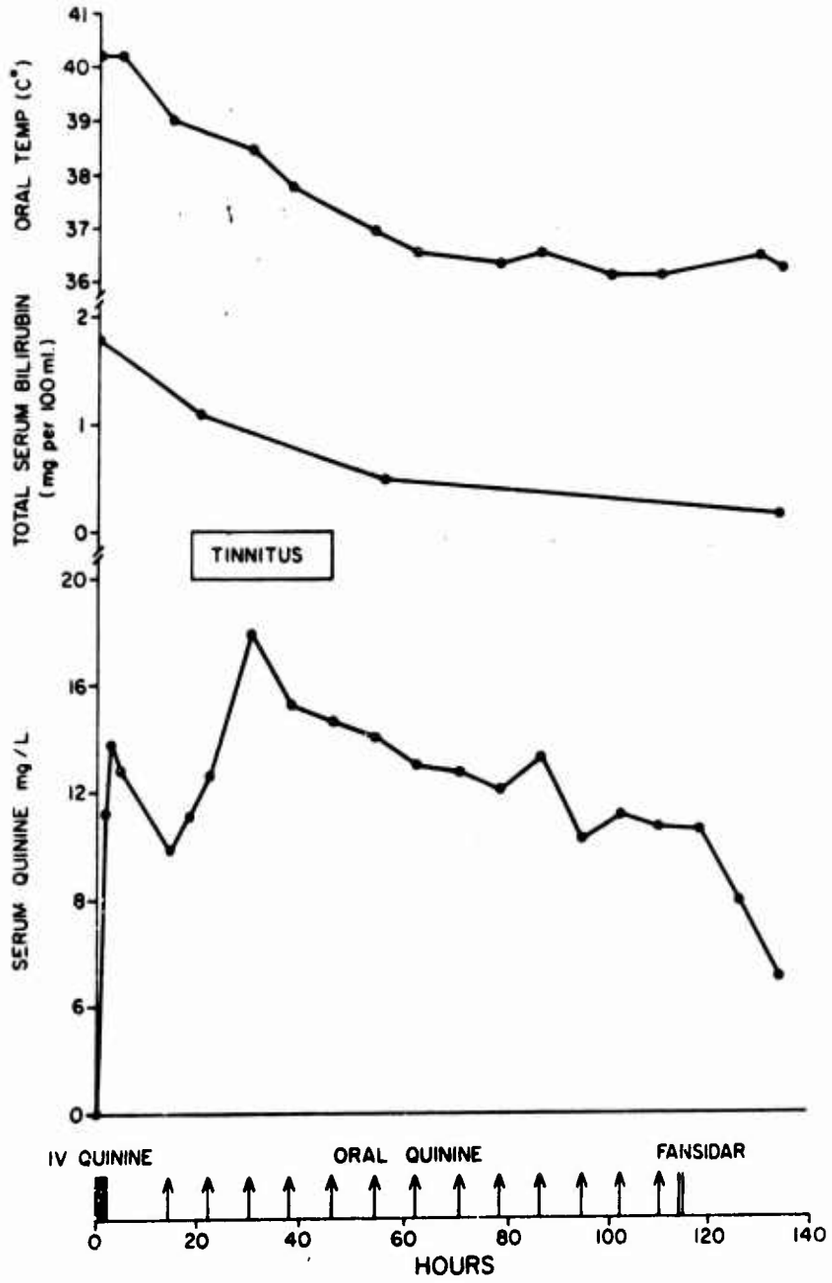

Figure 2. Routine quinine dose, 540 mg every 8 hours, produced a toxic serum concentration. There was a decrease in serum quinine despite constant dosage, associated with a decrease in total serum bilirubin.

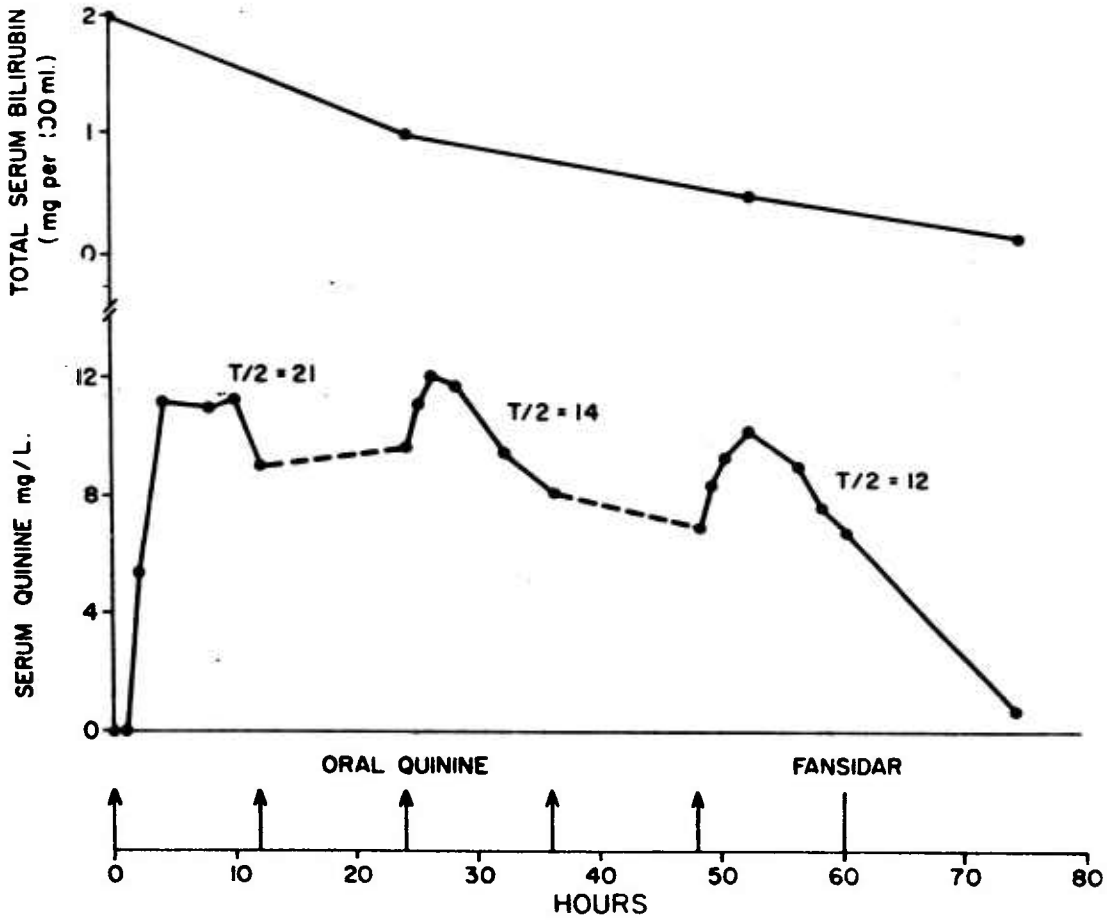

Figure 3. Clinical improvement and satisfactory serum quinine concentrations associated with 540 mg quinine every 12 hours. A decrease in the approximate half-life (T/2) of quinine was associated with decrease in total serum bilirubin.

probably the optimum dose for falciparum patients with severe renal, hepatic or cerebral complications. Thus the more seriously ill the patient, the lower should be the dose of quinine.

It is of interest that 15 percent of the patients (13/86) in the single dose study had detectable quinine in the serum on admission. This is an indication of the prevalence of therapy before admission. Quinine toxicity may be compounded if this possibility is not considered.

SUMMARY: Patients with chloroquine-resistant falciparum malaria were studied. In 35 patients serum quinine concentrations were determined 1, 2, 4 and 8 hours after the initial dose of quinine or after the beginning of an infusion. The patients were randomly assigned to one of five dose regimens: two hour intravenous infusion, four hour intravenous infusion, coated tablets, plain tablets and intramuscular injection.

Intravenous quinine was better absorbed than oral quinine from one to four hours but the serum levels were not significantly different at eight hours. A four hour infusion is the optimum method of administering the first dose of quinine. Intramuscular quinine produced a significantly greater serum quinine level than oral quinine at one hour but a significantly lower level at eight hours. Further patients were studied and it was shown that plain tablets produced a significantly greater mean serum quinine concentration than coated tablets one hour after dosing.

In Thai patients (average weight 50 kg), full dose quinine (540 mg base) every 8 hours often produced toxic serum concentrations, whereas dosing every 12 hours was optimal therapy but still produced cinchonism in 30% of patients.

15. Evaluation of a Direct Counting Technique for Malaria in Thailand

OBJECTIVE: To evaluate a direct quantitative technique for the determination of parasite densities in malaria in rural hospitals in Thailand.

BACKGROUND: Various methods have been used for the direct quantification of malaria parasites but none has been widely used in tropical countries. The technique discussed in this report was introduced by Earle and Perez in 1932.

DESCRIPTION: A measured volume of blood (e.g. 0.005 ml) is spread evenly on a measured rectangle (e.g. 3 x 15 mm) on a

slide. Thus the thickness of the film is known (0.11 mm). A grid is placed in one eye-piece of a binocular microscope and calibrated with a stage micrometer. Thus the volume of blood seen under 1 grid is known. For example, in the SEATO studies, about 1800 grids correspond to 1 cu.mm. of blood. Two types of staining are used. For outpatient screening 0.002 ml of blood are placed in the rectangle and stained with concentrated Giemsa (1:5 dilution) for five minutes. In patients admitted to the ward, 0.005 ml of blood are used and the thick film is stained with dilute Giemsa (1:50 dilution) for 30 minutes. Two rectangular thick films are prepared on inpatient slides. A circular thick film and a thin film are also prepared on all patients.

In this study the technicians were observed performing the parasite counts. There were five technicians on the study team. A stop watch was used to time the duration of the count. The following data were recorded for each determination of the parasite density. The name of the technician, the microscope used, the number of fields counted, the actual count obtained, the count per cu.mm. and the time elapsed in performing the count. A hand calculator was used for determining the parasite densities.

PROGRESS: The first factor assessed was the variability among technicians. Numerous duplicate counts on coded slides were performed by all the technicians. One technician counted with an accuracy of 3.4 per cent (Table 1) as determined on nine slides. The microscopes used were recalibrated frequently. No consistent difference in the two microscopes was observed.

The number of fields that need to be counted varies with the parasite count. For example, if the parasite count is 500,000 per cu.mm. there are about 250 parasites under each grid. The slide is scanned to find typical concentrations of parasites and with such a high count, a minimum of two fields have to be counted. At the other end of the scale a count of 500 requires the counting of at least 90 fields. Usually the number of fields counted is 2, 20, 90 or a larger number. Except for very low counts, about 200-500 parasites are counted. The accuracy of the technique is shown in Table 1. In this experiment the most accurate count was achieved in the patient with a parasite density of 10,000 (0.4 percent precision). The technique was less accurate with both high (4.1% precision), and low (11.1% precision) parasite densities. However, the accuracy of the technique was satisfactory through all ranges of the parasite count. This technician read the nine slides twice in an average of 3.8 minutes per slide (range 2.1 to 6.3 minutes). Numerous other experiments have shown that only about four minutes is needed to make an accurate count.

Table 1. Duplicate Counts on 9 Falciparum Malaria Slides by One Observer Using The Direct Quantitative Technique

Slide	1st (a) Reading	2nd (b) Reading	Mean (c)	c-a	Precision* $[\frac{c-a\%}{c}]$
1	220220	202748	211484	8736	4.1
2	188188	182364	185276	2912	1.6
3	46592	47593	47092	501	1.1
4	29757	29302	29529	228	0.8
5	10920	10829	10874	46	0.4
6	2020	2220	2120	100	4.7
7	1660	1900	1780	120	6.7
8	400	500	450	50	11.1
9	10	20	-	-	-
	Mean 55529	53052	-	-	3.4*

* This figure is the reproducibility from the mean or "PRECISION" and is an index of the accuracy of the technique.

SUMMARY: In rural hospitals in Thailand, a direct quantification technique has proved useful and accurate for the determination of parasite densities in malaria.

16. Treatment of Severe Malaria in Children

OBJECTIVE: To determine the optimum management of severe malaria in children.

BACKGROUND: During 1974 clearcut guidelines were deduced at Trad for the management of severe malaria in adults. Specifically the maximum safe daily dose of quinine in adults was 20 mg/kg. This confirms work performed in Malaysia 50 years ago but in the intervening period 30 mg/kg has emerged as the recommended daily dose. Also at Trad in 1974 we determined that the maximum safe daily fluid intake was 1500 ml in adult patients with falciparum malaria. During this study period, it became apparent that the mortality rate of severe falciparum malaria in small children was high. The onset of convulsions is a frequent and grave complication in small children. The management of severe malaria in children has received little attention in the literature. Another problem is the difficulty often encountered in setting up or maintaining an intravenous infusion because of the small veins in infants.

DESCRIPTION: The children described in this study were treated at the Trad Provincial Hospital in Southeast Thailand. They were first evaluated in SEATO outpatient clinic and then admitted to the ward. Most of the patients were treated between April and July 1974 since there was an unusually high proportion of sick children presenting to the hospital during this interval. The use of the metering chamber (capacity 100 ml) was introduced so that precise volumes could be infused into the children.

PROGRESS: The most difficult problems in management occurred in small children and infants. Children above the age of 12 years could be treated similarly to adults acept the dosage of any medication was best determined on a mg/kg basis. Therefore, only the data on children aged 12 years and less are included in Table 1. Most of the children noted in the Table were very seriously ill on admission. It is difficult to infuse an effective but not toxic dose of quinine, therefore details on the amount of quinine given as the first dose are shown in the Table. The recommended daily dose of quinine is not more than 30 mg/kg/day or 10 mg/kg every 8 hours. Most of the children received only infusion every 24 hour interval because of the difficulty in initiating and maintaining infusions.

Early in the study the question arose whether 5 mg/kg might not be a safer dose of quinine than 10 mg/kg in children. However, Case No. 630 and Case No. 684 were both admitted gravely ill and died a few hours after admission. They received smaller doses of quinine (5-6 mg/kg). Whatever dose of intravenous quinine was used, some children improved and others deteriorated; however, in general, 5 mg/kg appeared to be a safe and non-toxic dose of quinine. Some interesting case-histories are now given.

Cases 649-651. These three children, aged eight, five, and three years were siblings admitted at 1500 hours on 18 May 1974. Cases 649 and 651 were alert but toxic. Case No. 650, the three year old girl, was almost in coma. All three children received half-strength quinine, i.e. 0.5 mg/ml normal saline. Cases 649 and 650 received 5.0 mg/kg over about a four hour interval and 651 received 4 mg/kg. All the patients improved and oral quinine therapy was commenced the next day. Case 649 received one tablet of quinine at 0600, 1400 and 2100 hours, case 650 received 1/2 tablet crushed in water at 0600 and 1400 hours and case 651 received 1/2 tablet at 0600, 1400 and 2100 hours. All the patients then received a dose of Fansidar. All three patients were radically cured although two had a mild P. vivax infection on day 28. None of these children was desperately ill, but all responded well to a half-dose of intravenous quinine.

Case 686. This one year old child was admitted very breathless and crying. His pulse rate was 160 and respiratory rate 70. The liver and spleen were enlarged and hematocrit 24 percent. He was drinking satisfactorily. To avoid pulmonary edema, a small volume (50 ml) containing 50 mg quinine was infused over a five hour interval. Oral quinine was then administered as a 1/2 tablet crushed in water every 12 hours, for a total of six doses. A 1/2 tablet of Fansidar was then given. The child made a satisfactory recovery.

DISCUSSION: Severe falciparum malaria in small children has a serious prognosis. Intravenous quinine therapy should be administered slowly in small amounts. The appropriate initial dose is usually 5-10 mg/kg administered as an intravenous infusion. Satisfactory results have been achieved by giving maintenance therapy as oral quinine when the patient's condition has improved. In infants, 1/2 tablet plain quinine crushed in water can easily be administered by the mother every 12 hours under close professional supervision. Repeated small doses of intravenous quinine should be administered if the child remains severely ill.

Table 1. Details of Initial Dose of Quinine in Children Aged 12 Years or Less with Severe Falciparum Malaria

Case Number	Age (yr)	Weight (Kg)	Parasite Count	Hematocrit (%)	First Dose Quinine (mg)	Volume Infused (ml)	Infusion Time (Hours)	Initial Dose (mg/kg)	Comment
609	7	?10	458000	38	180	180	5.0	? 18	Died
613	9	19	160000	23	180	180	4.5	9.5	
630	12	?25	334000	16	125	125	2.5	? 5.0	Died
640	2	10	1200000	11	117	235	12.0	12.0	Died
644	4	14	95000	24	82	165	5.0	6.0	
648	11	21	430000	31	100	200	6.0	5.0	
649	8	20	333000	33	95	190	4.0	5.0	
650	3	11	178000	29	55	110	3.7	5.0	
651	5	14	238000	30	55	110	3.7	4.0	
660	1.5	8	54000	18	40	80	5.0	5.0	
665	2	13	113000	20	45	90	4.0	4.0	Not enough Quinine
681	11	?	56000	12	350	?	?	? 17	Died
683	4	14	370000	32	150	250	5.0	11	
684	1	5	450000	12	31	63	6.0	6.0	Died
685	4	14	210000	26	100	100	3.0	7.0	
686	1	8.5	53000	24	50	50	5.0	6.0	
F27	5	18	65000	39	180	180	2.0	10.0	
F28	11	28	287000	41	210	210	3.0	7.5	
F31	8	20	258000	32	180	180	4.3	9.0	

17. A Study of Falciparum Malaria in Vietnam Using Serum Quinine Concentrations

OBJECTIVE: To obtain further information on the relation between quinine dosage schedules and the serum level of the drug in patients treated for acute falciparum malaria.

BACKGROUND: Recent work in Thailand has suggested that quinine dosage every 12 hours is optimal therapy in falciparum malaria. Therefore this hypothesis was tested in a group of Vietnamese patients with falciparum malaria. The value of using serum quinine concentrations was also investigated.

DESCRIPTION: The study was performed on patients with malaria admitted to an infectious diseases ward of a general medical hospital in Saigon, South Vietnam. The patients were self-referred and presented with "fever" as their main complaint. A positive diagnosis of falciparum malaria was made when the thin smear on admission showed characteristic appearances of an acute P. falciparum infection.

Patients studied received an initial 600 mg dose of quinine given IV over a period of four hours in 500 ml of 5% dextrose in saline. Some patients continued to receive IV quinine at 12 hour intervals followed by a single dose of Fansidar (three tablets), while other patients were given oral quinine at 12 hour intervals followed by a single dose of Fansidar. Blood was drawn and the serum separated for quinine determinations at 0, 4, 8, 12 and 24 hours following the first quinine dose and also after the last quinine dose was administered.

The duration of fever and parasitemia were recorded and the severity of the malaria and any side-effects of the quinine were noted during the course of treatment.

PROGRESS: In November and December 1974, a total of 19 patients were studied. There were 12 male and 7 female patients with an age range of 13 to 63 years and a mean age of 28 years.

The mean duration of fever was 1.7 days and of parasitemia 3.2 days. The percentage parasite count on admission ranged from 0.1% to 36.0%. Nine patients had a count of 1.0% or less, three patients were between 1-3%, and three patients had between 3-6% parasitemia. High parasite counts of 36.0%, 35.0%, 12.8% and 12.0% were found in four patients.

Two patients died. One of these was a 42 year old man who developed tachypnea on the second hospital day after receiving

four doses of IV quinine at 12 hour intervals. He had a petchial rash on his face and limbs, gum bleeding and coughed up bloody sputum. His lungs were clear on auscultation and he was not orthopneic. His parasite count was 0.4%. The other patient was a 63 year old man with cerebral malaria and a parasite count of 35%. He became anuric, failed to respond to fluid loading and mannitol with furosemide and died before dialysis could be initiated. He received a single IV dose of 600 mg quinine in a four hour infusion.

Several patients developed mild nausea and vomiting and two patients developed sinus bradycardia which returned to normal sinus rhythm on stopping the quinine.

Serum Quinine Levels: Preliminary results are available. Patient No. 1 was aged 13 and weighed 29 kg. He received 1200 mg quinine (a double dose) as his first dose. The serum quinine peaked at 18 mg per L at four hours after dosing and then decreased rapidly with a half-life of about six hours. The patient received a total of four doses of intravenous quinine every 12 hours followed by a dose of pyrimethamine with sulfadoxine (Fansidar). Following the last dose of quinine his serum level peaked at 12 mg per cent. Despite the satisfactory levels of serum quinine obtained, he returned with a positive smear on day 15.

Patient No. 22 was anuric. His serum quinine concentration peaked at 17 mg per L following the first dose of quinine. Despite the anuria, the serum quinine then decreased rapidly with an approximate half-life of about eight hours. When the serum quinine levels have been completed, and overall analysis will be presented.

18. Evaluation of Experimental Antimalarial Drugs in Rhesus Monkeys Infected with Plasmodium cynomolgi

OBJECTIVE: To evaluate the effectiveness of selected experimental drugs against Plasmodium cynomolgi malaria in rhesus monkeys. Results of these studies in subhuman primates are used in the U.S. Army antimalarial drug development program to guide the design of further animal experiments and to aid in the selection of drugs for human trials.

DESCRIPTION: These are a continuation of studies initiated in 1971, and reported with details of methodology in the SEATO Medical Research Laboratory Annual Reports for 1972-1974. These studies include an evaluation of blood schizontocidal activity of candidate compounds in rhesus monkeys inoculated intravenously with 5×10^8 parasitized erythrocytes obtained from donor monkeys infected with Plasmodium cynomolgi strain B. Test drugs are

administered daily by stomach tube for seven days beginning four days after the parasite inoculation. Suppression of parasitemia is indicative of blood schizonticidal activity, and monkeys in which parasitemia fails to reappear for one month after splenectomy at 30 days are considered cured. Drug tolerance studies, in which a minimum tolerated dose is established and major toxic effects characterized, are also conducted in rhesus monkeys.

This year pilot studies to establish a rhesus monkey test system to evaluate candidate compounds for causal prophylactic or radical curative activity have been initiated, using methodology patterned after that developed in other laboratories. Techniques for infecting Anopheles balabacensis with Plasmodium cynomolgi strain B and for reproducibly infecting rhesus monkeys with infective sporozoites are being refined.

RESULTS:

Blood Schizonticidal Tests: This year 19 experimental antimalaria drugs were evaluated for blood schizonticidal activity. Minimum curative doses are indicated in Table 1. A number of the newer 8-aminoquinolines, particularly WR 182232 have greater activity against blood schizonts and are less toxic than primaquine. Two novel compounds, WR 194965 and WR 204165 have excellent antimalarial activity. A study performed with a formulated mixture of sulfadiazine and WR 158122 (a 2,4-diaminoquinazoline) suggests that these compounds are synergistic in combination. Minimum curative doses for the individual components of the mixture were 100 and 1.0 mg/kg respectively.

Tolerance Tests: Drug toxicity studies were conducted with six compounds. Results are summarized in Table 2. Hepatic toxicity has been encountered in two 8-aminoquinolines (WR 181023 and WR 182232), and in two benzamidines (WR 4931 and WR 199385).

Sporozoite Induced Tests: The suitability of Anopheles balabacensis for mass production of infective P. cynomolgi strain B sporozoites has been established. In a series of preliminary experiments, this mosquito has been demonstrated to be hardy and an aggressive feeder on rhesus monkeys. Engorgement rates have regularly been above 85%, and 90% or more of the engorged mosquitoes have developed moderate to heavy sporozoite infections. Baseline studies with premaquine are in progress, and the testing of experimental compounds in prophylactic and radical curative regimes in the sporozoite - induced system will be initiated in the coming year.

Table 1. Summary of Blood Schizonticidal Tests in Rhesus Monkeys

Type of Compound	WRAIR Drug Number	Minimum Curative Dose (mg/kg/day)
4-Aminoquinoline	1544	10.0
8-Aminoquinoline	2975 (Primaquine)	NC[1] (31.6)
	6020	NC[1] (100.0)
	181023 (lot 1)	10.0
	181023 (lot 2)	31.6
	182232	3.16
	182234	10.0
4-Quinoline-methanol	184806	10.0
9-Phenanthrene-methanol	181018	31.6
4-Pyridine-methanol	182231	10.0
Sulfonamide	4629 (Sulfalene)	100.0
	4873	NC[1] (100.0)
Miscellaneous	5473	3.16
	5949 (Trimethoprim)	100.0
	25979 (Nitroguanil Hydrochloride)	31.6
	190830	100.0
	194965	3.16
	204165	3.16
Combination Study	7557 (Sulfadiazine) 10:1 158122	1.0:0.1

[1]Not curative. The compound had suppressive activity, but did not cure at the maximum dose tested. The maximum tested dose is indicated in parentheses.

Table 2. Summary of Drug Tolerance Studies in Rhesus Monkeys

Compound Number	Maximum Tolerated Dose (mg/kg/day)	Principle Toxic Effect
WR 4931	< 3.16 (I.M.)	Liver Damage
WR 172435	< 316 (Oral)	Emesis
WR 181023	< 10 (Oral)	Liver Damage
WR 182232	< 31.6 (Oral)	Liver Damage
WR 184806	10 (Oral)	Emesis
WR 199385	< 3.16 (I.M.)	Liver Damage

SUMMARY: Six antimalarial compounds have been evaluated for toxicity in rhesus monkeys, and 19 for blood schizonticidal activity against P. cynomolgi strain B. Sporozoite-induced test systems are being developed to permit the evaluation of causal prophylactic and radical curative activity of antimalarial drugs in rhesus monkeys.

19. Relationship between Erythrocytic Adenosine Triphosphate (ATP) Level and Human Malaria

OBJECTIVE: To establish a quantitative assay of ATP in human erythrocytes for the determination of normal erythrocytic ATP levels in a Thai population and to determine its relationship to malaria infection.

BACKGROUND: It is known that there is considerable variation in the levels of erythrocytic ATP between individuals in a population, and that this level is constant in healthy individuals (96, 97). Comparative studies in American Negroes and Caucasians indicated the existence of different mean quantities of erythrocytic ATP between these two groups. Since the gene pool of the American Negro is derived from an African Negro stock exposed to malaria for many generations, the lower mean levels of ATP in this group suggests selection pressure caused by malaria. Further studies revealed that there is a strong positive correlation between the erythrocytic level of ATP and P. falciparum parasitemia (98). In human as well as simian infections, high ATP levels were directly associated with relatively high peak parasite counts (99). It has been suggested that the protective mechanism against malaria infection may result from the following:

a. Erythrocytic ATP levels of the host play an important role in supporting the initial increase of parasitemia. With the lower level of ATP, a retardation of the primary increase in parasitemia is seen, resulting in a less severe clinical course of infection.

b. The role of ATP in maintaining metabolism and viability of living cells indicates that erythrocytes with low ATP levels are less capable of maintaining their viability. This would result in the inability of the intraerythrocytic asexual parasites to develop completely, and the parasitized erythrocytes may rupture prematurely.

The purpose of this study is to investigate erythrocytic ATP levels in Thai populations continuously exposed to malaria infection, and to compare them with populations from nonendemic areas.

DESCRIPTION: A technique for quantitative assay of erythrocytic ATP utilizing a firefly luminescence method described by Stanley and Williams (100) was utilized. A calibration curve of ATP was obtained by adding an aliquot of fresh extract of desiccated firefly lanterns to various known concentrations of ATP in phosphate buffer pH 7.4. The resulting light pulses were counted in the liquid scintillation spectrometer.

In most experiments, heparinized blood was used for the quantitative assay. Immediately after venipuncture, the blood was precipitated with trichloroacetic acid and maintained at - 70°C for assay of ATP. It was found that delaying precipitation of the blood resulted in a significant decrease in ATP levels (101). To prevent this loss ACD solution at pH 5 was used as an anticoagulant (102) when immediate processing of blood specimens was not possible. One milliliter of blood was added to EDTA, mixed, and kept at 4°C (wet ice) for cyanmethemoglobin determination.

Populations from Bangkok and Lumpoon representing a nonendemic group were compared with an endemic group from Chonburi and Prachinburi. In addition to these two groups, a number of newborn infants from Women's Hospital in Bangkok was included in this study as a control.

RESULTS: As shown in Table 1 the mean erythrocytic ATP level of the nonendemic group was 3.78 micromoles/gram hemoglobin, and that of the endemic group was 3.47 micromoles/gram. In newborn infants, the ATP level was found to be higher than that of adults. This finding in newborn infants agreed with that reported by Gross et al (103), although the assay methods utilized were different. With the firefly luminescence technique, the results were found to be slightly higher than those produced by the hexokinase, G-6-PD technique. There was no significant variation of the mean levels of erythrocytic ATP in either group. Birthplaces and residential areas were used for comparison in this study; however, both groups shared a common gene pool. It appears that the quantitative level of erythrocytic ATP is under genetic control of a multifactorial type and this finding is more suggestive of a genetic control than an environmental control.

Table 1. A Comparison of Erythrocytic ATP Levels in Thai Populations Residing in Nonendemic and Endemic Malarial Areas

Subject Population	No. Case	Micromole ATP/gm Hb			Hb gm/100 ml.	
		Range	Mean		Range	Mean
Newborn						
Bangkok	120	3.2 - 7.3	4.83		11.2 - 18.5	14.96
Nonendemic Areas						
Bangkok	98	2.62 - 5.8	3.77	3.78	8.6 - 17.5	14.4
Lumpoon	84	2.66 - 5.0	3.79		7.6 - 17.3	13.7
Endemic Areas						
Chonburi	29	2.19 - 4.3	3.06	3.47	8.7 - 14.3	10.81
Prachinburi	198	2.91 - 5.2	3.89		6.6 - 17.3	13.64

Project 3A762759A829MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 336 Field studies on drug resistant malaria

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6495	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	337
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Synthesis of Antimalarial Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012100 Organic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (in thousands)
FISCAL YEAR	PRECEDING 75	7.2	350
	CURRENT 76	7.2	350

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3551

20. GENERAL USE

Foreign intelligence not considered

21. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Rothe, W. E., COL

NAME:

22. KEYWORDS (Provide EACH with Security Classification Code)

(U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Chemical Syntheses

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective is to manage, integrate, and provide technical direction for both a contract and in-house program to obtain potentially active antimalarial compounds for military use through rational organic syntheses.

24. (U) Necessary research areas are defined, proposed research evaluated, ongoing research guided, evaluated, and integrated with the other program elements. Technical advice is obtained through an Ad Hoc Study Group on Medicinal Chemistry. Contract research progress is monitored through site visits and reports and information exchanged by contractors through technical meetings.

25. (U) 74 07 - 75 06 The solid leads uncovered in the area of prophylactic and radical curative agents have continued to be exploited. Tentative structure/activity generalizations can now be made with respect to such activity in the 6- and 8-amino-quinolines, quinaldines and lepidines. One lepidine, WR-181,023, is targeted for an IND in FY-76. Although synthetic work in the aminoalcohol area has been terminated for over a year, the key compounds that emerged have been proceeding through advanced screening. An IND has been prepared on one quinolinemethanol, WR-184,806, and two pyridinemethanols are targeted for INDs, one, WR-180,409, in FY-77 and the other, WR-172,435, in FY-78. A Mannich base, WR-194,965, which emerged from the synthesis program in this area, now terminated, is targeted for a FY-77 IND. Other than the radical curative agents mentioned above the active synthesis areas are of a probing nature; while many active compounds have been uncovered no clinical candidates have emerged. During the year 675 compounds were obtained as the result of the rational synthesis program, of which 319 were targets. An additional 5465 were submitted under the no-dollar agreement as well as 561 gifts and 233 purchased. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 337 Synthesis of Antimalarial Drugs

Investigators:

Principal: Thomas R. Sweeney, Ph.D.
Associate: COL William E. Rothe, VC; Richard E. Strube, Ph.D.; Edgar A. Steck, Ph.D.; Bing T. Poon, Ph.D.; June A. Page; Daniel L. Klayman, Ph.D.; LTC Peter S. Loizeaux, VC; 1LT Thomas S. Woods, CmlC; SP5 David H. Jun; Ruby N. Boyd; SP4 Joseph Bartosevich; CPT John A. Scovill, Ph.D.; SP4 Linda M. Cutts.

The Contract Chemical Synthesis Program

Although synthetic work in the aminoalcohol area has been terminated for over a year, three of the key compounds that have emerged from that work have been proceeding through advanced screening. An IND has been prepared on one quinolinemethanol, WR-184,806, and two piperidine-methanols are targeted for IND's, one, WR-180,409 in FY-77 and the other WR-172,435, in FY-78. The synthesis in the Mannich base area was also terminated at the beginning of this year and the best compound from that effort, WR-194,965 has been started along the development path with an IND targeted for FY-77.

At the end of FY-75 there were fourteen active synthesis contracts to be carried into FY-76. One new contract is expected to be let as a result of an approved proposal. During the year nine contracts were terminated. In addition to the above-mentioned contracts, three preparations laboratories, used chiefly to resynthesize, on a larger scale, selected compounds that are needed for testing in large animals, toxicological studies, and clinical investigations were active during the year and will be carried into FY-76. Also active was one contract to synthesize radioactively tagged compounds and one to analyze and confirm purity and identity of compounds and compositions to be used in preclinical and clinical studies. The cooperative agreement for the screening of compounds continued to be a fertile source of new compounds. Six new agreements were consumated during the year. Twenty-four papers in the chemical literature were published during the year as a result of work done in the synthesis program.

The most intensive area of research during the year has been concerned with prophylactic and radical curative compounds. The solid leads that had been uncovered have been undergoing development and it is now possible to make tentative structure/activity generalizations with respect to the aminoquinolines. Although activity can be demonstrated in the 6-aminoquinolines, lepidines, and quinaldines and the

8-aminonaphthyridine series, it is at a much higher drug level then that in the 8-amino analogs. The 7-aminoquinolines seem to be unpromising. The evaluations now underway plus the evaluation of a number of compounds that are now being synthesized should allow the selection of a few new IND radical curative candidates. An interesting modification recently begun and which has precedent in the 4-aminoquinoline series, is the introduction of an 8-aminoalkylhydrazino side chain. In contradistinction to the modification of the side chain in the 8-aminoquinolines, there is being synthesized a number of compounds in which the ring system will be modified. The new ring systems will include naphthalene, isoquinoline, quinazoline, benzimidazole, and cinnoline. The first one synthesized, the 5-methoxynaphthalene analog of pamaquine, appears to be of sufficient interest to warrant development of the lead. Finally selected compounds are being resolved into their optical antipodes because of the distinct difference in toxicity exhibited by the antipodes of primaquine.

Synthesis in the area of folate antagonists has continued to be phased out; only those areas which represent work in the pipeline or seem to have exceptional promise are to be continued. Thus the following synthesis areas were phased out during the year: 1. 2,4-diamino-6-[(anilino)methyl]pyrido[2,3-d]pyrimidines; 2. 2,4-diamino-6-[(benzyl)methylamino]pteridines; 3. 6-[(anilino)methyl]-2,4-pteridinediamines; 4. 2,4-diamino-6-[(heterocyclic)methyl]pteridines; 5. 2,4-diamino-6-[(aryloxy and arylthio)methyl]pteridines; 6. 1,3-diamino-[1]benzothieno-[3,2-f]quinazolines; 7. 1,3-diamino-12H-[1]benzothiopyrano[3,2-f]quinazolines; and 8. 2,4-diamino-5,6,7,8-tetrahydroquinazolines. Compounds in the above areas numbered 1, 4, 5, 6, and 7 were largely devoid of activity. Most of the compounds in area 3 were inactive but there were exceptions. Area 2, on the other hand, yielded highly active compounds, and one, WR-199,361, proved to be one of the most active compounds known against P. berghei in mice. No further work will be done in areas 2 and 3 unless such is indicated as the result of testing against drug resistant strains. Also phased out during the year was work on the 3-amino-7-arylthio (and 7-benzylamino)-1,2,4-benzotriazines because of the poor activity of the target compounds. The high inhibitory power of the quinazoline analog of methopterin against thymidylate synthetase, a tetrahydrofolate-dependent enzyme, plus its good oral activity against P. berghei, prompted the synthesis of compounds in which the glutamate moiety was incorporated into side chain of the 6-arylthio-2,4-diaminoquinazoline structure. Conversely, folic acid analogs were synthesized in which the glutamic acid portion of the molecule was altered. Neither of these types of compounds, unfortunately, were active. The only research projects remaining active in the antifol area are small efforts in the 1,3-diamino-14H-naphtho[2',3';5,6]thiopyrano[3,2-f]quinazolines, the 5,7-diamino-3-[(anilino)methyl]pyrimido[5,4-e]-as-triazines, the 1,3-diamino-7-benzyl-8,9-dihydro-7H-pyrrolo[3,2-f]quinazolines and the 1,3-diamino-7-benzyl-7,8,9,10-tetrahydropyrido[3,2-f]quinazolines. Some work is also continuing in the modification of the amino group in

some of the more active 2,4-diamino-6-substituted quinazolines.

The synthesis of purine nucleosides as potential antimalarial agents based upon the modest but definite antimalarial effects of Cordycepin, Ara-A, and the N^6-methyl-$N^{\prime}$-nitroso derivative of Ara-A has been largely phased out. Research had concentrated on modification of the groups on N^6 in several series of nucleosides, viz., those in which the sugar moieties were ribosyl, arabinosyl, 3-deoxyribosyl and 2-deoxyribosyl. In addition, a sizable effort was devoted to the synthesis of C-nucleosides. Unfortunately, the unpromising biological test results in this area along with the difficult and expensive chemistry involved militated against continuation of the research. One project which will continue into the next year in order to get the desired target compounds is concerned with the synthesis of 3'-alkyl cordycepin analogs. The alpha isomers have been synthesized; the beta isomers should be forthcoming during the coming year.

A synthesis program to develop the appreciably high suppressive activity of Clindamycin and its 1'-demethyl-4'-despropyl-4'-pentyl analog was terminated during the year. The reported side effects of Clindamycin, the activity of a private company in the area and the difficulty of the chemistry all indicated that the costs would outweigh the benefits if the work were to be pursued.

Work on the synthesis of potential hypoxanthine-guaninephosphoribosyl transferase inhibitors as antimalarial agents has been progressing during the year and will continue.

Synthesis in the 2,5-bis-(4-guanylphenyl)furane and cyclic congenor area has been halted. While the compounds were quite active against T. rhodesiense the activity against P. berghei was disappointing.

Probing actions are continuing in the area of the 1,1'-(ethanediylidenediimino)-bis[2-(substituted phenyl)guanidines] and related compounds, the (2-benzimidazoyl)guanidines, the 3-amino-6-aryl-1,2,4,5-tetrazines and the 3-amino-5-aryl-4H-1,2,4-triazoles. The tetrazines, as a class, are showing interesting, although not outstanding, activity.

Because it was thought that additional work would be warranted in several classes of compounds which had been investigated to a limited extent earlier, syntheses of a number of amino-trichloromethyl-as-triazines, -pyrimidines, and -thiadiazoles has been started.

A synthesis project which yielded a large number of mesoionics allied to purinone was terminated because of lack of activity of the target compounds.

The project for the synthesis of oxazinediones will continue into next year with special emphasis on the synthesis of fluorinated compounds related structurally to the active 5-fluoroorotic acid.

Work on the synthesis of amino acids and dipeptides as isoleucine and methionine antagonists has been terminated because of the lack of activity of the target compounds.

The Preparations Laboratories

During the year, for the purposes mentioned earlier, 21 compounds were requested from the preparations laboratory and 26 received. Of the 21 requests, 5 were for quantities of 1 kg or more, 7 for quantities of 100 to 999 grams and 9 for quantities of 1 to 99 grams. Of the 26 compounds received 4 were in quantities of 4 kg or more, 8 in quantities of 100 to 999 grams and 14 in quantities of 1 to 99 grams. With respect to radiolabelled compounds, 6 were requested during the year and 7 received. In addition, 20 samples of radiolabelled compounds were made available to contractors for antimalarial research.

The Analytical Laboratory

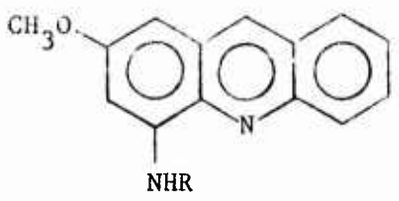
The analytical facility handled 50 requests for investigation of purity, identity and/or stability including bulk compounds and formulations.

Acquistion of Compounds

During FY-75 a total of 675 compounds were received as the result of the rational synthesis research, 319 of these were target compounds. In addition, 5465 compounds were received under the cooperative industry-government agreement, 561 were received as gifts and 233 were purchased.

The Organic Laboratory Synthesis Program

On the basis of their structural relationship to the radical curative 8-aminoquinolines, a project has been undertaken to prepare a series of 4-substituted-amino-2-methoxyacridines. In order to



accomplish this, the synthesis of 4-amino-2-methoxyacridine was developed which involved the condensation of 4-chloro-3-nitroanisole with anthranilic acid. The product, on treatment with phosphoryl chloride, yielded 9-chloro-2-methoxy-4-nitroacridine. Whereas, it was found possible to remove the chloro group via the p-toluenesulfonyl-

hydrazide and reduce the nitro group step-wise, a hydrogenation technique was devised in which both steps were consolidated. Because of the high insolubility of the 2-methoxy-4-nitro-9-(p-toluenesulfonyl-hydrazine)acridine in the reduction medium and the other production considerations, only limited quantities of the desired 4-amino-2-methoxyacridine could be prepared using the facilities at WRAIR. At present, the compound is being produced on a larger scale at the Cordova Chemical Co. Upon receipt by us, the amino group of the compound will be substituted with side chains common to the most active 8-aminoquinolines.

Based on a lead obtained in the screening program, a series of 13 additional thiosemicarbazones containing adamantyl, 2-, 3-, and 4-pyridyl, as well as aryl groups has been synthesized and submitted for testing. A recent subseries under investigation is based on 4-substituted thiosemicarbazides having novoldiamine and related chains. In this connection a novel method for the synthesis of thiosemicarbazones is being developed which involves the reaction of an S-methyl-thiocarbazate with an aldehyde or ketone followed by treatment of the resulting azomethine with an appropriate amine to yield a thiosemicarbazone.

The reaction of 2-amino-2-thiazoline with benzoylisothiocyanate was studied and was found to give three products, namely: 1-benzoyl-3-(2-thiazolin-2-yl)-2-thiourea, 2-benzamido-2-thiazoline thiocyanic acid salt, and a bicyclic compound, 6,7-dihydro-2-phenyl-4H-thiazolo[3,2-a]-triazine-4-thione. The latter is related to some triazine antimalarials and is now being screened.

Ten grams of arsphenamine was prepared for the schistosomiasis program.

An investigation into the scission of S-S bonds, begun under the antiradiation drug development program, was completed. It was demonstrated that sodium hydrogen selenide, generated by dissolving elemental selenium in sodium borohydride, is an extremely effective disulfide and organic thiosulfate cleaving reagent-superior to sodium borohydride alone. This technique affords selenium-free thiols in high yield from disulfide and thiosulfate precursors.

The synthesis of azaadamantane antimalarials was interrupted by Lt. Thomas S. Woods' separation from the Army in December 1974.

Data Processing

During the past year activity in data processing has centered around efforts to bring all data systems in-house and to up-grade these systems to take advantage of increased disc processing capabilities.

1. Inventory

At the start of FY-76, a new inventory system, which has been in the parallel processing stage, will be fully operational. The new system is totally disc oriented and will result in improved running time and fewer possibilities of error because of decreased tape handling. Other specific improvements include the printing of names and addresses on reports sent to submitters, the automatic generation of shipping lists for routine samples which reduces turnaround time and keypunch errors, the automatic generation of name and company sequenced submitter lists, the expansion of the P,C,S code to include, for both open and discreet compounds, codes for the return samples, blinds, and combination studies and the simplification of error correction procedures.

2. Chemistry

The conversion of the chemistry system to the CDC 3500, after many setbacks, has finally begun. Phase A is now in production, all input to the current system is in a format compatible with the new system. The final systems design has been completed and will be submitted for approval by the end of FY-75. In addition the initial conversion of the data base is scheduled for June 1975. Program development of the basic system is due to be completed by December 1975 with the remaining development and file building to be finished within fiscal 76.

3. Biology

The biology computer system continues to expand at a rapid rate. Test results on more than 12,000 chemical compounds have been added during FY-75. The system remains tape-bound for routine processing at the close of FY-75, however, the rewriting of the biology system programs for disc operation on the in-house computer has been completed. Parallel processing will start 1 July 1975. Standard codes have been established for major biological parameters thereby facilitating comparison of test results between systems, searches for specific types of test information, and statistical analysis of test results. Edits, updates and print reports for the many test systems have also been standardized.

Programs have been designed so that new test systems may be added without any major modifications to the operating system.

The interface of the new biology system with the current chemistry system is under development. The interface with the new chemistry system referred to above should be completed during FY-76.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 337 Synthesis of Antimalarial Drugs

Literature Cited.

Publications:

1. Klayman, D.L. and Woods, T.S.: Unequivocal Structure Assignment of the Products of the Reaction of 2-Amino-2-thiazoline and Its Analogs with Carbethoxy Isothiocyanate, J. Org. Chem., 39, 1819 (1974).

2. Griffin, T.S. and Klayman, D.L.: The Selenium Analogs of Biuret, J. Org. Chem., 39, 3161 (1974).

3. Woods, T.S. and Klayman, D.L.: Cleavage of Sulfur-Sulfur Bonds with Sodium Hydrogen Selenide, J. Org. Chem., 39, 3716 (1974).

4. Klayman, D.L. and Copeland, E.S.: The Design of Antiradiation Agents, in "Drug Design," Vol. 6, E.J. Ariens, ed., Academic Press, 1975, pp 82-142.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6435	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	338
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Protective Immunity in Malaria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	1.0	100
FISCAL YEAR CURRENT 76	1.0	100

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Immunity; (U) Antigens; (U) Protozoa; (U) Tropical Medicine; (U) Antibodies

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The objective of this work unit is to elucidate the protective mechanisms involved in immunity to malaria, a disease which has repeatedly impeded military operations, and to investigate the feasibility of immunoprophylaxis against this disease.

24 (U) The approach used in these studies is to study both in animal models and through the use of in vitro techniques the response elicidated by the malaria parasite on the immune system, and to determine the relative roles of cellular and molecular mediators in these processes.

25 (U) 74 07 - 75 06 Studies on mouse models have resulted in evidence that the effect of antibody on malaria parasites in vivo is profound but cannot eradicate the infection; antigenic variants arise which kill the animals. Experiments in primates also indicate a very profound effect of antibody but in this case the animals survive challenge. Monkeys with experimental malaria develop changes in complement components during the terminal development of disseminated intra vascular coaglulation. Cryopreserved malaria parasites have been cultured for up to several days. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1974 to 30 June 1975.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 338 Protective immunity in malaria

Investigators.
Principal: LTC Carter L. Diggs, MC, M.D.
Associate: MAJ David R. Ahr, MC, M.D.; J.P. Bingham; John Bussey; Louis T. Cannon, Sr., B.S.; James Dillon, B.S.; Barbara J. Flemmings, B.S.; CPT Fred Hines, VC, DVM; MAJ Herber Lindsley, M.D.; Al von Doenhoff, B.S.

1. Culture of Plasmodium falciparum

Objective. These studies are designed to develop methods for the culture of cryopreserved P. falciparum in erythrocytes in order that effector mechanisms of immunity can be explored in vitro.

Description. Methods have previously been developed for cryostorage of P. falciparum infected erythrocytes which allow retrieval from the frozen state with an acceptably low degree of lysis. (See WRAIR Annual Report FY 1974). These parasites are highly infectious and can synthesize protein in vitro in short term (5-6 hours) experiments. The present studies are concerned with extension of this period of in vitro functional integrity to allow maturation of parasites and invasion of new host erythrocytes; development through the complete cycle (over a period of about 48 hours) will be necessary to explore the effects of mediators of immunity an schizonts and merozoites.

Progress. Protein synthesis proceeds in an almost linear fashion for at least six hours in the absence of serum, but then rapidly falls off and has essentially stopped by 16 hours (Table I). The influence of human and fetal calf serum is also indicated in Table I, and it is clear that human serum allows a very substantial improvement in the capacity of the cells to synthesize protein with the period of linear increase in counts extending up to 48 hours. Fetal calf serum is very much less effective. The activity is absent in cultures containing chloroquine.

Discussion. Extension of the period during which P. falciparum can synthesize encourages optimism that development of the cryopreserved parasites through the complete cycle may be attainable. Additional studies must be performed to determine if this is the case.

2. Relationship of antigenic variation to the efficacy of antibody mediated protection against P. berghei.

Objective: These studies were conducted to explore the possibility

that multiple doses of antibody can prevent fatality in P. berghei infections in mice.

Description. Immune serum for these experiments was obtained from random bred Walter Reed mice immunized with irradiated P. berghei parasitized erythrocytes and challenged with non-irradiated parasites which had survived the resulting low grade parasitemia for thirty days thereafter (See WRAIR Annual Report FY 1974 for details of the immunization method). Experimental mice were infected with $2x10^4$ non-irradiated parasites IV and at the same time given 0.5 ml of immune (or normal) serum IP. The serum treatment was repeated daily for nine days; parasitemia and mortality were monitored daily.

Progress: Immune serum exerted a protective effect as measured by both the prepatent period and the time to death (Table 2). However, 100% mortality was observed regardless of treatment. Of great interest is the fact that some animals exhibited parasitemia even during the period when immune serum was being administered daily. To study the antigenic specificity of the parasites causing death in animals which received immune serum, the experiment was replicated using the same serum but parasites isolated from an immune serum treated mouse. The results are shown in Table 3. It is evident that prepatency and time to death was similar in the two experimental groups.

Discussion. These experiments demonstrate the failure of immune serum developed in response to immunization and chronic infection to eradicate parasites of the homologous strain in spite of its clear cut antiparasitic effect. It is suggested that this failure is not due to a quantitative deficiency since parasites arose in passively immunized animals in spite of continued serum treatment of the same intensity as that which delayed onset of patency. A shift in antigenic specificity of the parasites during the course of infection in passively immunized animals was demonstrated. The mechanisms which allow survival of actively immunized mice in spite of the development of antigenic variants which kill passively immunized animals are largely unknown. Their elucidation is of central importance to an understanding of immunity to malaria.

3. Complement components Plasmodium falciparum infections.

Objective: This study was designed to determine if an alternate complement pathway is activated during acute malaria in owl monkeys. These animals develop disseminated intravascular coagulation (DIC) and it has been proposed that this syndome might be the result of a chain of events initiated by activation of components of the complement system.

Description. Eight monkeys were used in this preliminary study. Radial immunodiffusion assays for C3, C4 and C3 proactivator (C3PA),

assays of hemolysis of unsensitized sheep erythrocytes by monkey serum pretreated with cobra venom factor (a measure of C3PA and earlier components of the properdin system), assays of clotting factors, and platelet counts were performed on all animals. Four monkeys were then infected with P. falciparum and the same parameters monitored over their short course of infection prior to fatal termination.

Progress. Serum concentrations of C3 and C3PA appeared to rise slightly between prebleeding and early parasitemia and to then undergo a modest fall as parasitemia increased. No systematic change in C4 concentrations were discerned. There was considerable variation in the values obtained for individual monkeys so that taken as a group, there was no clear cut difference in the range of values for C3, C4 and C3PA in infected vs control animals or in animals before vs after infection (Table 4). The apparent concentrations of all three components were higher than those observed in human sera, most likely because the antisera used were prepared against human components; these cross reacting antisera would be expected to require more antigen for equivalence than if homologous antisera were available.

All three animals which were assayed for cobra venom factor induced hemolysis showed depressed values. Two monkeys which each had 4 units of hemolytic activity had parasitemias of 6 and 24% whereas the animal with 2 units had 34% of the erythrocytes parasitized. No results were obtained on the fourth animal due to an insufficient volume of the serum sample.

All four animals had markedly depressed clotting factors and severe thrombocytopenia indicative of DIC.

Discussion. The observed fall in cobra venom induced hemolytic activity indicated that these monkeys developed abnormally low concentrations of one or more components of the properdin system coincident with heavy parasitemia. The less impressive changes in C3PA concentrations as estimated immunochemically might indicate the presence of a circulating inactive product of C3PA. A similar explanation might apply to the apparent lack of change in the concentrations of C3 and C4; previous studies of other malarias have clearly demonstrated decreases C4 although C3 seems to be spared in the instances where it has been studied. Gel diffusion experiments to assess the possibility of qualitative antigenic changes are planned. If altered components are present, their possible association with circulating immune complexes and/or erythrocyte stromata will be suggested. The mechanism by which the properdin system is altered in the monkeys cannot be determined from the data available. Activation by erythrocyte stroma, through immune complex activation of the classical pathway or by direct activation through immune complexes must all be considered.

Although DIC and alternate complement pathway activation seem to coexist in these animals, no evidence for a causal relationship between the two has been obtained. Studies to provide additional evidence relevant to alternate pathway activation are planned. If the results of these studies confirm those already obtained, it will be of great interest to determine whether or not depletion of complement components prior to infection can block the development of DIC.

Table I

Effect of serum on protein synthesis by P. falciparum in vitro

hours of culture	mean CPM ± 95% confidence limits Serum		
	None	Fetal Calf	Human
0	140 ± 90	100 ± 50	70 ± 20
5	1820 ± 60	1340 ± 170	1570 ± 250
16	2630 ± 70	2720 ± 210	4780 ± 320
24	2730 ± 70	2890 ± 430	5540 ± 330
48	2620 ± 70	3670 ± 150	10950 ± 1360

Table 2

Delay in development of P. berghei ("wild") in mice given homologous immune serum

Treatment	Prepatent period days Median (range)	Time to death, days Median (range)
Immune Serum	5.5 (4-7)	18.5 (17-22)
Control Serum	*4	11.5 (11-14)

*All became patent on day 4

Table 3

Lack of delay in development of P. berghei ("variant") given serum from mice immune to P. berghei ("wild").

Treatment	Prepatent Period days Median (range)	Time to death, days Median (range)
Immune serum	2.9 (2-3)	16 (14-18)
Control serum	*3	16.5 (10-19)

*All became patent on day 3

Table 4

Ranges of values of selected parameters of the complement system in owl monkeys injected with *P. falciparum*

Day of Infection	-16 to -9		3		6-10	
Assay	Controls	Infected	Controls	Infected	Controls	Infected
Cobra venom factor induced hemolysis, units/ml	8-17	7-20	6-18	7-16	9-12	2-4 (3)
C3PA* mg%	240-890	230-620	310-590 (3)	310-760	320-1010	190-620
C3* mg/%	180-380	210-540	200-540	310-540	200-340	140-320
C4*	30-50	27-48	40-45 (3)	22-53	32-57	15-52
%Parasitemia	___	___	___	<1	___	1-39%

*Based on the use of human serum standards (See text).

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 338 Protective immunity in malaria

Literature Cited.

Publications:

1. Diggs, C.L. and Osler, A.G.: Humoral Immunity in Rodent Malaria III: Studies on the Site of Antibody Action. J. Immunol. 114: 1243, 1975.

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PROJECT 3A162110A830
MILITARY DOG IMPROVEMENT

Task 00
Military Dog Improvement

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6441	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NA	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A830	00	055
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Development and Evaluation of Improved Biological Sensor Systems

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

001700 Animal Husbandry 01180 Operations

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
07 00	CONT	DA		C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	5	265
FISCAL YEAR CURRENT 76	5	280

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

ASSOCIATE INVESTIGATORS

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NAME: Lees, CPT,G.E.; Leighton, 1LT E.A.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Detector dog; (U) Selective breeding; (U) Mines; (U) Trip Wires; (U) Ambush

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To better protect the combat soldier by genetic development of a more intelligent and sensually acute detector dog that is free of hip dysplasia and is temperamentally better suited for detecting the presence of the enemy than is now generally available.

24. (U) Critically evaluated AKC registered German Shepherd Dogs were purchased as foundation stock. The progeny of these and subsequent generations are closely evaluated by recognized tests designed to reveal the superior individuals. These are in turn used as breeders.

25. (U) 74 07 - 75 06 Thirty nine litters produced 205 weaned puppies. Present kennel population is 210 dogs. During the year 211 dogs were transferred to such using agencies as the DOD Dog Training Center, Lackland AFB, the US Army Scout Dog Platoon, Ft. Benning, and WRAIR. An additional 23 dogs were retained as breeders. Continued progress in improving temperament, trainability and freedom from hip dysplasia was evidenced by our fourth generation dogs. Of the first 20 of these to reach one year of age, none were rejected for poor temperament or trainability; only four for hip dysplasia. Computer derived heritability estimates for hip dysplasia and temperament in this kennel were 19% and 70% respectively. These estimates will be used to appropriately weight the available information on an individual dog and its relatives in determining their breeding value. The frozen semen bank was tested by inseminating 16 bitches with reconstituted semen. Eight conceptions occurred resulting in 34 fetuses. Consultant visits were made by authorities in the fields of genetics and canine hip dysplasia.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval

DD, FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A162110A830 MILITARY DOG IMPROVEMENT

Task 00 Military Dog Improvement

Work Unit 055 Development and evaluation of improved biological sensor systems

INVESTIGATORS.

Principal - COL Merida W. Castelberry, VC
Associates - MAJ Jeffrey M. Linn, VC
CPT Thomas G. Nyland, VC
CPT George E. Lees, VC
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OBJECTIVE. To better protect the combat soldier by genetic development of a more intelligent and sensually acute detector dog that is free of hip dysplasia and is temperamentally better suited for detecting the presence of the enemy than is now generally available.

BACKGROUND. Despite the large number of pet dogs in the nation, the acquisition of suitable working dogs for military purposes is always a problem. This is especially true during wartime because of the greatly increased demand. This project was authorized during the Vietnam conflict for the purpose of developing a line of more proficient military working dogs and to assist in relieving the shortage of acceptable dogs.

APPROACH. Critically evaluated AKC registered breeding stock purchased especially for this purpose was selectively bred to produce superior progeny. These are in turn closely evaluated by recognized tests designed to reveal the superior individual. Linebreeding combined with progeny testing of each generation is being used to accomplish the objective.

PROGRESS.

A. Breeding Program.

1. Forty litters produced 208 weaned puppies.

2. Present kennel population is 205 animals.

3. Disposition of 234 dogs was made as follows:

Walter Reed Army Institute of Research	137
DOD Dog Training Center, Lackland AFB, TX	45
Retained for Breeding	23
US Army Scout Dog Platoon, Ft. Benning, GA	12
US Customs	6
Civilian Police Departments	4
The Seeing Eye, Inc.	3
US Army Mobility Equipment Rsch and Dev Center	2
US Capitol Police	1
Letterman General Hospital	1

B. Special Projects.

1. The continuing puppy diarrhea study being conducted with Veterinary Division, Walter Reed Army Institute of Research (WRAIR), revealed that German Shepherd puppies on high starch diets demonstrated:

a. chronic diarrhea
b. frank starch in feces
c. malabsorption of xylose
d. mucosal injury of small intestine
e. many trichomonads and giardia

German Shepherd puppies on starch-free diets showed:

a. no diarrhea
b. no mucosal injury of small intestine
c. rarely trichomonad or giardia presence in feces
d. malabsorption of xylose

A gluten-free diet did not lessen diarrhea, the intestinal lesions, or enable the gut to normally absorb a pentose. The diarrhea was not a result of a gluten enteropathy. Diarrhea and mucosal injury was eliminated in puppies on starch-free diets, suggesting either a carbohydrase deficiency or a mucosal injury from by-products of starch hydrolysis. However, dramatic malabsorption of xylose was evident in puppies on starch-free diets, suggesting impaired functioning of the small intestine not evident by morphological changes. The frequent occurrence of trichomonas and motile giardia in abnormal stools was interpreted to be a result rather than a cause of the diarrhea, since numerous giardia were seen in duodenal washings of Shepherd pups as well as inbred hounds. The diarrhea was not related to overgrowth in the small intestine of the predominant aerobic bacteria, entrobacteriaciae and entrococci. For detailed report see 1975 annual report of Veterinary Division, WRAIR.

2. The canine liquid nitrogen frozen and stored semen bank, established in 1973, was tested by inseminating 16 test bitches with reconstituted semen. Eight conceptions occurred. Four of these were spayed 30 days following their last insemination to permit their shipment to the DOD Dog Training Center at Lackland AFB, TX. Twenty viable fetuses were present in these pregnancies. The other four pregnant bitches were permitted to whelp normally with a total of 14 puppies being delivered. These are being raised and evaluated for military service in the same manner as their naturally conceived peers.

The two oldest collections tested, from different males, had been stored for 505 and 462 days respectively. Although the motility and morphology of the sperm cells from each dog were quite good, conception did not occur in either trial. The remaining 14 collections tested were from eight other males and were in storage an average of 41 days. Two pregnancies, by different males, resulted from semen frozen for 66 days.

3. We are attempting a surgical repair of canine hip dysplasia using part of a procedure described by Brinker (1). Since we radiograph our dogs at 5, 8 and 11 months of age, we are able to detect early onset of dysplastic signs such as joint laxity and initial osseous remodeling. The procedure consists of osteoctomizing the greater trochanter, reattaching it slightly distal and caudal to its former position, and utilizing the normal pull of the gluteal muscles to further seat the femoral head in the acetabulum. A preliminary series of ten surgeries is planned with four recently completed.

4. The canine panosteitis study being made in collaboration with Veterinary Division, Letterman Army Institute of Research (LAIR) was continued. A clinically positive male dog was sent to that organization for their examination and subsequent addition to their panosteitis breeding colony.

C. Veterinary Medicine. Preventive medicine remains an integral part of the total health care program. An essential element is the immunization program. At two weeks of age, a puppy receives measles vaccine to provide early cross-immunity to canine distemper and distemper-hepatitis, leptospirosis vaccine is administered at 11 and 14 weeks of age, and annually as a booster thereafter. Two changes from last year's program have been made to provide increased protection. First, a six week's vaccination has been added since puppies are taken out of the compound to be socialized earlier. In addition we have changed to an improved leptospirosis vaccine giving a longer duration of immunity. The rabies vaccination program consists of modified live virus vaccine given at thirteen weeks, one year, and every three years thereafter. A vaccination at six months has been dropped and annual boosters extended to every three years in view of latest national recommendations. A comprehensive parasite control program is maintained to detect, treat, and eliminate external and internal parasites. Emphasis is placed on interrupting the life cycle of the parasite by rigid sanitation procedures and avoiding the use of drugs where possible. Common external parasites include the demodectic mange mite and the American Dog Tick, while internal parasites are Giardia, coccidia, and roundworms. We are experiencing an increase in heartworm indidence in our colony which may call for prophylactic medication in the near future.

Major surgical procedures during the past fiscal year include 45 ovario-hysterectomies, one caesarean section, one fracture repair, one patent ductus arteriosus repair, one elbow hygroma removal, one ununited anconeal process repair, one intestinal torsion repair and two intestinal intusssusception. In addition, six intestinal biopsies and four hip surgeries were conducted as part of research projects. Consistent with the policy to continually improve health care services, the Division acquired a blood chemistry analyzer to add to diagnostic ability and monitor progress during treatment. In addition we now have the capability of testing for Brucella canis and performing an improved test for the detection of canine heartworm.

D. Genetics. To provide reliable information for the critical evaluation of the breeding program, a thorough statistical analysis of hip scores and intermediate evaluation scores has been obtained.

Radiographic evaluations on the hips of 875 German Shepherd dogs X-rayed between 1968 and 1975 were available for analysis. Generally radiographs were taken at 5, 8 and 11 months of age, but on some dogs only X-rays at the 5th month or 8th month were available. This because they were not considered further usually because of poor temperament. Each radiograph was evaluated by Dr. Wayne Riser of the University of Pennsylvania College of Veterinary Medicine. The interpretation of scores used to classify each hip is given in Table 1.

TABLE 1. INTERPRETATION OF HIP RATING SCORES

Score	Meaning
1	Grade IV
2	Grade III
3	Grade II
4	Grade I
5	Borderline
6	Near Normal
7	Less than Ideal but Within Normal Limit
8	Normal for Age and Breed
9	Excellent

Because each hip was graded independently of its opposite side, the lowest score of the two scores given to a set of hips was used as the individual's hip score for analysis. Also, only the most current radiograph was used.

Data for the analysis of intermediate evaluation scores was available on 443 dogs evaluated between 1971 and 1974. The intermediate evaluation score is a subjective evaluation of an individual dog's temperament given by Mr. Larry Liljedahl of this Division. The score is on a scale from 1 to 9 with higher scores reflecting more desirable temperament.

Least squares techniques for data with unequal subclass frequencies outlined by Harvey (2) were used to compute the appropriate analyses of variance. Heritability estimates were obtained by calculating components of variance using techniques described by Henderson (3).

Preliminary analyses of hip scores examined the importance of both fixed and random effects. Fixed effects considered were sex, age of the animal when radiographed (age), year-season in which the animal was radiographed (Y-S), and the animal's generation (gen). Two factor interactions considered among the fixed effects were sex by age, sex by Y-S, sex by gen, age by Y-S, and age by gen. Random effects considered were sires and dams within sires. All fixed main effects and interactions were non-significant except for age and the age by Y-S interaction. These two effects were eliminated from the final model, however, because there appeared to exist a correlation between sires producing dysplastic dogs and the age when the dogs were X-rayed. The model used for the analysis of hip scores was:

$$Y_{ijk} = \mu + s_i + d_{ij} + e_{ijk}$$

where Y_{ijk} = the hip score for the k^{th} dog produced from mating the j^{th} dam with the i^{th} sire

μ = the overall mean

s_i = the effect of the i^{th} sire

d_{ij} = the effect of the j^{th} dam when mated with the i^{th} sire

and e_{ijk} = the associated error

The analysis of variance and constant estimates of the expected mean squares for the hip score is given in Table 2. Effects due to sires and dams within sires both were significant ($P<.01$).

TABLE 2. ANALYSIS OF VARIANCE AND COEFFICIENTS FOR EXPECTED MEAN SQUARES FOR HIP SCORES

Source	df	Mean Square	Coefficients for Expected Mean Squares σ_e^2	$\sigma_{D/S}^2$	σ_S^2
Sires	23	11.3905**	1.0	5.1519	35.0351
Dams/Sires	166	5.3845**	1.0	4.5203	
Remainder	685	2.7259	1.0		

** P<.01

Preliminary analyses of the intermediate evaluation scores examined the importance of sex, year-season in which evaluated (Y-S), generation, handler and two factor interactions between sex and Y-S and between sex and generation as fixed effects. Random effects were sires and dams within sires. Results from the preliminary analyses showed that all of the fixed effects and the two factor interactions considered were non-significant (P>0.05). The model used to analyze the intermediate evaluation score was:

$$Y_{ijk} = \mu + s_i + d_{ij} + e_{ijk}$$

where Y_{ijk} = the intermediate evaluation score observed on the k^{th} dog produced from mating the j^{th} dam with the i^{th} sire

μ = the overall mean

s_i = the effect of the i^{th} sire

d_{ij} = the effect of the j^{th} dam when mated with the i^{th} sire

and e_{ijk} = the associated error

The analysis of variance and coefficients of the expected mean squares for intermediate evaluation are given in Table 3. Differences due to sires and dams within sires were significantly different (P<.01).

TABLE 3. ANALYSIS OF VARIANCE AND COEFFICIENTS FOR EXPECTED MEAN SQUARES FOR INTERMEDIATE EVALUATION

Source	df	Mean Square	Coefficients for Expected Mean Squares σ^2_e	$\sigma^2_{D/S}$	σ^2_S
Sires	12	25.1914**	1.0	5.8679	32.3829
Dams/Sires	73	4.9353**	1.0	5.0298	
Remainder	358	2.1786	1.0		

** P<0.01

Using the mean squares and the corresponding coefficients for the variance components, estimates of the variance components for both the hip scores and the intermediate evaluation scores were calculated. The variance component estimates are given in Table 4 along with the associated estimates of heritability for both traits.

TABLE 4. ESTIMATES OF COMPONENTS OF VARIANCE AND HERITABILITY

Source	Hip Score		Intermediate Evaluation Score	
	Variance Component	h^2 [1]	Variance Component	h^2
Sires	0.1641	0.19	0.6115	0.73
Dams	0.5660	0.66	0.5481	0.66
Remainder	2.7259	- --	2.1786	- --

[1] Calculated from $4(\text{Variance Component})/\sum_{i=1}^{3} \text{Variance Component}_i$

To help interpret the results of the analyses of Tables 2 and 3, an examination of the variance components is necessary. Falconer (4) and others have shown that the sire component of variance is an estimate of one-fourth the additive genetic variance while the dams within sires component of variance estimates one-fourth the additive genetic variance plus one-fourth the dominance genetic variance plus the variance due to common environment. Algebraically these equalities may be represented by:

$$\sigma^2_S = 1/4\ V_A$$

$$\sigma^2_{D/S} = 1/4\ V_A + 1/4\ V_D + V_{Ec}$$

where V_A = additive genetic variance

V_D = dominance genetic variance

V_{Ec} = variance due to common environment

A large difference between σ^2_S and $\sigma^2_{D/S}$ where $\sigma^2_{D/S}$ is larger than σ^2_S indicates that the effects due to common environment plus dominance are quite large. This difference is very apparent in the analysis of hip scores where the difference between the $\sigma^2_{D/S}$ and σ^2_S components is more than twice as large as the σ^2_S component. Maternal effects usually arising from the influence due to a common litter environment seem to be very important in explaining the variation observed in hip scores. A separate analysis of weaning weight as a measure of the dam's milking ability is now being completed to help examine the source of the maternal influence in the variation of the hip score. The heritability estimate for hip score, using only the sire component of variance to estimate the additive genetic variance, is then 0.19.

With regard to the intermediate evaluation score, however, Table 4 shows that the $\sigma^2_{D/S}$ component is actually smaller than the σ^2_S component indicating that the effects other than additive genetic effects are not important in explaining the observation. This observation then allows the averaging of the sire and sire components of variance to estimate the additive genetic variance. The heritability estimate for intermediate evaluation score from this analysis is then 0.70.

Work is now being completed, in cooperation with the Division of Biometrics, WRAIR, to use the heritability estimates for both hip scores and intermediate evaluation scores in estimating the relative breeding values of individual animals to the overall breeding program. Due to the relatively low heritability of the hip score, all available information on an individual's relatives - maternal and paternal half-sibs, full sibs, and progeny - must be combined to provide the best possible estimate of the individual's breeding value. WRAIR's CDC 3500 is now being programmed to provide this information. On the other hand, the relatively high heritability of the intermediate evaluation score makes an individual's own phenotype the best predictor of his genotype. Consequently, only the individual's own intermediate evaluation score will be used to estimate the breeding value for intermediate evaluation.

E. Puppy Evaluation. Experience is emphasizing the value of exposing puppies up to 12 weeks of age to novel situations. Accordingly, a section of three enlisted personnel was set up whose function is to "socialize" and evaluate three week old to twelve week old puppies. Agreement was reached with the Child Day Care Center at Edgewood Arsenal to bring these young puppies to the center and let the children who have parental consent and

a signed release play with them. As part of the effort to expose the puppies to crowds of people, three transportable pens have been obtained. At the morning and noon rush hours the pens are set up at congested areas and people encouraged to play with and to pet the puppies.

Evaluation of the individual dogs sensual acuity, steadfastness, and trainability is a continuing effort from the thirteenth week to the eleventh month of age. This is accomplished by means of rag play, line agitation, simple building search, and field scouting problems.

G. Visits.

1. One hundred and eighteen visitors were received by this organization during the period of this report. They included the Deans of the nation's veterinary schools, the United Kingdom Military Attache, and members of various civilian law enforcement canine corps officials. A Thailand Army Veterinary Corps officer made a 30-day training visit.

2. Consultant visits were made by Dr. W. H. Riser (hip dysplasia) and Mr. E. A. Hart (breeding and blood lines).

H. Equipment - An Ames BMD Blood Analyzer was received.

DISCUSSION. Two outstanding late third generation litters were whelped during late June 1974. These whelpings produced 12 dogs which are now 13 months old. Without exception they have almost perfect hips, outstanding working dog temperament, and are quick and willing to learn. These are the first litters in which all of the members met all the requirements of this project to the degree represented by these animals. Two males and six females have been retained for breeding purposes. The remaining four were males which were sent to the DOD Dog Training Center. Two of the bitches recently whelped a total of eight puppies. A third is now pregnant. These breeders and their progeny are expected to play a significant role in the future development of this project.

The frozen semem successes are the first reported in the German Shepherd Dog breed, and among the first reported in dogs. Although the question of long term storage of frozen semen remains open, short term storage and the ability to air ship frozen semen appears practical. Tight controls and sanction by the American Kennel Club would, of course, be required.

The new programming for the computer gives an added dimension and capability not previously available to the breeding program of this organization. The multiplicity of problems associated with selectively breeding against hip dysplasia, poor temperament, low intelligence, and genetic defects can now be solved much more accurately and far more quickly than heretofore.

PRESENTATIONS. Presentations concerning the work of this Division were made by an associate investigator to the organizations listed below:

A. American Association for Laboratory Animal Science (National Capital Area Branch), Edgewood Area, Aberdeen Proving Ground, 11 February 1975.

B. American Association for Laboratory Animal Science (national Capital Area Branch), Richmond, VA, 13 May 1975.

CONCLUSION. Man's ability to successfully breed for a desired result is well known. The development of a truly superior military working dog is progressing well.

Project 3A162110A830 MILITARY DOG IMPROVEMENT

Task 00 Military Dog Improvement

Work Unit 055 Development and evaluation of improved biological sensor systems

Literature Cited.

References:

1. Brinker, W.O., Flo, G., and Cross, J.F. 1968. Acetabuloplasty as Corrective Surgical Procedure in Selected Cases of Congenital Hip Dysplasia in the Dog. Sci. Proc., 35th Annual Meeting, AAHA, 260-262.

2. Harvey, W.R. 1960. Least-Squares Analysis of Data with Unequal Subclass Numbers. ARS 20-8.

3. Henderson, C.R. 1953. Estimation of Variance and Covariance Components. Biometrics 9:226

4. Falconer, D.S. 1960. Introduction to Quantitative Genetics. Ronald Press Company. New York, New York.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6444	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADE	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A830	00	056
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with security classification code)

(U) Diseases of Military Animals

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 005900 Environmental Bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	6	150
FISCAL YEAR CURRENT 76	3	412

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security classification code) (U) Military Dogs; (U) Ehrlichia canis; (U) Tropical Canine Pancytopenia; (U) Babesia; (U) Coronavirus; (U) SV5; (U) Canine Respiratory Disease

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23. (U) To define, study, diagnose, and control known and potential infectious diseases of military dogs. A major effort is directed toward the etiology, pathogenesis, treatment and control of canine ehrlichiosis and related diseases which jeopardize the military dog program. Additional studies concern the epidemiology, diagnosis, treatment, and control of other disease agents affecting the military dog.

24. (U) Conventional epidemiological, pathological, and microbiological methods are employed; unconventional procedures are developed as needed.

25. (U) 74 07 - 75 06 The cell-mediated immune (CMI) response following infection with E. canis was greater in inbred hounds than in German Shepherd dogs. German Shepherds that developed severe chronic ehrlichiosis did not respond to as great a degree as other infected dogs. Antilymphocyte serum administration caused no detectable influence on the CMI or humoral responses. A serologic survey of dogs in Phoenix, AZ, showed 11.5% to have ehrlichial antibodies, with the incidence rate being highest in German Shepherds (23.8%). The first case of naturally occurring ehrlichiosis above the 38th parallel in the US was detected. Parainfluenza SV5 was serologically detected in 81% and W191R canine calicivirus in 10% of sick dogs during two epizootics of respiratory disease at Lackland AFB. Bordetella bronchiseptica and Mycoplasma sp. were isolated during an outbreak of respiratory disease among puppies at Edgewood Arsenal. Chronic diarrhea in adult German Shepherds was associated with dramatic 24 hrs loses of Na^+ and water, but apparently unrelated to enteric pathogens or digestion of starch, fat, or protein. The condition in German Shepherd puppies was associated with malabsorption and complicated by inadequate starch hydrolysis. A parvovirus was isolated from some of these puppies, but the significance of the virus was not established. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 74 - 30 June 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498-1 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A162110A830 MILITARY DOG IMPROVEMENT

Task 00 Military Dog Improvement

Work Unit 056 Diseases of Military Animals

Investigators:
Principal: LTC Edward H. Stephenson, VC
Associate: Leonard N. Binn, PhD; COL Earl W. Grogan, VC; LTC Paul K. Hildebrandt, VC; MAJ John L. Brown, VC; MAJ Richard D. deShazo, MC; CPT John R. Boyce, VC; CPT George E. Lewis, Jr., VC; Joseph V. Osterman, PhD; Ernest S. Windham, MS; Albert R. Warner, Jr.; Irene E. Hemelt, AB; Ruth L. Marchwicki, BS; Raymond E. Hayhoe; COL Merida W. Castleberry, VC; SP5 Arshad Hussain; SP5 Dolores White; SP4 Richard Federman; SP4 Denise Peak; PVT Eva L. Kuc; PVT Lorraine M. Rusnov

Description.

To define, study, diagnose, and control known and potential infectious diseases of military dogs. A major effort is directed toward the etiology, pathogenesis, treatment, and control of canine ehrlichiosis (tropical canine pancytopenia) and related diseases which jeopardize the military dog program. Additional studies concern the epidemiology, diagnosis, treatment, and control of other disease agents affecting the military dog.

During the reporting period, research activities have included: (1) the immune response to Ehrlichia canis in experimentally infected dogs, (2) a retrospective epidemiologic survey of an apparent Ehrlichia canis epizootic in Phoenix, Arizona, (3) Ehrlichia canis infection in a dog in Virginia, (4) respiratory disease in recruit military dogs, (5) diarrhea in military dogs, (6) viral studies of the diarrhea syndrome in military dogs, and (7) respiratory disease in puppies of the Biosensor military dog colony. Some of the investigations reported were done jointly with Dr. Miodrag Ristic and other investigators at the University of Illinois, Urbana, IL.

1. Immune response to Ehrlichia canis in experimentally infected dogs.

Dogs infected with Ehrlichia canis developed a humoral antibody response, as determined by the indirect fluorescent antibody (IFA) test (49), but the response did not prevent persistent infection. Antibodies have been detected as early as 7 days post inoculation

and continued to increase for months following infection (13).

Treatment with adequate doses of tetracycline has been shown to be effective in clearing persistent E canis infection in dogs. However, dogs cleared of the infection were fully susceptible to reinfection and disease with the homologous strain despite high titers of circulating antibody.

Infections with E canis also were characterized by hypergammaglobulinemia (13,14,64) and plasmacytosis (32,33,60). On the basis of such findings Burghen et al. suggested that changes seen in severe ehrlichiosis may have an immunopathologic basis (14). Buhles et al. concluded that the continued increase in serum antibodies in conjunction with the development of hypergammaglobulinemia and plasmacytosis probably reflected persistence of the organism and possibly represented a mechanism analogous to the excessive production of immunoglobulins in Aleutian disease of mink, lymphocytic choriomeningitis, African swine fever, and equine infectious anemia (13). The development of severe chronic ehrlichiosis, however, can not be explained entirely on the basis of immunopathologic findings. Other factors are apparently involved since dogs with mild chronic ehrlichiosis also had elevated levels of antibodies and serum gammaglobulins. When chronically infected dogs were treated with tetracycline and subsequently reinoculated with the homologous strain of E canis, the antibody response of dogs that had had severe chronic disease differed from those that had had mild chronic disease. Also, there were differences in the clinical and hematologic responses. Dogs that previously had severe chronic disease responded to challenge as if they previously had not been exposed to E canis, whereas dogs that had had mild chronic disease appeared to have a degree of resistance to E canis (13). These findings indicated a degree of immunologic unresponsiveness in dogs that developed severe chronic ehrlichiosis, which possibly involved the cell-mediated immune system.

The purpose of this investigation was to analyze the role of the cell-mediated immune system in the pathogenesis of canine ehrlichiosis.

The isolant of E canis used was originally recovered from a German Shepherd dog naturally infected in Southeast Asia (3) and had been maintained by passage in laboratory-maintained inbred hounds. Passage of the isolant in splenectomized dogs showed the agent to be free of Babesia spp. and Hemobartonella spp.

A total of 20 dogs were used, 15 were German Shepherd dogs and 5 were inbred hounds. Two of the German Shepherd dogs previously had been infected with E canis and subsequently cleared of the agent by

tetracycline therapy. The remaining dogs had not been exposed to E canis, as determined by a negative serologic response. Three of the German Shepherd dogs were used as uninfected controls.

Each of the principals was inoculated intravenously with 5 ml of fresh, whole blood obtained from a dog in the acute phase of ehrlichiosis.

All of the dogs were examined and rectal temperatures were measured daily. Blood samples were obtained every day for hematological evaluation. White blood cell (WBC) and red blood cell (RBC) counts were performed with an electronic cell counter. Thrombocyte counts also were performed electronically. Packed cell volumes (PCV) were determined by standard microhematocrit methods. One hour erythrocyte sedimentation rates (ESR) were determined in Winthrobe tubes. Hemoglobin was determined by the cyanmethemoglobin method. Routine differential cell counts were done on blood smears stained by a modified Wright's technique. Buffy coat smears were stained by the Giemsa method for detection of E canis morulae.

Prior to inoculation with E canis all of the German Shepherd dogs were sensitized to 2-4-dinitrochlorobenzene (DNCB) using a modification of the procedure of Joseph et al. (40). Each dog was anesthetized with sodium thiamylal (Surital, Parke-Davis, Detroit, MI) and an area on the medial aspect of the left thigh, close to the popliteal lymph node, was shaved and cleaned with alcohol. A sensitizing dose of 2,000 μg of DNCB dissolved in propylene glycol at 60 C was injected intradermally. Challenge inoculations were made 35 days later. Aliquots of 25, 50, and 100 μg of DNCB dissolved in acetone were applied on the surface of the skin on the medial aspect of the same thigh, using plastic rings to confine the substrate until the acetone had evaporated. Reactions were observed 24, 48, and 72 hrs after application and the final reaction was graded after 72 hrs, according to the method of Frey et al. (29). A second challenge dose was administered 60 days after inoculation with E canis. Positive reactions consisted of areas of induration and erythema measuring 0.6 to 1.9 cm in diameter at the test site.

Each of the German Shepherd dogs was sensitized to the Bacillus Calmette-Guerin (BCG) prior to inoculation with E canis. The BCG preparation used was lyophilized (Bacillus Calmette-Guerin, lot number IL 74 (S) 21, Institute for TB Research, Chicago, IL) and the ampule was estimated to contain 2 to 8 x 10^8 viable units. It was reconstituted to 2 ml with sterile distilled water. A sensitizing dose of 0.1 ml was injected intramuscularly in the right thigh about 2.5 cm distal to the popliteal lymph node. Thirty-five days later each dog was challenged with intradermal injections

of 1:100, 1:500, and 1:1,000 dilutions of Old Tuberculin (OT) (Tuberculin, Old, Lot 995511D, stock 4-491-1, Parke-Davis, Detroit, MI). The reactions were observed 24, 48, and 72 hrs post injection. A second challenge injection series was given 60 days after inoculation with E canis. A reaction was considered to be positive when an area of induration and erythema measuring 0.6 to 1.9 cm in diameter developed at the test site 72 hrs after injection.

The German Shepherd dogs were inoculated intradermally with 0.1 ml of E canis antigen prior to and 60 days after inoculation with E canis.

Ehrlichia canis in the 72nd passage in primary monocyte cultures was used for preparation of specific antigen for the leukocyte migration inhibition (LMI) test. When approximately 90% of the culture cells contained E canis morulae, as determined by the Giemsa staining technique, each 30 ml Falcon tissue culture flask (Falcon Plastics Division, Bioquest, Los Angeles, CA) was frozen at -70 C. The contents of each flask were thawed at room temperature and the cell suspensions were pooled. Each flask was rinsed with 2 ml Eagle's minimum essential medium (MEM) and this volume was added to the cell suspension pool. The cell suspension was sonicated (Branson S-75 Sonifier Cell Disruptor, Heat Systems-Ultrasonics, Inc., Plainview, LI, NY) using a power output of 100 watts for 2 5-second intervals interrupted by a 5-second period. The sonicated suspension comprised the antigen preparation and was stored in 2 ml aliquots at -70 C. Uninfected monocyte cultures were treated in a like manner and were used for control purposes.

Leukocytes for the LMI test were harvested from 40 ml of blood obtained from each dog. The blood was drawn into a syringe that contained 500 units of sodium heparin, U.S.P., and 20 ml of 3% dextran sulfate in 0.85% NaCl. The syringe was placed in a vertical position with the needle end up and the erythrocytes were allowed to settle for 1 hr, then the plasma was removed and centrifuged at 1,000 x g for 20 min at 4 C. The cell pellet was washed twice in Hank's balanced salt solution. After the second wash, the cells were suspended in 40 ml of RPMI medium 1640 and the leukocytes were separated by using a Ficoll-Hypaque isopycnic gradient as previously described (58). Following centrifugation of the gradient at 400 x g for 30 min at 4 C, the leukocyte band was removed and the cells were washed twice in 40 ml of RPMI medium 1640 by centrifuging at 400 x g for 30 min. The final cell pellet was suspended in 11 ml of RPMI medium 1640 that contained 20% fetal calf serum, 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 0.25 µg of amphotericin B/ml. The final cell concentration was 2.3×10^7 cells/ml. Cell viability, as determined by trypan blue dye exclusion, was 98%. The cells were

divided into equal aliquots. E canis antigen, 1.0 ml, was added to 1 portion, while the second aliquot served as a non-sensitized control. Both aliquots were preincubated for 4 to 6 hrs in an atmosphere of 10% CO_2 at 37 C, according to the method of Gorski et al. (30). After preincubation the cells were centrifuged at 600 x g for 20 min. The supernatant was decanted and saved. Siliconized 25 µl capillary tubes were filled with cells and centrifuged, according to the method of David and David (21). Leukocytes from uninfected dogs were used as normal controls and were processed in the same manner. Two capillary tube stumps containing cells were placed in each chamber. Three to 4 chambers were prepared from capillary tubes containing sensitized cells and 3 to 4 were prepared from unsensitized cells. Migration chambers were incubated for 18 to 24 hrs in a 10% CO_2 atmosphere at 37 C. The leukocyte migration distances were read at 50X magnification with the aid of a micrometer. Mean percent migration in the presence and absence of antigen was determined. Percent inhibition of migration was calculated as previously described (50).

Four infected dogs, 2 German Shepherd dogs and 2 inbred hounds, were treated with antilymphocyte serum (ALS) (Antilymphocyte serum, lot 57-109F, Microbiological Associates, Inc., Bethesda, MD). Canine erythrocytes were isolated by the Ficoll-Hypaque technique and were added to the ALS. After a 1 hr adsorption period at 37 C, the erythrocytes were removed by centrifugation at 600 x g for 20 min. Adsorbed ALS was given intravenously on days 92, 95, 99, 102, 105, 109, and 112 post inoculation (PI) with E canis. Each dog received 10 mg of protein/kg of body weight/treatment.

Results of challenge reactions to DNCB, before and after inoculation with E canis, are given (Table 1). Seven of the 12 (58.3%) DNCB sensitized dogs were positive to the chemical before inoculation with E canis. Two of the 7 positive dogs converted to negative after infection. Three of the 6 dogs that were negative prior to ehrlichial infection had a positive reaction to DNCB following inoculation with ehrlichiae. All of the dogs sensitized to BCG, except dog 120, had positive skin reactions when challenged with OT prior to E canis infection; however, only 2 of the 12 dogs (16.7%) had positive reactions following infection. None of the dogs developed detectable skin reactions to E canis antigen, introduced intradermally, before or after inoculation with the agent.

Each of the 17 dogs inoculated with E canis developed typical signs of ehrlichiosis 12 to 17 days PI and ehrlichial morulae were observed in mononuclear cells during the acute phase of the disease. Five of the dogs had thrombocyte counts below 50,000 cells/cmm of blood 90 days PI and were considered to have severe chronic disease, whereas the remaining 7 German Shepherd dogs and all inbred hounds had

thrombocyte counts greater than this value and were categorized as having mild chronic disease (Table 2).

Inoculated German Shepherd and inbred hound dogs each developed a humoral antibody response to E canis. Antibody titers ranged from 1:320 to 1:20,480 (Table 2). There were no detectable differences in IFA titers between the 2 breed of dogs. Similarly, no differences were observed between German Shepherd dogs with severe chronic disease and those with mild chronic disease.

Base line LMI values for the dogs prior to inoculation with E canis ranged from 15 to 18% inhibition of leukocyte migration. All 20 dogs were negative for migration inhibition in the presence of ehrlichial antigen before inoculation. Likewise, control antigen prepared from E canis-free dogs did not inhibit the migration of leukocytes. Positive inhibition reactions developed in 7 of the 12 (58.3%) German Shepherd dogs (Table 2). Most of the dogs developed the positive response by 28 days PI. Among the 5 dogs that developed severe chronic disease, 2 showed a cell-mediated response and had an average of 28.8% inhibition of leukocyte migration. Of the remaining 7 German Shepherd dogs, which had mild chronic disease, 5 showed a cell-mediated immune response with a mean inhibition of migration of 52.7%. Four of the 5 (80%) infected inbred hounds developed a positive response within 28 days PI and returned to preinfection baseline levels by 42 days PI. The average percent of migration inhibition was 62.0%.

The effect of ALS administration in E canis-free inbred hounds 416 and 466, E canis-inoculated inbred hounds 474 and 477, and E canis-inoculated German Shepherd dogs 118 and 123 are given (Fig 1,2,3). Administration of ALS did not affect the thrombocyte count in German Shepherd dog 118 but caused a transitory and inconstant thrombocytopenia in dog 123. In infected inbred hounds ALS administration resulted in a marked, but transitory thrombocytosis. A mild, fluctuating thrombocytopenia occurred in the 2 ALS-treated, uninfected inbred hounds.

Preliminary investigations for this study suggested that E canis possibly could induce immunosuppression in infected dogs since the organism infects and persists in the mononuclear cells, which are directly or indirectly involved in cell-mediated and humoral immune responses. Two antigens, DNCB and BCG, that were known to induce a cell-mediated immune (CMI) response were used to test this hypothesis.

Results of skin reactions indicated that there was a measurable diminution in the delayed hypersensitivity response to DNCB and old tuberculin (OT) of BCG in the sensitized German Shepherd dogs following infection with E canis (Table 1). Since less than 50% of the dogs

became negative to DNCB after infection with E canis, it appeared that specific immunosuppression was not induced by the infectious agent.

The clinical response of German Shepherd dogs to infection with E canis resulted in either a severe chronic syndrome or a mild chronic state, whereas inbred hounds only developed the mild chronic syndrome (13). An explaination has not been advanced for this divergence in clinical responses between and among breeds of dogs, although Smith (55) showed that the thrombocytopenia in canine ehrlichiosis in both breeds was the result of extensive platelet destruction. It was postulated that the immunologic response to ehrlichial infection differed in the inbred hound and German Shepherd dogs. A definite difference in the CMI response by the two breeds was demonstrated. Only 58.3% of the German Shepherd dogs elicited a response, while a positive response occurred in 80% of the inbred hounds, as shown by the LMI test. The increased percentage of inbred hounds developing a CMI response could explain the apparent differences in clinical responses between the two breeds of dogs. Only 2 of the 7 German Shepherd dogs that developed a positive CMI response were classified as having severe chronic ehrlichiosis, thus the variable CMI response in German Shepherd dogs may be the reason why some German Shepherd dogs proceed to the severe chronic form of the disease. German Shepherd dogs that were classified as having the mild chronic form and developed a CMI response had a mean migration inhibition of 52.7% as compared to a mean migration inhibition of 28.8% for dogs that were in the severe chronic phase of the disease. The mild chronic phase of the disease may be related to a more efficient immune response, while dogs that elicit a lesser CMI response proceed to the more severe form. The mean value for the LMI test in the inbred hound was 62.0%, which was slightly greater than that observed in the German Shepherd dogs with mild chronic disease (52.7%). Why some of the dogs infected with E canis did not develop a CMI response may be attributed to the fact that there is a severe pancytopenia in many cases of canine ehrlichiosis and few lymphocytes would remain in the vascular system to be effective in the LMI test.

It was postulated that administration of ALS to dogs, German Shepherd or inbred hounds, would cause a dog in the mild chronic phase of the disease to be converted to the severe chronic phase. ALS administration in 2 uninfected inbred hounds induced fluctuating degrees of thrombocytopenia, which was similar to the alterations induced by ALS in German Shepherd dog 123, that had mild chronic ehrlichiosis. These results agreed with the findings of Ward (62) that ALS treatment exerted a strong antithrombocyte activity. ALS administration in a German Shepherd dog with severe chronic

ehrlichiosis, on the other hand, did not produce noticeable anti-thrombocyte activity. Treatment of E canis-infected inbred hounds with ALS produced a marked, but temporary, thrombocytosis after the 3rd and 4th injections of ALS. Following the 4th injection, fluctuating degrees of thrombocytopenia were observed. The results for the 2 breeds are conflicting; therefore, interpretation is speculative.

ALS is a potent immunosuppressant of cell-mediated immunity and it has been shown to cause accelerated deaths in viral infections, enhance viral oncogenesis, and induce death in mice infected with Babesia microti (1,35,65). ALS also causes a diminution of the humoral immune response to thymus-dependent antigens, such as sheep erythrocytes and bovine serum albumin (43). Treatment of hamsters with Mycoplasma pneumoniae and rats with lymphocytic choriomeningitis using ALS has had beneficial results (47,57). Use of ALS in E canis-infected dogs did not have a detrimental or beneficial effect. None of the dogs in the mild chronic phase of the disease converted to the severe chronic phase. Further, alleviation of the thrombocytopenia in the inbred hounds was temporary and could not be considered to have been therapeutically useful.

The cell-mediated immune response in 12 German Shepherd and 5 inbred hounds infected with E canis was studied using the LMI test. Of the German Shepherd dogs, 58.3% developed positive responses, while 80% of the inbred hounds became positive. German Shepherd dogs that developed the severe chronic form of the disease did not respond to as great a degree. The CMI responses declined with time and had disappeared in most of the dogs by 147 days PI.

Serum antibody titers, as measured by the IFA test, increased with time and remained at elevated levels. Two German Shepherd dogs and 2 inbred hounds were given ALS for a 3 week period commencing at day 92 PI. There was no detectable influence of ALS on the CMI responses and IFA titers in either breed, and it was not possible to convert a dog with a mild chronic infection to the severe chronic form of the disease. The CMI response developed by most of the inbred hounds and some of the German Shepherd dogs was thought to have contributed to prevention of progression of the disease to the severe phase.

2. A retrospective epidemiologic survey of an apparent Ehrlichia canis epizootic in Phoenix, Arizona.

Canine ehrlichiosis primarily occurs in tropical and subtropical areas of the world and is caused by Ehrlichia canis. The agent has been isolated from dogs in Algeria (23), Southeast Asia (39), Aruba (Netherlands Antilles) (12), Puerto Rico (39), and the Virgin

Islands (39). Serologic diagnosis of ehrlichiosis has been made in dogs from various regions of the United States and the organism was isolated from dogs in Oklahoma (25), Arkansas (27), Texas (48), and Florida(39).

Previous diagnosis of canine ehrlichiosis in the United States has been limited to sporatic, isolated cases. On 15 November 1974, it was learned that approximately 20 dogs had been examined and treated in the Maryvale Animal Hospital, Ltd, Phoenix, Arizona, for varying degrees of epistaxis during the preceeding 60 days. Most of the dogs had many of the clinical signs and hematologic alterations typical of severe chronic ehrlichiosis. Also, there had been a severe tick infestation problem in the Phoenix area, which had abated during the week of 3 November 1974 due to a freeze and heavy snowfall.

Preliminary investigations verified the occurrence of ehrlichial infections in the Phoenix area. Serum samples were obtained from 3 dogs that had shown typical signs of canine ehrlichiosis. The samples were from a Cocker Spaniel, a German Shepherd-cross, and a Scottish Terrier. Each of the sera was serologically positive for E canis antibodies, as determined by the indirect fluorescent antibody (IFA) test (49).

Further evidence of the extensiveness of the disease in the Phoenix area was obtained when a military dog buying team from Lackland AFB, Texas, went to Phoenix, Arizona, to purchase recruit dogs for military use. A total of 54 dogs was purchased, transported to Lackland AFB training facilities and accepted for training as sentry dogs. Serum samples were obtained from the dogs on 4 March 1975 at the request of the Dept Vet Diag Svcs, Div Vet Med, for testing for E canis antibodies. Eleven dogs (20%) had positive antibody titers for E canis.

The purpose of this study was 4-fold: (1) define the extent of the canine ehrlichiosis epizootic that occurred in the Phoenix area; (2) correlate, to the extent possible, the number of cases of frank disease with the number of unrecognized or undiagnosed cases; (3) isolate the causative strain of E canis from 1 or more active cases; and (4) delineate an endemnic area of ehrlichiosis from which purchase of dogs for military use must be made only after extensive medical evaluation.

Blood samples for serum preparation were collected in the Phoenix area from dogs of various breeds and ages. Serum was obtained from dogs that had one or more episodes of epistaxis or other typical signs of ehrlichiosis. Also, serum was obtained at random from dogs that did not have a medical history which would indicate ehrlichial

infection. The sera were tested for ehrlichial antibodies by the indirect fluorescent antibody (IFA) test (49).

Sera were collected in 2 increments during a 2-month period. A total of 379 samples were obtained from 54 breeds of dogs throughout the Phoenix area. Serologic analysis has not been completed; therefore, only partial results are available and final analysis of the data must await additional testing, although certain trends seem to be emerging.

Results for 209 of the sera are shown (Table 3). A preponderance of the dogs that were seropositive were in the working group of dogs (16/104). Of these dogs, 15 of the seropositives were from German Shepherd or German Shepherd-mix breed dogs for an incidence rate of 23.8% in that breed (Table 4). Positive sera, however, were detected in each of the breed groups, involving a total of 9 breeds (Dachshund, Beagle, Rhodesian Ridgeback, Boston Terrier, Cocker Spaniel, Scottish Terrier, Poodle, German Shepherd, and Samoyed). The incidence rate in all breeds, less German Shepherd, was 6.2% (9/146).

The predominance of the seropositive sera were obtained from dogs that had been ehrlichiosis suspects (16/70). Only 8 of the 139 sera (5.8%) obtained from nonsuspect dogs had ehrlichial antibodies.

There was no significant difference in the incidence of seropositive sera from males (13.0%) as compared with females (9.6%). The age of the dogs that were positive ranged from 12 weeks to 13 years of age. The occurrence of seropositive dogs was distributed over the entire Phoenix area, with no predominance of cases in any one sector.

A susceptible German Shepherd dog was inoculated with whole blood obtained from 1 of the German Shepherd dogs purchased in Phoenix and transported to Lackland AFB. The inoculated dog developed a typical, acute infection with E canis and the causative organism was isolated. The infected dog did not progress past the mild chronic form of the disease.

Previous investigations have shown that once a dog is infected with E canis the infection persists (13,26,38,53), unless a therapeutic regimen of tetracycline (30 mg/lb body wt/day for 14 days) is administered (3,13). Based on the results of this survey and those of the 54 dogs procurred for military use, it would appear that approximately 20% of the German Shepherd dog population in the Phoenix area was infected with ehrlichiae as compared to an apparent incidence rate of 6% in the remainder of the breeds combined. A possible explaination for the increased incidence of disease among German Shepherd dogs is the diminished cell-mediated immune response that occurs in German Shepherd dogs when infected with E canis (see pre-

vious section). A reduced ability of dogs of this breed to respond may allow ehrlichiae to become established and induce disease more readily, as compared with other breeds of dogs.

It also was interesting to note that of the 15 seropositive German Shepherd dogs 10 were suspected of having ehrlichiosis and 5 were nonsuspects. The suspect cases probably were in the severe chronic phase of the disease since they were exhibiting typical signs of severe chronic ehrlichiosis. Nonsuspect dogs would have to be considered to have been in the mild chronic phase of ehrlichiosis. Previous investigations in this laboratory (unpublished data) have shown that approximately 66% of the German Shepherd dogs experimentally infected with E canis progress to the severe chronic state within 100 days post inoculation and 33% of the German Shepherds and all of the inbred hounds infected do not progress past the mild chronic phase. Thus, survey data obtained to date corroborates the laboratory findings in German Shepherd dogs. The observation of 6 seropositive dogs among the suspect cases of the remaining breeds and 3 seropositive dogs in the nonsuspect cases was not expected. Two explainations for the occurrence of more apparent cases of severe chronic ehrlichiosis than mild severe cases can be forwarded. One, the strain of E canis infecting dogs in the Phoenix area may be more virulent than ehrlichial strains previously encountered, although this hypothesis is doubtful as the German Shepherd dog inoculated to isolate the ehrlichial strain involved did not develop severe chronic ehrlichiosis. One additional consideration, however, must be taken into account when evaluating this initial hypothesis. Enhanced virulence may require passage of the ehrlichiae through the vector, Rhipicephalus sanguineus, whereas artificial transmission via a syringe may result in decreased virulence. Two, the combination of infection with E canis and the high, dry altitude of the Phoenix area may have resulted in an increased number of cases of epistaxis than had been observed in tropical and subtropical areas.

More definite conclusions possibly can be made upon completion of the survey. Nevertheless, a significant proportion of the dogs in Phoenix, Arizona, were serologically positive for E canis antibodies and each dog that was positive must be considered to be infected. This reservoir of persistently infected dogs provides an endemic source of ehrlichiae for future epizootics of ehrlichiosis. The military significance of such an endemic area was exemplified when the military dog buying team bought the 54 German Shepherd dogs in the Phoenix area. Following completion of training, the dogs would have been transferred to bases and posts worldwide; therefore, a reservoir for potential epizootics elsewhere would have resulted, which could have caused serious biopolitical implications.

A serologic survey of dogs in the Phoenix, Arizona, area was made. Preliminary results showed 24 of 209 dogs (11.5%) had ehrlichial antibodies, as determined by the IFA test. The incidence rate was highest in German Shepherd dogs (23.8%) as compared to 6.2% in the remaining breeds of dogs. A significant difference in disease rates between sexes was not observed. Dogs 12 weeks to 13 years of age were seropositive. The causative agent was isolated by subinoculation of whole blood from a seropositive dog into a susceptible German Shepherd dog.

3. Ehrlichia canis infection in a dog in Virginia

Canine ehrlichiosis was diagnosed in a 9-year-old, female, Collie-cross dog that had been whelped in Northern Virginia and had not been transported outside the area. This was the first naturally occurring case of Ehrlichia canis infection detected above the 38th parallel in the United States. A detailed report of the case has been accepted for publication (56).

4. Respiratory disease in recruit military dogs.

For the past decade, studies have been performed on the etiology and epizootiology of respiratory disease in recruit military dogs. From 1966 to 1968, epizootics of respiratory disease occurred at the DOD dog procurement and training centers at Lackland AFB (LAFB), Texas, and at the scout dog training center at Ft Benning, Georgia (11,16). The outbreaks of disease affected approximately 25% of the dogs and seriously disrupted training and deployment. Parainfluenza SV5 was recovered from affected dogs and the virus was shown to be highly communicable (9). Although more than 80% of newly recruited dogs were susceptible to SV5 infection, the respiratory disease rate declined markedly from 1969 to 1972. In the summer and fall of 1973 epizootics of respiratory disease occurred in the recruit military dogs at LAFB and at Kadena AFB, Okinawa (Annual Progress Report, 1 July 1973-30 June 1974). Parainfluenza SV5 was recovered again from affected dogs and more than 75% of the dogs had a rise in specific antibody titer. In addition, a calicivirus, W191R, was recovered from the dogs at LAFB. Rises in antibody titer to W191R occurred only in dogs at LAFB during the summer of 1973.

Two additional epizootics of respiratory disease occurred at LAFB in 1974. This report summarizes studies on the etiology and epizootiology of these outbreaks. In addition, further information is presented on the incidence of SV5 antibody in newly recruited dogs.

In 1974, respiratory disease occurred in recruit military dogs at LAFB during the winter-early spring and late summer-early fall.

The last detectable case of disease was observed in October 1974. During each of these periods, approximately 20% of the dogs were affected and more than 80% of the sick dogs had a rise in titer to SV5 virus (Table 5). In addition, 10% (7/70) of the affected dogs had a rise in titer to the W191R canine calicivirus. All but 1 of the latter dogs also had a rise in titer to SV5 virus.

In each of the 2 disease periods, only a few cases of respiratory disease occurred within the first month. A SV5 infection was detected in 1 of the 2 initial dogs to become ill in each disease period. Only a few dogs developed respiratory disease initially, then there was a period of about 2 weeks before additional cases of disease developed. The majority (70%) of the cases occurred within a 2-week period in the latter portion of each outbreak. The rate of inapparent SV5 infections was also lower during the initial portions of each outbreak.

Studies on the incidence of SV5 antibody in newly recruited military dogs are summarized (Table 6). The percentage of dogs arriving with SV5 antibody increased from 9.4 to 10.3% in 1969 and 1970, recpectively, to a peak of 26.3% in 1973. The findings suggested an increased rate of SV5 infection in 1973. Each year dogs were received from more than 80% of the contiguous states with 1 or more SV5 seropositive dogs originating from 37% or more of these states. Over the 6 year period 15.9% of 1803 dogs were SV5 serotest positive. Dogs were received from all 48 contiguous states with 1 or more seropositive dogs coming from 39 states. The findings suggested that parainfluenza SV5 infections had occurred throughout the United States for many years. However, it is important to emphasize that approximately 85% of the recruit dogs were susceptible to SV5 infection.

Studies of the relationship between age and incidence of SV5 antibody in newly recruited military dogs are summarized (Table 7). There was a progressive increase in the percentage of seropositive dogs with age, from 3.7% in dogs under 12 months to 25.7% in dogs greater than 30 months of age.

The data reported provides additional evidence for the role of SV5 infection in respiratory disease of recruit military dogs. In 1974 more than 80% of the sick dogs had a rise in SV5 antibody titer. The etiologic significance of the W191R calicivirus infections in dogs with respiratory disease is unknown. However, W191R virus infections were detected in only 10% of the sick dogs and all but 1 of these dogs also developed SV5 antibody. In previous studies at LAFB, W191R infections also occurred at a lower rate than SV5 infections.

Over a 10 year period at LAFB and Ft Benning, Georgia, there has been an association between SV5 infection and epizootic respiratory disease in military dogs. Morever, serologic studies on newly recruited dogs have consistently indicated that nearly 85% of the dogs were susceptible to SV5 infection. These findings clearly indicated the need for a parainfluenza SV5 vaccine to prevent this respiratory disease in military dogs. Following the licensing of a parainfluenza vaccine in December 1974, a controlled field trial was initiated in May 1975 to evaluate this vaccine in recruit military dogs at LAFB. The study is being performed in conjunction with the veterinary staff at the DOD military dog center at LAFB.

Further studies were conducted on the etiology and epizootiology of respiratory disease in recruit military dogs at LAFB, Texas. During the year, 2 epizootics of respiratory disease occurred, which affected 79 dogs. Serologic test results indicated that SV5 virus infected 81% and W191R canine calicivirus infected 10% of the sick dogs. These results supported previous observations of the association of SV5 infection and epizootic respiratory disease in recruit military dogs. The results of serosurveys of newly recruited dogs from 1969 to 1974 indicated that more than 70% of the dogs arriving each year were susceptible to SV5 infections. The large number of SV5 susceptible dogs purchased each year and the occurrence of SV5 associated epizootic respiratory disease indicated the need for a SV5 vaccine. Studies are in progress to evaluate the safety and potency of a newly licensed SV5 vaccine in recruit military dogs.

5. Diarrhea in military dogs.

Studies began last year (Annual Progress Report 1 July 1973-30 June 1974) on diarrhea affecting a closed colony of 225 German Shepherd dogs at the Division of Biosensor Research, Aberdeen Proving Ground, MD. Mushy to watery stools were regularly observed in most dogs. The episodes of diarrhea had no clear cut onset or termination, and bacteria, chlamydiae, or viruses could not be implicated as the cause of the diarrhea. A coronavirus (1501R) was isolated from a fecal specimen of 1 of 15 puppies in 5 study litters. Serum antibody conversions or changes in titer to this virus by neutralization test were observed in mothers of all 5 litters and 11 of 14 study puppies within 3 months after weaning. Although 1501R is serologically related to another coronavirus (1-71), which has been shown to cause diarrhea in experimentally infected inbred hound puppies, there were no obvious clinical correlations between German Shepherd dogs with diarrhea and the coronavirus infection. Additionally, there were no clinical correlations with the known cycling of coccidial infections (Isospora rivolta followed by I. canis), nor

with any histopathology attributable to coccidia. Watery, frothy stools always were associated with large numbers of trichomonads and giardia. Sprue-like lesions were observed in the jejunum and particularly the ileum of affected dogs. Since endoparasites and microorganisms did not appear to be etiologically related to the diarrhea, feeding trials and stool analyses were conducted to further define the diarrhea.

In one feeding trial, 3 litters of German Shepherd puppies, at the time of weaning, were each placed on different diets. Each litter contained 5 puppies. One diet of 50% cooked rice and canned dog food lacked gluten, a protein in cereal grains which causes enteropathies in some humans, but not described for dogs. The second diet lacked starch, and contained by weight, one part each of fish meal, soy protein concentrate, dextrose, and soybean oil with 0.1 part cellulose and 0.15 part mineral/vitamin supplement. The third litter consumed the usual puppy diet (Annual Progress Report, 1 July 1973 - 30 June 1974), which included the constant availability of a dry, commercial puppy chow. Each litter was fed its respective diet twice daily. Stool consistencies were recorded daily, and stools were examined weekly for pH, parasites, starch, and fat. Weight gains were recorded weekly. The d-xylose absorption test was conducted as described by Hill et al. (34) when the puppies were 8 or 11 weeks of age. Plasma concentrations of d-xylose were assayed by the method of Roe and Rice (51). Results were compared with those obtained from similar tests conducted on 11, age-matched Beagles fed a commercial diet similar to that usually consumed by German Shepherd puppies. Approximately 1 week after the xylose absorption tests, enterotomies were performed on 2 puppies from each litter. Saline washes of duodenum, mid-jejunum, and terminal ileum were quantitatively cultures for evidence of overgrowth of "normal" enteric bacteria (enterobacteriaciae and enterococci). Similar titrations of intestinal washings were performed on 4 age-matched inbred hounds with firm stools. Biopsy pieces were removed from terminal jejunum and ileum and fixed in cold, buffered formalin for light microscopy.

Weekly weight gains from 4 to 8 weeks of age were greatest in the litter fed the usual diet with ad lib feeding (1 lb 6 oz to 2 lb 9 oz) followed by the rice-fed litter (7 to 14 oz), then the litter on the starch-free diet (6 to 12 oz). Stools were mushy, or watery and frothy from the litters on the starchy diets, whereas they were black, formed, and tarry from the liter on the starch-free diet. The ranges of mean, weekly pH values for the litters on the usual, rice, and starch-free diets were, respectively: 5.3 to 6.7, 4.6 to 6.1, and 5.8 to 7.1. Spirochetes, trichomonads, and giardia were numerous in mushy, watery stools. These parasites were rarely observed in the feces from the puppies on the starch-free diet. Other parasites, although occasionally observed, were

never numerous. Fat droplets were occasionally observed in stools from puppies on the starchy diets, but were not observed in feces from the puppies eating the starch-free diet. All puppies in each of the 3 litters showed abnormally low and delayed absorption of d-xylose when compared with age-matched inbred hounds similarly assayed (Fig 4b, 4c). Surprisingly, the puppies with the formed, parasite-free stools on the starch-free diet showed the poorest absorption of d-xylose. In contrast to the poor xylose absorption exhibited by the German Shepherd puppies, inbred hound puppies absorbed the sugar in a manner comparable to that described for adult Beagles and German Shepherds by Hill et al. (34) (Fig 4a). The inbred hound puppies showed an earlier rise in plasma xylose concentrations (30 min to 60 min vs 60 min to 90 min) compatible with the intubated vs ingested method of administration. When these dogs were compared on the basis of the mean total accumulated absorption of d-xylose (Table 8), there were no differences ($p > 0.5$) between the inbred hound puppies (220 mgm %) examined in this study and the adult Beagles (220 mgm %) and German Shepherds (210 mgm %) examined by Hill et al. (34). By contrast, there was a significant difference ($p < 0.01$) between these values vs those (141 mgm %) measured in the German Shepherd puppies.

Jejunal and ileal biopsies were taken from 2 German Shepherd puppies of each litter when they were 10 weeks old. Biopsies from the puppies on the two starchy diets showed: (1) reduced villous to crypt ratios ($< 1:1$), (2) lymphocytic infiltration in the lamina propria, (3) cuboidal epithelial cells, and (4) discharged goblet cells. By contrast, biopsies from the 2 German Shepherd puppies on the starch-free diet showed an intestinal morphology comparable to that observed in inbred hound puppies. The villous to crypt ratios were 2:1, the epithelial cells were columnar, the goblet cells were full, and the lamina propria was not remarkably infiltrated with lymphocytes.

Bacterial cultures of saline washes from the small intestines of the 6 German Shepherd and 4 inbred hound puppies showed no evidence of overgrowth ($> 10^8$ organisms per ml) with the predominant, aerobic enteric bacteria, enterobacteriaciae and enterococci. These bacteria in all puppies numbered from (1) 10^2/ml to 10^5/ml in the duodenum, (2) 10^2/ml to 10^7/ml in the jejunum, and (3) 10^2/ml to 10^8/ml in the ileum. Interestingly, motile giardia were readily observed in duodenal washes from all dogs.

In summary, German Shepherd puppies on high starch diets showed: (1) chronic diarrhea, (2) frank starch in the feces, (3) numerous trichomonads and giardia in the feces, (4) malabsorption of xylose, and (5) mucosal injury of the small intestine. German Shepherd puppies on the starch-free diet showed: (1) no diarrhea, (2) rarely

trichomonads or giardia in the feces, (3) no mucosal injury of the small intestine, but (4) very poor absorption of d-xylose. The gluten-free, rice diet did not lessen the diarrhea, the intestinal lesion, or enable the gut to normally absorb a pentose. The diarrhea was not the result of a gluten enteropathy. Diarrhea and mucosal injury was eliminated in puppies on the starch-free diet, suggesting either a carbohydrate deficiency in the German Shepherd puppies or mucosal injury from by-products of starch hydrolysis. However, dramatic malabsorption of xylose was evident in the German Shepherd puppies on the starch-free diet, suggesting impaired functioning of the small intestine not evident by morphological changes. The frequent occurrence of trichomonads and motile giardia in abnormal stools was interpreted to be a result rather than a cause of the diarrhea, since numerous giardia were seen in duodenal washings of all the German Shepherd and inbred hound puppies. The diarrhea was not related to overgrowth in the small intestine of the predominant, aerobic bacteria.

Since it appeared that diet profoundly affected the diarrhea in German Shepherd puppies, and reports by Kronfeld (42) and McCuiston (45) concluded that German Shepherds do not adequately digest dog food with a high cereal content, chemical analyses were performed on fecal samples from inbred hounds and German Shepherds for fat, protein, and particularly carbohydrate constituents as an indirect measurement of digestive function. Adult dogs were used in this experiment to avoid the complication of juvenile carbohydrase deficiencies reported for human infants (4,5,6,44) and other animals (17,22,31,63), and because mushy to watery stools persist in adult German Shepherds. Additionally, commercial diets contain coarsely ground grains, principally corn, which are frequently observed "undigested" in the feces. Consequently, 3 German Shepherd and 2 inbred hounds were fed pellets of the usual coarsely ground diet (Respond 2,000), and 4 German Shepherds and 2 inbred hounds were fed the same food after micronization to approximately 100 μ and re-pelleting. Analytical methods for crude protein and fat are described in the Official Methods of Analysis of the AOAC for Animal Feeds (37), and lactic acid was analyzed by a modification of the gas-liquid chromatographic extraction method from the same source. Starch was analyzed by a method used by the USDA meat analysis laboratories, and the hemicellulose analysis was a gravimetric modification from the same source (59).

Expectedly, stools from inbred hounds were formed and relatively firm, whereas those from German Shepherds were unformed and mushy. Both breeds showed surprisingly comparable digestive efficiency for fat, protein, starch, and hemicellulose, and both breeds more efficiently digested the fat and starch from the ground feed (Table 9). Concentrations of lactic acid, a colonic by-product of

carbohydrate fermentation by bacteria, were significantly higher in feces of German Shepherds (0.38% of fecal dry matter) vs inbred hounds (0.13% of fecal dry matter). Since lactic acid by itself is not thought to be contributory to diarrhea (2), the significance of the higher lactic acid concentrations in German Shepherd vs inbred hound stools is not clear. Several mushy stools collected from German Shepherds were subjected to additional carbohydrate analyses. Water soluble stool extracts not precipitable as starch showed no evidence of reducing sugars by Benedict's test before acid hydrolysis. After acid hydrolysis, reducing sugars were readily detected. These reducing sugars were glucose, a monomer of starch, and arabinose, xylose, and galactose, which are monomers of hemicellulose. Silica gel, thin layer chromatographic assays of both hydrolyzed and nonhydrolyzed stool extracts from German Shepherds did not reveal lactose or sucrose.

Results from this feed trial showed that adult German Shepherds with chronic diarrhea digested starchy diets as efficiently as inbred hounds without diarrhea, indicating that German Shepherds were not deficient in pancreatic α-amylase or intestinal glycosidases. Humans with congenital disaccharidase deficiencies pass stools of low pH, hith osmolality, and lactic acid concentrations and with detectable concentrations of disaccharide. Even though disaccharides were not detected in the stools of German Shepherds with diarrhea, the high concentrations of stool lactic acid suggested an abnormal intestinal fermentation of carbohydrate.

In conjunction with the constituent analyses of stools, investigations were started for the comparison of the carbohydrase activity of dogs suffering from diarrhea and of unaffected dogs. The starch degradative enzymes to be assayed are: 1-4 amylase E.C. 3.2.1.1, amyloglucosidase E.C. 3.2.1.3, maltase E.C. 3.2.1.20, isomaltase E.C. 3.2.1.20, and sucrase 3.2.1.26. A comprehensive literature search revealed many available analytical methods, most of which were unsatisfactory for comparative use because they had no standard or universal units of activity (7,8,15,24,28.41,46,61). Consequently, methods utilizing dye-starch conjugates as substrates were rejected. The Dahlquist assay (18) which was based on reducing sugars liberated from starch was chosen to measure the high concentrations of amylase present in the pancreas, and because its unit of activity was defined in accordance with recommendations of the Joint Sub-Commission on Clinical Enzyme Units of the International Union of Biochemistry and the International Union of Pure and Applied Chemistry. The sensitive glucose oxidase assay (19,54) based on the amount of glucose liberated by carbohydrate hydrolysis was chosen to detect the low levels of disaccharidases and amyloglucosidase present in intestinal mucosa, and because its unit of activity was also acceptable for comparative purposes. Initially, standard commercial enzyme preparations were

utilized to standardize assay conditions. Pancreatic and intestinal specimens have been obtained from "normal" non-German Shepherd dogs sacrificed in the course of experimental protocol authorized for other investigations.

Duodenum, jejunum, and ileum were measured and 6.0 cm sections were opened longitudially, gently washed with diluent (0.02 M $CaCl_2$ in 0.85% NaCl), and stored at -60 C in pre-chilled, labelled plastic bags. Frozen pancreatic specimens were weighed, minced in 20 volumes of diluent, sonicated for 2 min, diluted 1:10, and stored at -60 C. The mucosa was scraped from the frozen intestinal piece with a glass slide, weighed, minced in 4 volumes of diluent, sonicated for 1 min, appropriately diluted, and stored at -60 C.

For the Dahlquist assay, an appropriate dilution of specimen containing less than 2.8 units/ml was incubated at 25 C for 3 min with the starch substrate. The reaction was stopped by adding the 3,5-dinitrosalicylic acid color reagent, boiled for 10 min, cooled, and diluted with 5 volumes of distilled water. The optical density of the reaction mixture at 530 nm was compared with that of a 1, 2, and 3 mg maltose standard and corrected for background with a control mixture containing starch in distilled water.

For the glucose oxidase assay, an appropriate dilution of specimen yielding less than 100 µg of glucose was incubated at 37 C for 1 hr with the disaccharide substrate. After dilution with distilled water, the reaction was stopped by boiling for 2 min. An aliquot was incubated at 37 C for 30 min in the presence of the peroxidase-glucose oxidase enzymes and the o-dianisidine color reagent. The optical density of the reaction mixture at 450 nm was compared with that of a standard glucose series (0 to 100 µg). Boiled specimens were assayed for estimating contaminating tissue concentrations of glucose.

The Dahlquist assay, in our experience, has been relatively easy to conduct. Evaluated on specimens from 10 "normal" dogs, observed values have been reproducible within a two-fold range (Table 10, 11). By contrast, several technical difficulties required correction with the more sensitive glucose oxidase assay before satisfactory, reproducible results were obtained. Contaminating glucose in the starch substrate, and contaminating maltases in the glucose oxidase reagent yielded unacceptably high background levels of activity. Utilizing Tris buffer as an enzyme diluent and extraction of starch with isopropyl alcohol resolved these problems, and observed levels of enzyme activity were reproducible within 2 to 3-fold ranges.

Results showed high concentrations of α-amylase in the pancreatic specimens and low concentrations in the washed mucosa. Maltase and sucrase were present in similar quantities. It was noted that a 5:1 ratio of maltase to sucrase was frequently observed in adult animals (20,36). The difference between our results and those in the literature may be due to juvenile enzymatic incompetence. The low amyloglucosidase activity may reflect the same phenomenon. Enzymatic comparisons between adult German Shepherds and inbred hounds are planned.

The diarrhea of German Shepherds was further defined by comparing 3 successive 24 hr fecal specimens from 2 adult inbred hounds and 2 German Shepherds by total weight, and by the volume, osmolality, and electrolyte (Na^+, K^+, Cl^-, and HCO_3^-) concentrations of the stool water. Fecal specimens from these dogs had been examined previously for fat, protein, and carbohydrate constituents. The dogs were consuming Respond 2000, ad lib, and were watched and monitored constantly during the collection period. Stools were thoroughly scraped off of a clean, dry run immediately after voiding, placed in clean, tared, paint cans, stored at 4 C, and processed immediately after each 24 hr period. Firm inbred hound stools were mechanically mixed, centrifuged at 47,000 x g for 20 min, and the clear, dark amber supernatant was removed for analysis. The voluminous, runny German Shepherd stools were homogenized on a paint shaker for 30 min, and centrifuged at 1,000 x g for 30 min for preliminary clarification. This supernatant was re-centrifuged at 10,000 x g for 30 min, and the clear, light amber supernatant was removed for analysis. The osmolality of stool water was obtained by freezing point depression and was expressed as milliosmoles per kg of water (mosmol/kg). The Na^+ and K^+ concentrations were obtained by flame photometry and expressed as milliequivalents per liter (meq/l). The Cl^- concentration in meq/l was obtained by a chloridimeter, and the HCO_3^- concentration in meq/l was estimated by the formula: $Na^+ + K^+ - Cl^- = HCO_3^-$.

These observations showed a dramatic and significant difference between German Shepherds and inbred hounds (Table 12,13). Fecal volume, moisture, frequency, and electrolyte concentrations of inbred hound stools fall within expected normal ranges for dogs. Surprisingly, normal ranges for the osmolality of canine feces were not available for comparative purposes. Fecal specimens from German Shepherds were consistently 100 milliosmoles lower than those from inbred hounds, suggesting that the diarrhea was not principally osmotic. The moisture content of German Shepherd feces (81%) was consistently and significantly ($p < .01$) higher than that of inbred hounds (67%). The comparative loss of water and electrolytes between the two breeds on a 24 hrs basis (Table 14) was even more dramatic. The loss of water and Na^+, respectively, was 10-fold and 20-fold higher for German Shepherds than

inbred hounds. The greater loss in K^+ and Cl^- in stool water of German Shepherds may reflect that lost by solvent drag. The apparent dramatic loss of HCO_3^- in stool water of German Shepherds may be misleading since it was not measured directly. The HCO_3^- value is useful in indicating a substantial anion gap, probably filled by organic anions as well as HCO_3^-. These observed differences between German Shepherds and inbred hounds in 24 hr stool frequency, volume, water, and electrolytes represent the most distinguishing quantitative features of the diarrhea yet studied.

A number of helpful conclusions can be summarized from the observations on the diarrhea described above. Although enteric infections with coronavirus and coccidia occurred in rapid sequence in the weanling age puppy, chronic diarrhea occurred in the adult dog not heavily infected with these organisms. The frequent occurrence of trichomonads and motile giardia in abnormal stools were interpreted to be a result rather than a cause of the diarrhea. Frank starch was readily detected in the acidic feces of German Shepherd puppies fed rice or commercial puppy diets containing high cereal grain contents. By contrast, and in spite of reports to the contrary, adult German Shepherds were as capable of digesting starch as were inbred hounds. Two interesting but inexplicable observations were: (1) the sprue-like lesions in the jejunum and ileum of puppies fed the high starch diets that were absent in the puppies on the starch-free diet: and (2) the sporadically high lactic acid (> 0.5% of the dry matter) concentrations in stool water of adult German Shepherds, but not inbred hounds. Speculatively, the products of carbohydrate hydrolysis may be poorly absorbed in the German Shepherd and lead directly or indirectly to mucosal injury. Further, these by-products might be fermented in the colon to lactic acid and be undetectable in the stools as disaccharides or monosaccharides. However, in humans with osmotic, fermentive diarrhea associated with congenital carbohydrase deficiencies, stool disaccharides are detectable. Also, the osmolalities of stool water are higher in humans with carbohydrase deficiencies than those without. Again, affected German Shepherds had consistently lower stool osmolalities than unaffected inbred hounds. These observations suggest that the diarrhea was not principally osmotic. The puppy litter on the gluten-free diet had persistent diarrhea, mucosal injury in the small intestine, and malabsorbed xylose, indicating that the syndrome was not a gluten enteropathy, a condition never described for dogs. Finally, the German Shepherd puppies on the starch-free diet passed fairly formed stools and showed relative normal mucosal architecture in the small intestine, but absorbed xylose very poorly. This observation suggested a physiological disfunction in the small intestine. Strengthening this hypothesis were the observed gross losses of water and Na^+ in the 24 hr stool waters of German Shepherds vs inbred hounds. All these date suggested that the small intestine of the affected

German Shepherds may be in a net secretory state, which may be one of the underlying mechanisms for the chronic diarrhea.

6. Viral studies in the diarrhea syndrome in military dogs.

Virus studies

Results of initial virus isolation tests on bitches and their pups with normal and "abnormal" stools were summarized (Annual Progress Report, 1 July 1973-30 June 1974). The recovery of Reo-like viruses from pup 1490 and its bitch 1102 was reported and also the isolation of a third agent from pup 1501. The third agent, 1501R, produced intranuclear inclusion bodies in the Walter Reed Canine cell (WRCC) line. This report summarizes studies on the identification of the 3 isolates and the results of serologic tests to determine their occurrence in the Biosensor Colony. In addition, the isolation of a coronavirus from a pup with a diarrheal syndrome is described. Serologic test findings indicate that this coronavirus often infects many pups of the colony.

Hemagglutination-inhibition (HI) tests with reference anti-reovirus types 1, 2, and 3 serums were carried out to identify the 1490 and 1102 isolates. Only reovirus type 2 antiserum inhibited the isolates. Serum specimens from bitch 1102 inhibited the hemagglutinins of reovirus type 2 and the isolates at low titers. However, serum specimens from pup 1490 did not inhibit reovirus 2 or the isolates. Attempts to reisolate the 1102 virus from the original serum was unsuccessful and attempts to reisolate the 1490 agent could not be made because the specimen had been exhausted.

The 1501R agent produced cytopathic effects (CPE) that resembled those of the minute virus of canines (MVC). Examination of ultrathin sections of 1501R infected cell cultures revealed full and empty virions in the nucleus that measured 15 to 20 nm in diameter. At times, the virions were arranged in crystalline arrays. The virions resembled parvoviruses in size and morphology. Antiserum against the MVC neutralized 1501R virus at high titers. The 1501R agent hemagglutinated rhesus monkey erythrocytes at 50 C and the hemagglutination was inhibited by anti-MVC serum. These findings indicated that 1501R virus was antigenically related to the MVC. Although serum specimens from pup 1501 were not available, a litter mate, pup 1504, had a rise in HI antibody titer to MVC and 1501R virus. The latter findings provided further evidence for the validity of the isolation.

Additional virus isolation tests led to the recovery of a coronavirus, 1505R from a fecal specimen of a 7-week-old pup with diarrhea. Giant cells were evident in infected primary dog kidney cells. The infected cells did not hemadsorb guinea pig erythrocytes.

The 1505R virus also produced CPE in canine A-72 and Denky canine cell cultures, but not in the WRCC line. Multinuclear giant cells were evident in hemotoxylin and eosin stained infected cell cultures.

Results of studies of the chemical and physical properties of 1505R virus are summarized (Table 15). The virus was inactivated by chloroform treatment but was resistant to storage at pH 3.0 for about 3 hours. Growth of the agent was not inhibited by 5-iodo-7-deoxyuridine (IUDR) treatment. The agent passed through 450 and 220 nm membrane filters without loss of titer. However, a 2.3 log reduction in titer occurred when 1505R virus was passed through the 100 nm filter and virus could not be recovered from the 50 nm filtrate. These findings indicated that 1505R was an enveloped RNA virus between 50 and 100 nm in diameter.

To further identify this isolate ultrathin sections of infected cell cultures were examined electron microscopically in collaboration with Dr. A. Strano, Armed Forces Institute of Pathology. Enveloped particles, varying from 70 to 100 nm in diameter, were evident in vesicles in the cytoplasm. The cores of the virions ranged from 40 to 70 nm in diameter with an envelope ranging from 10 to 20 nm in diameter. The majority of the virions were 90 nm in diameter, with a core of 60 nm and an envelope of 15 nm. The virions were formed by budding into cytoplasmic vesicles. On the basis of these observations, the 1505R agent could be classified as either a coronavirus or a member of the Bunyamwera virus group.

Neutralization tests were done with reference antiserums against enveloped RNA viruses recovered from dogs. The 1505R agent was neutralized by swine transmissible gastroenteritis (TGE) virus antiserum and not by serum against canine distemper, SV5, measles and lymphocytic choriomeningitis viruses. Additional neutralization tests with reference coronavirus antiserums confirmed the neutralization observed with TGE virus serum, but none of the other calf, mouse, rat, chicken, and pig (strain HEV) coronavirus antisera neutralized 1505R virus. Convalescent serum from a pup experimentally infected with a canine coronavirus, 1-71, also neutralized 1505R virus. The 1-71 virus is antigenically related to TGE virus. These findings indicated that 1505R was a coronavirus antigenically related to TGE virus of swine and canine coronavirus 1-71.

Serologic tests were performed to determine the occurrance of infections with the Biosensor isolates and related viruses. In addition, neutralization tests to detect canine herpesvirus infections were done, as this virus produces diarrhea in young pups. Prior to whelping, antibody to reovirus type 2, MVC, coronaviruses, and canine herpesvirus were detected in 1 or more of the 5 bitches

(Table 16). Antibodies to the 3 coronaviruses were present in the serum of almost all of the bitches, but antibodies to the other agents occurred less frequently. Increased antibody titers to canine herpesvirus and to the reoviruses were not detected, suggesting that infections with these viruses did not occur. The HI test results indicated that 15 to 31% of the pups and 20% of the bitches were infected with 1501R virus of the MVC. Infections with a coronavirus appeared to be very common as approximately 80% of the pups had a rise in titer and all surviving pups and bitches had significant levels of coronavirus neutralizing antibodies.

The serologic test findings provided additional evidence for the validity of the parvovirus (1501R) and coronavirus (1505R) isolations. The high percentage of pups that had a coronavirus infection and previous findings which indicated that a similar canine coronavirus isolate could induce gastroenteritis in neonatal inbred hounds suggested that the coronavirus may play a significant role in the diarrhea syndrome at the Biosensor colony. However, further epizootiological and pathogenicity studies are required to evaluate the significance of these coronavirus infections.

Further virus studies were performed on German Shepherd pups with a diarrhea syndrome at the Biosensor colony. Virus isolation and serologic test findings indicated that these pups were infected with a parvovirus that was antigenically related to the minute virus of canines and a coronavirus that was related to transmissible gastroenteritis virus of swine and a dog coronavirus isolate. Almost all of the pups had serologic evidence of infection with the coronavirus and approximately a third with the parvovirus.

7. Respiratory disease in the pups of the Biosensor military dog colony.

During August and September of 1974 an epizootic of upper respiratory disease (URD) occurred in 53 of 78 (68%) pups at the Division of Biosensor Research, Aberdeen Proving Ground, MD. The affected pups were derived from 14 of 15 litters born between 23 May and 9 July 1974. The first case of URD in a litter usually occurred when the pups were 9 to 10 weeks of age and new cases continued to occur for 2 to 3 week period. The most frequent signs of disease observed were nasal discharge, cough, and anorexia. The duration of illness was usually 1 week. Six pups had 2 episodes of disease and 1 pup died of pneumonia. Laboratory studies were conducted to detect possible bacteriological and viral etiological agents. Bordetella bronchiseptica was recovered from nose and/or throat swab specimens of each of 10 pups with signs of URD. The bordetella cultures were sensitive to tetracycline, solymycin, gentamycin, polymyxin, neomycin, and chloromycin; and resistant to

penicillin, gantricin, furadantin, and bacitracin. Transmissible hemadsorbing agents were recovered from the nose and/or throat of 7 of 12 affected pups in primary dog kidney and the Walter Reed Canine Cell (WRCC) cultures. The hemadsorption observed differed from that produced by parainfluenza SV5 in that it was "focal" and not general throughout the cell cultures. The hemadsorption agent was sensitive to aureomycin treatment. The latter findings suggested the possible presence of mycoplasma. Following cultivation in artificial media, typical mycoplasma "fried-egg" colonies were isolated from hemadsorption positive cultures. The mycoplasma colonies also hemadsorbed guinea pig erythrocytes. A representative isolate was identified as M cynos by Dr. J. Tully, National Institutes of Health. Further studies are in progress to identify the isolates.

Serologic tests to detect viral infections in pups with URD are summarized (Table 17). More than 90% of the affected pups had antibodies to both the canine coronavirus 1505R and parvovirus minute virus of canines (MVC). Significant increased antibody titers occurred in 29% of the pups to 1505R and 40% to the MVC. Many of these affected pups also had increased antibody titers to the antigenically related coronaviruses, transmissible gastroenteritis virus (5/26) and canine coronavirus 1-71 (10/27). More than 85% of the 78 pups in the 15 litters examined had neutralizing antibody to the 1505R coronavirus. Serologic evidence of canine herpes and SV5 infections were not observed.

Following the isolation and antibiotic sensitivity testing of Bordetella bronchiseptica, the treatment of pups with URD was changed to Delta Albaplex (Upjohn Co., Kalamazoo, MI) from penicillin-streptomycin mixture or Azimycin (Schering Corp., Kenilworth, NJ). Delta Albaplex contains tetracycline which was effective against both bordetella and mycoplasma. The pups rapidly improved with the new antibiotic treatment. However, additional measures to improve the husbandry of the pups also were taken; therefore, the recovery could not be solely attributable to the new antibiotic therapy.

This study summarized clinical and laboratory observations on an epizootic of respiratory disease in weanling pups at the Biosensor Colony. The outbreak was of added interest because of the large number of affected pups, lack of response to the previously successful antibiotic treatment, and the absence of respiratory disease in older dogs of the colony. Early in the epizootic, the possible introduction of parainfluenza SV5 was of great concern. The recovery of Bordetella bronchiseptica from each of the sick pups was of significance as this organism is commonly regarded as an important secondary invader. However, Wright and his co-workers (66) have presented evidence that B bronchiseptica can produce

a contagious upper respiratory infection in weanling pups. Although Mycoplasma spp are readily recoverable in artificial media from the nose and throat of dogs, this report describes the first recovery of mycoplasma in cell cultures from dogs with respiratory disease. The role of mycoplasma in canine respiratory disease has not been fully resolved. Rosendal (52) has recovered 5 species of mycoplasma from the lungs of dogs with pneumonia but not from the lungs of normal dogs, and he felt that these observations provided evidence that mycoplasma play a role in the etiology of canine pneumonia. Further field observations and pathogenicity studies are required with pure cultures of mycoplasma to evaluate the role of various Mycoplasma spp in canine respiratory disease. Although other factors may be involved, the prompt response of affected pups to tetracycline administration suggested that either the bordetella or the mycoplasma or both contributed to the disease observed.

The viral serologic test findings provide further evidence for the occurrence of coronavirus and parvovirus infections in the Biosensor colony. These observations confirm and extend the studies made on these pups with the diarrheal syndrome. The findings indicated these 2 viruses may be enzootic in the colony. The role of these 2 agents in the diarrhea syndrome and respiratory disease require additional study.

An epizootic of upper respiratory disease occurred in 68% of 78 pups at the Biosensor colony at Edgewood Arsenal, MD. Bordetella bronchiseptica and Mycoplasma spp. were recovered from affected dogs. The mycoplasma was isolated originally from cell cultures which exhibited focal hemadsorption. Viral antibody test findings indicated that canine parvovirus and coronavirus infections were common in pups of the colony. Serologic evidence of parainfluenza SV5 and canine herpes virus infections were not found. The affected pups had a prompt beneficial response to a tetracycline containing drug which was effective against the bordetella and mycoplasma isolates.

Table 1. Skin reaction of German Shepherd dogs sensitized to and challenged with 2,4-dinitrochlorobenzene (DNCB) and old tuberculin (OT), before and after exposure to Ehrlichia canis.

	DNCB[a]		OT[b]	
Dog No.	Pre exposure	Post exposure	Pre exposure	Post exposure
56	Negative	Positive	Positive	Negative
59	Positive	Positive	Positive	Negative
113	Positive	Negative	Positive	Negative
114	Positive	Negative	Positive	Negative
115	Positive	Positive	Positive	Negative
116	Positive	Positive	Positive	Negative
117	Positive	Positive	Positive	Negative
118	Negative	Negative	Positive	Negative
119	Negative	Negative	Positive	Negative
120	Negative	Positive	Negative	Positive
122	Negative	Positive	Positive	Positive
123	Positive	Positive	Positive	Positive

[a]Sentized dogs challenged with 100 ug of DNCB. A positive reaction consisted of an area of erythema and induration that measured approximately 0.6 to 1.9 cm in diameter at the test site after 72 hrs.

[b]Sensitized dogs challenged with 1:1,000 dilution of OT. A positive reaction consisted of an area of induration and erythema measuring 0.6 to 1.9 cm in diameter at the test site after 72 hrs.

Unsensitized dogs 124, 125, and 126 were negative when tested with DNCB and OT.

Table 2. Maximum leukocyte migration inhibition (LMI) responses and indirect fluorescent antibody (IFA) titers in dogs inoculated with Ehrlichia canis.

Dog No.	Breed	LMI[a]		IFA[b]		Clinical Disease[c]
56	German Shepherd	26.0	(24 days)	10,240	(62 days)	Severe
59	German Shepherd	76.0	(24 days)	10,240	(48 days)	Mild
113	German Shepherd	0		20,480	(91 days)	Severe
114	German Shepherd	0		20,480	(91 days)	Mild
115	German Shepherd	75.0	(51 days)	2,560	(27 days)	Mild
116	German Shepherd	0		5,120	(75 days)	Severe
117	German Shepherd	56.6	(53 days)	2,560	(48 days)	Mild
118	German Shepherd	31.6	(52 days)	5,120	(55 days)	Severe
119	German Shepherd	0		10,240	(69 days)	Mild
120	German Shepherd	31.1	(21 days)	10,240	(98 days)	Mild
122	German Shepherd	0		10,240	(75 days)	Severe
123	German Shepherd	25.0	(54 days)	10,240	(75 days)	Mild
473	Inbred Hound	37.0	(72 days)	10,240	(48 days)	Mild
474	Inbred Hound	67.8	(24 days)	10,240	(112 days)	Mild
475	Inbred Hound	57.6	(24 days)	10,240	(55 days)	Mild
476	Inbred Hound	0		5,120	(62 days)	Mild
477	Inbred Hound	85.6	(23 days)	5,120	(48 days)	Mild

[a]Largest percent of LMI and days PI when observed.

[b]Greatest IFA titer measured and days PI when observed.

[c]Classification of disease form, mild chronic or severe chronic, 90 days PI. Dogs with thrombocyte counts of 50,000 cells/cmm or less were considered to have severe chronic disease and dogs with thrombocyte counts greater than 50,000 cells/cmm were categorized as having mild chronic disease.

Table 3. Ehrlichia canis antibodies in dogs in Phoenix, Arizona.

Breed Group	Suspect Ehrlichiosis	Non-suspected Ehrlichiosis	Total
Hound	2/6[a] (33.3)[b]	1/16 (6.3)	3/22 (13.6)
Non-sporting	1/2 (50.0)	0/3 (0.0)	1/5 (20.0)
Sporting	1/6 (16.7)	0/30 (0.0)	1/36 (2.8)
Terrier	1/9 (11.1)	0/12 (0.0)	1/21 (4.8)
Toy	0/3 (0.0)	1/12 (8.3)	1/15 (6.7)
Working	11/43 (25.6)	5/61 (8.2)	16/104 (15.4)
Mix Unknown Origin	0/1 (0.0)	1/5 (20.0)	1/6 (16.7)
TOTAL	16/70 (22.9)	8/139 (5.8)	24/209 (11.5)

[a]Number serologically positive/total tested.

[b]Percent serologically positive.

Table 4. *Ehrlichia canis* antibodies in the working group of dogs in Phoenix, Arizona.

	Suspect Ehrlichiosis	Non-suspect Ehrlichiosis	Total
Working Group	11/43[a] (25.6)[b]	5/61 (8.2)	16/104 (15.4)
Working Group less German Shepherd	1/16 (6.3)	0/25 (0.0)	1/41 (2.4)
German Shepherd and German Shepherd-X	10/27 (37.0)	5/36 (13.9)	15/63 (23.8)

[a]Number serologically positive/total tested.

[b]Percent serologically positive.

Table 5. Parainfluenza SV5 and calicivirus W191R antibody studies of military dogs with respiratory disease at Lackland AFB 1974.

Dates of Onset of Respiratory Disease	No. Affected/ Total (%)	No. with increased neutralizing antibody titer/total tested (%)	
		SV5	W191R
23 Jan-16 Apr	39/194 (20)	33/39 (85)	3/35 (9)
22 Aug-22 Oct	40/211 (19)	27/35 (80)	4/35 (11)
Total	79/405 (20)	60/74 (81)	7/70 (10)

Table 6. Canine parainfluenza SV5 antibody in newly recruited military dogs.

Year	No. Dogs Examined	No. with SV5 antibody (%)	Origin of Dogs: No. of States (%)[a]	Origin of Dogs: No. States with Seropositive Dogs (%)
1969	434	41 (9.4)	46 (95)	18 (39)
1970	387	40 (10.3)	44 (92)	20 (45)
1971	237	43 (19.0)	42 (87)	17 (40)
1972	205	33 (16.0)	41 (85)	15 (37)
1973	369	97 (26.3)	40 (83)	27 (68)
1974	171	34 (19.3)	37 (77)	16 (43)
Total	1803	288 (15.9)	48 (100)	39 (81)

[a]Contiguous states of the United States.

Table 7. Relationship of age and incidence of canine parainfluenza SV5 antibody in newly recruited military dogs.

Age (Months)	No. with SV5 antibody / Total tested	Percent
< 12	1/27	3.7
12-18	82/763	10.7
19-24	66/384	17.2
25-30	65/340	19.1
> 30	73/284	25.7
Total	287/1798	16.0

Table 8. Values[a] of accumulated assays for the plasma absorption of d-xylose[b] at selected time intervals after administration of d-xylose to German Shepherds and other dogs.

Time interval after administration	No. of assays during time interval	mg of d-xylose per 100 ml of plasma			
		Mature[c]		Puppy[d]	
		Beagle	German Shepherd	Inbred Hound	German Shepherd
0 to 30 min.	2	46	45	59	22
0 to 60 min.	3	110	107	116	58
0 to 180 min. (total)	6	220	210	220	141

[a]Accumulated values expressed as arithmetic means

[b]Dose was 0.5 gm per kg body weight

[c]Extracted from Hill, F.W.G., et al. 1970. Vet. Rec. 87:250-255. Xylose ingested. Six Beagles in column 3; 5 German Shepherds and 5 other breeds in column 4.

[d]Xylose administered by stomack tube. Eleven inbred hounds in column 5, and 15 German Shepherds in column 6.

Table 9. Carbohydrate, fat, and protein content[a] of fecal specimens from adult dogs fed a commercial dry diet.[b]

Fecal Composition	German Shepherd[c]		Inbred Hounds[d]	
	Coarse diet	Ground diet	Coarse diet	Ground diet
	Mean % (range)	Mean % (range)	Mean % (range)	Mean % (range)
Total fat	2.0 (1.5) (3.1)	1.3 (0.6) (2.5)	2.0 (1.5) (3.0)	1.1 (0.5) (1.6)
Protein	25.3 (22.2) (30.0)	27 (23.6) (31.9)	27.6 (26.2) (29.0)	30.9 (26.5) (39.2)
Starch	3.5 (1.9) (4.4)	2.4 (1.8) (3.1)	3.0 (2.6) (4.2)	2.1 (2.0) (2.3)
Hemicellulose	6.6 (5.8) (8.6)	7.8 (6.0) (10.4)	8.2 (7.7) (8.9)	7.3 (6.7) (8.3)
Lactic acid	0.38 (0.037) (0.54)	0.38 (0.04) (1.21)	0.13 (0.03) (0.24)	0.06 (0.02) (0.12)

[a]Carbohydrate, fat, and protein values are percent of dry fecal weight.

[b]Respond 2000, Div of Ageway, Syracuse, NY.

[c]Analyses on 8 to 10 fecal specimens collected from 3 dogs on the coarse diet and 4 dogs on the ground diet.

[c]Analyses on 4 fecal specimens collected from 2 dogs on the coarse diet and 2 dogs on the ground diet.

Table 10. Amylolytic activity of pancreatic and jejunal homogenates from "normal" puppies.

Dog No.	Pancreatic α-amylase[a] μ mol maltose per min per mg protein (gm wt tissue)	Total intestinal amylase[a] μ mol maltose per min per mg protein (gm wt tissue)	Amyloglucosidase[b] μ mol glucose per min per mg protein (gm wt tissue)
1	19.4 (2,502)	not done	0.00079 (0.058)
2	17.1 (2,146)	0.01 (1.05)	not done
3	26.8 (3,616)	0.007 (5.67)	not done
4	20.2 (2,722)	0.08 (6.09)	0.00056 (0.044)
5	22.9 (3,088)	not done	0.00043 (0.056)
6	26.8 (3,440)	0.03 (2.82)	0.00043 (0.047)
7	18.3 (2,596)	0.008 (0.82)	0.00080 (0.084)
8	30.8 (3,267)	0.02 (2.28)	0.00087 (0.062)
9	27.6 (4,097)	0.01 (1.86)	0.00083 (0.097)
10	16.3 (2,444)	0.02 (1.94)	0.00104 (0.118)
Mean	22.6 (2,992)	0.03 (2.82)	0.00073 (0.075)

[a]Dahlquist method of assay. Total intestinal amylase represents absorbed pancreatic α-amylase plus the brush border amyloglucosidase.

[b]Glucose oxidase method of assay.

Table 11. Sucrase and maltase activity of jejunal homogenates from "normal puppies."

Dog No.	Sucrase[a] μ mol glucose per min per mg protein (gm wt tissue)	Maltase[b] μ mol glucose per min per mg protein (gm wt tissue)
1	0.73 (54.1)	0.62 (45.8)
2	0.10 (8.1)	0.11 (9.1)
3	0.43 (31.2)	0.29 (20.9)
4	0.22 (17.0)	0.23 (18.2)
5	0.55 (51.1)	0.49 (45.3)
6	0.33 (35.6)	0.38 (41.3)
7	0.47 (48.9)	0.36 (38.2)
8	0.44 (31.1)	0.61 (43.5)
9	0.35 (40.7)	0.38 (44.9)
10	0.53 (60.4)	0.46 (51.6)
11	0.42 (53.0)	0.39 (49.3)
Mean	0.41 (39.2)	0.39 (37.1)

[a]Dahlquist method of assay; sucrose jejunal homogenate incubated 30 min.
[b]Glucose oxidase method of assay.

Table 12. Twenty-four hour fecal volume, moisture, osmolality, and electrolytes for 2 German Shepherds fed a dry commercial diet, ad lib.

Observations \ Dog no. 24 hr Collection	G. Shepherd 1561 (66 lbs)			G. Shepherd 1674 (62 lbs)			24 hour Mean
	29 Apr.	30 Apr.	1 May	29 Apr.	30 Apr.	1 May	
No. stools	11	9	5	12	10	8	9
Stool weight (gm)	1,951	1,656	934	2,831	1,672	1,187	1,705
Percent moisture	83.0	83.3	77.6	82.7	79.2	78.5	80.7
Osmolality (mosmol/kg)	512	528	574	504	568	570	543
meq/liter							
Na^+	81	90	94	72	83	78	83
K^+	17	17	8	13	12	10	13
Cl^-	20	16	20	13	15	17	17
HCO_3^-	78	91	82	72	80	71	79
Feed consumed (oz)	31	30	30	50	42	35	36[a]
Consistency	Runny	Runny	Soft formed Cow flop	Runny	Runny	Soft formed Cow flop Runny	

[a]German Shepherds consumed 0.56 oz feed/lb body wt/24 hrs
German Shepherds passed 12 oz dry wt stool/36 oz feed consumed/24 hrs (1:3).

Table 13. Twenty-four hour fecal volume, moisture, osmolality, and electrolytes for 2 inbred hounds fed a dry commercial diet, ad lib.

Observations \ Dog no. / 24 hr Collection	Inbred hound 474 (32 lbs)			Inbred hound 488 (26 lbs)			24 hour
	29 Apr	30 Apr	1 May	29 Apr	30 Apr	1 May	Mean
No. stools	1	1	3	0	2	2	1.5
Stool weight (gm)	92	166	351	-	185	199	166
Percent moisture	63.5	67.3	70.5	-	67.3	65.1	67.1
Osmolality (mosmol/kg.)	693	621	672	-	626	633	649
meq/liter Na^+	39	43	57	-	42	33	43
K^+	19	6	7	-	15	14	12
Cl^-	21	19	17	-	21	19	19
HCO_3^-	37	43	47	-	36	33	49
Feed consumed (oz)	0	11	11	19	13	15	12[a]
Consistency	Firm/ Formed	Firm/ Formed	Firm/ Formed	-	Firm/ Formed	Firm/ Formed	

[a]Inbred hounds consumed 0.41 oz feed/lb body wt/24 hrs.
Inbred hounds passed 2 oz dry wt stool/12 oz feed consumed/24 hrs (1:6).

Table 14. Amount[a] of sodium, potassium, chloride and bicarbonate[b] ions by volume of fecal water voided in 24 hour periods from 2 German Shepherds and 2 inbred hounds fed a dry commercial diet, ad lib.

Dog	24 hour period	Stool water[c] (ml)	Na^+	K^+	Cl^-	HCO_3^-
Inbred hound 474	29 April	58	2	1	1	2
	30 April	112	5	0.7	2	5
	1 May	247	14	2	4	12
Inbred hound 488	29 April	0	0	0	0	0
	30 April	125	5	2	3	5
	1 May	130	4	2	3	4
	Mean	112	6	1	3	6
German Shepherd 1561	29 April	1,619	131	28	32	126
	30 April	1,379	124	23	22	126
	1 May	725	68	6	15	59
German Shepherd 1674	29 April	2,341	169	30	30	169
	30 April	1,324	110	16	20	106
	1 May	932	73	9	16	66
	Mean	1,387	112	19	23	109

[a]Amount in milliequivalents.

[b]Meq of HCO_3^- estimated indirectly by $Na^+ + K^+ - Cl^-$

[c]Obtained by multiplying gm wt stool/24 hr X % H_2O.

Table 15. Chemical and physical properties of 1505R isolate from Biosensor pups.

Treatment	Virus	$1/T_{50}$ ($\log_{10}$)		
		Not treated A	Treated B	Change (B-A)
Chloroform	1505R	4.5	≤ 1.0	≥ 3.5
	ICH	6.5	6.5	0.0
	C. Herpes	5.3	≤ 1.0	≥ 4.3
pH 3.0 for 3 hrs at 25°C	1505R	4.8	4.5	0.3
	ICH	6.3	6.3	0.0
	C. Herpes	4.4	≤ 2.0	≥ 2.4
Filtration[a] 450 nm	1505R	5.0	4.8	0.2
220 nm			4.5	0.5
100 nm			2.7	2.3
50 nm			≤ 1.0	≥ 4.0
IUDR ($10^{-3.5}$M)	1505R	5.6	5.5	0.1
	ICH	6.1	1.4	4.7
	SV5	7.1	7.1	0.0

[a]Membrane filter (Millipore Corp.)

Table 16. Neutralization (N) and hemagglutination-inhibition (HI) tests for virus infections in Biosensor bitches and pups.

Virus strain	Test	No. bitches with antibody before whelping/Total (%)	No. with increased antibody titers/Total (%)	
			Bitches	Pups
Canine parvovirus				
MCV	HI	2/5 (40)	1/5 (20)	4/13 (31)
1501R*	HI	0/5 (0)	1/5 (20)	2/13 (15)
Coronavirus				
TGE	N	4/5 (80)	1/5 (20)	12/14 (86)
1-71	N	5/5 (100)	0/5 (0)	12/15 (80)
1505R*	N	5/5 (100)	3/5 (60)	11/14 (79)
C. herpes				
D004	N	1/5 (20)	0/5 (0)	0/10 (0)
Reovirus				
type 1	HI	0/5 (0)	0/5 (0)	0/13 (0)
" 2	HI	3/5** (60)	0/5 (0)	0/13 (0)
" 3	HI	0/5 (0)	0/5 (0)	0/13 (0)
1490*	HI	2/5 (40)	0/5 (0)	0/13 (0)
1102*	HI	2/5 (40)	0/5 (0)	0/13 (0)

*Biosensor colony isolate

**HI titer of 1:20 or greater

Table 17. Viral antibody studies of pups with upper respiratory disease at the Biosensor Colony.

Virus strain	Test[a]	No. pups with antibody/total (%)	No. pups with increased antibody titers/total (%)
Coronavirus 1505R	N	32/35 (91)	10/35 (29)
Parvovirus MVC	HI	24/35 (96)	10/25 (40)
Parainfluenza SV5 3x84	N	0/31 (0)	0/24 (0)
Canine herpes D004	N	0/25 (0)	0/25 (0)

[a]N = Neutralization, HI = Hemagglutination-inhibition.

Figure 1. Effect of antilymphocyte serum (ALS) on the thrombocyte counts of Ehrlichia canis-free inbred hounds 416 and 466.

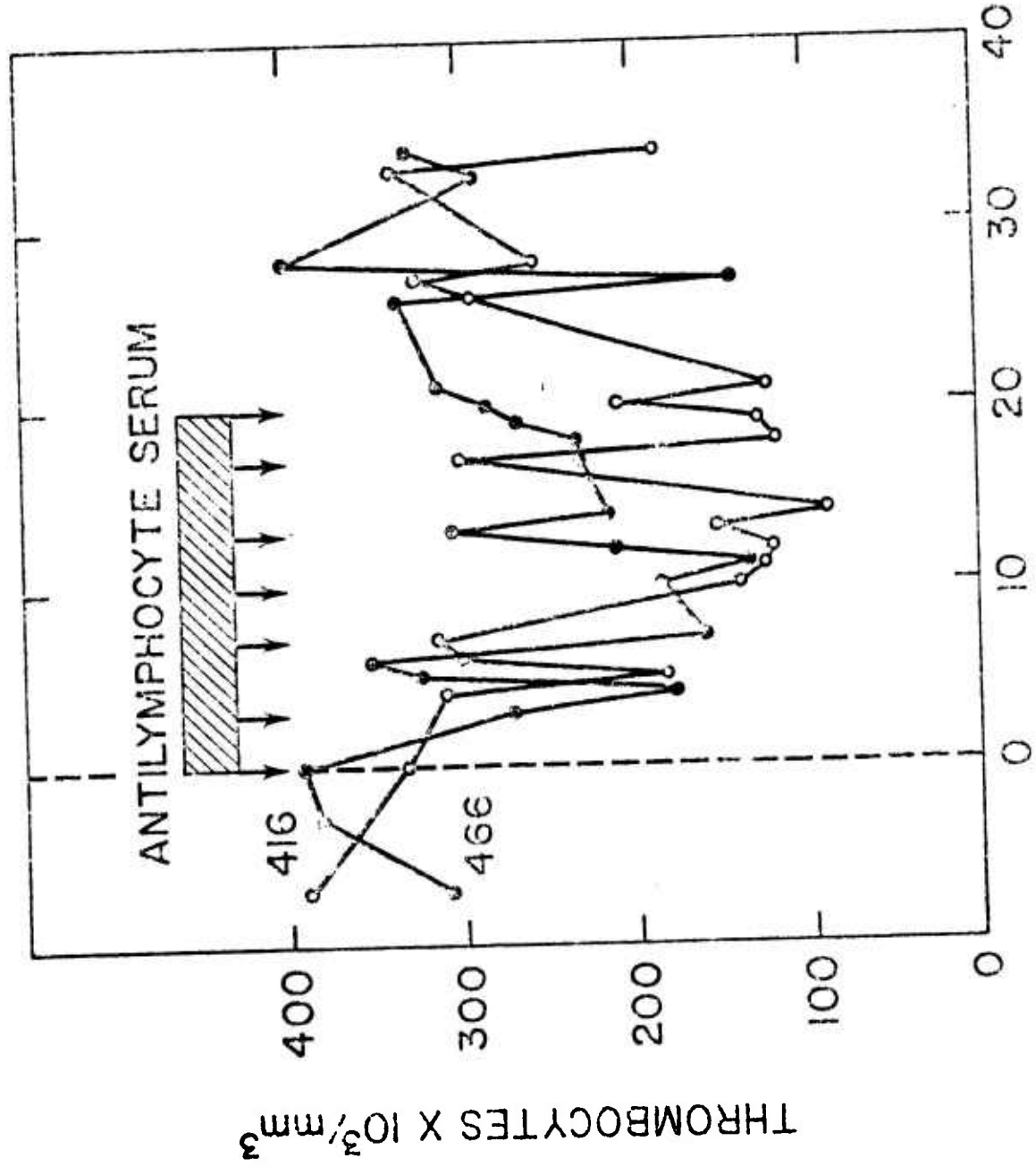


Figure 2. Effect of antilymphocyte serum (ALS) on the thrombocyte counts of Ehrlichia canis-infected inbred hounds 474 and 477.

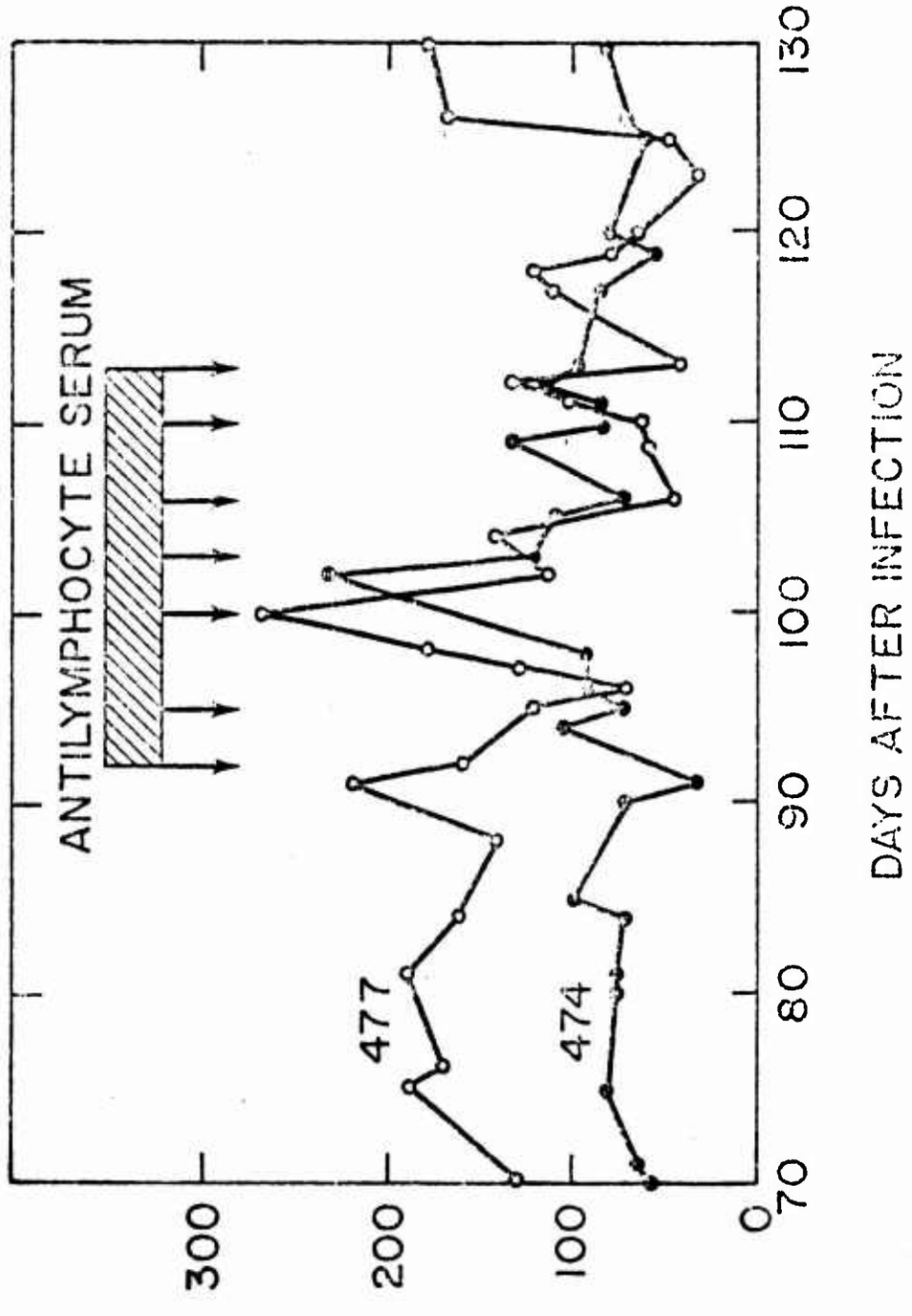
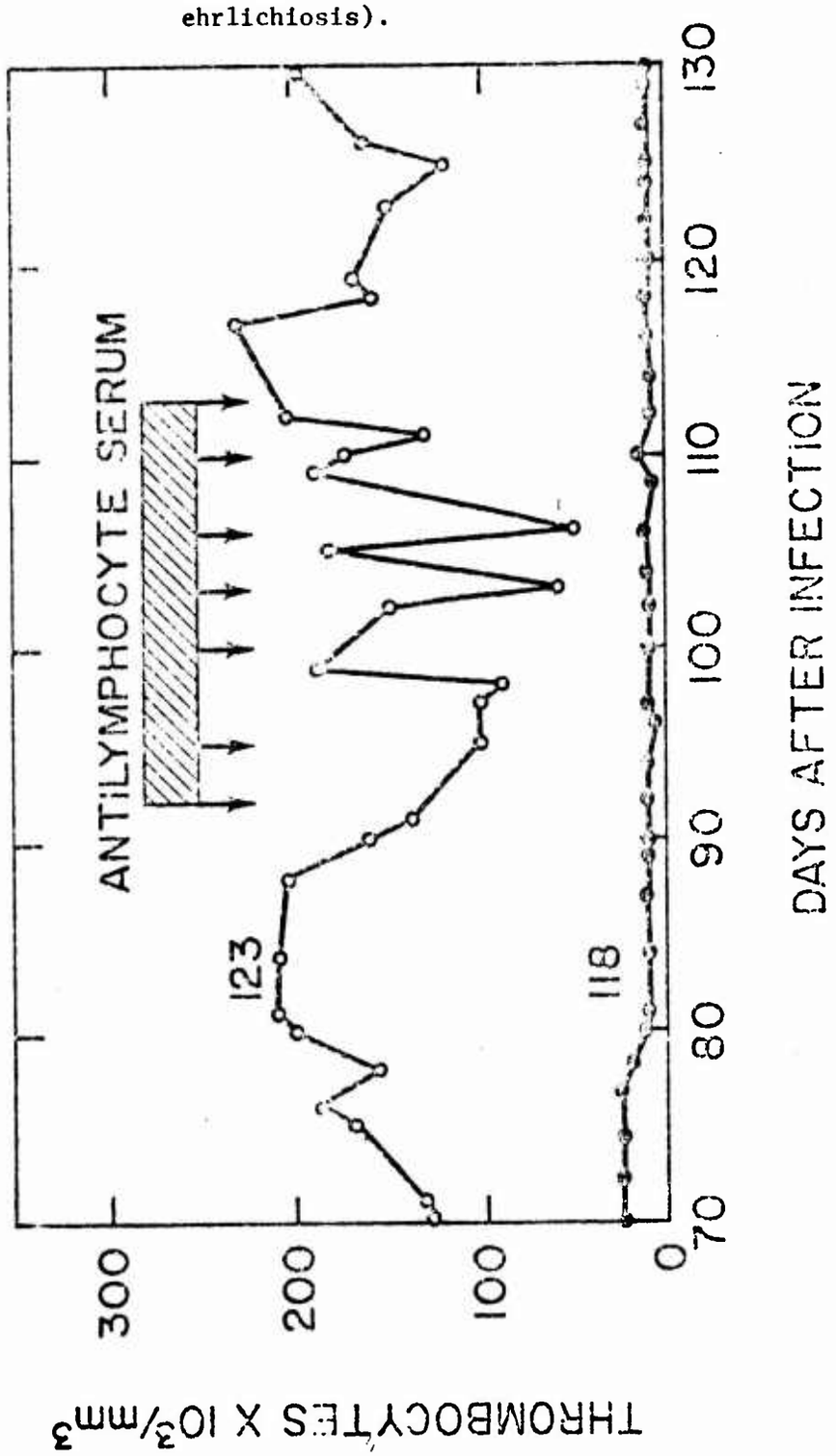
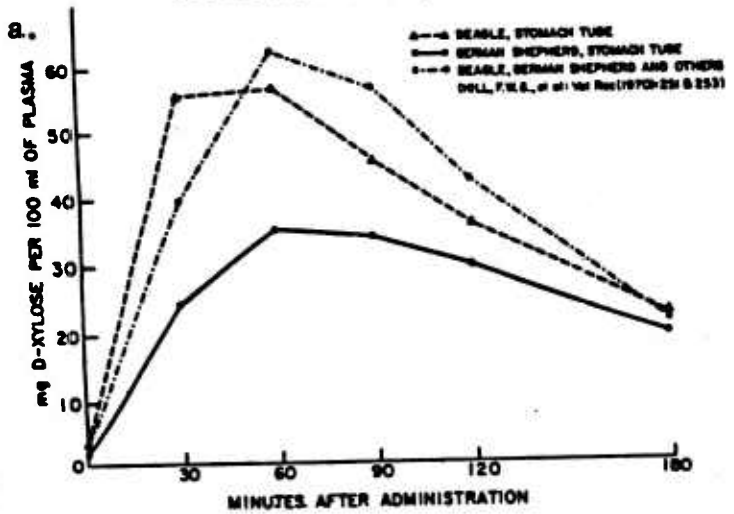


Figure 3. Effect of antilymphocyte serum (ALS) on the thrombocyte counts of Ehrlichia canis-infected German Shepherd dogs 118 (severe chronic ehrlichiosis) and 123 (mild chronic ehrlichiosis).



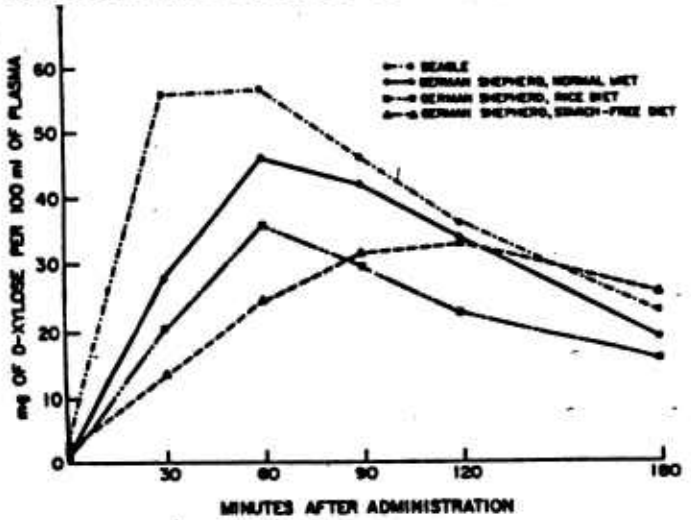
MEAN VALUES FOR PLASMA ABSORPTION OF D-XYLOSE AMONG SEAGLES AND GERMAN SHEPHERDS

a.



MEAN VALUES FOR PLASMA ABSORPTION OF D-XYLOSE AMONG BEAGLE PUPPIES AND THREE LITTERS OF GERMAN SHEPHERD PUPPIES

b.



MEDIAN VALUES FOR PLASMA ABSORPTION OF D-XYLOSE AMONG BEAGLE PUPPIES AND THREE LITTERS OF GERMAN SHEPHERD PUPPIES

c.

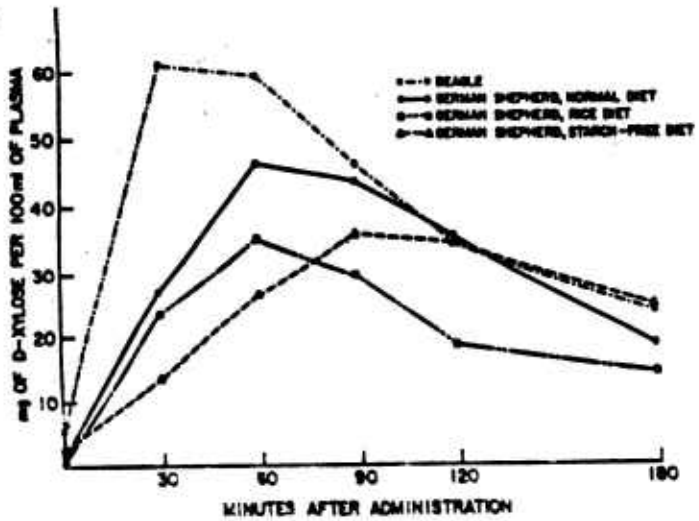


Figure 4

Project 3A162110A830

Task 00 Military Dog Improvement

Work Unit 056 Diseases of Military Animals

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PROJECT 3A762759A831
TROPICAL MEDICINE

Task 00
Tropical Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6525	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO /CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A831	00	070
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Provide with Security Classification Code)

(U) Anti-schistosomal Drug Development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	1.1	175
FISCAL YEAR CURRENT 76	1.1	200

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: University of Brasilia

ADDRESS: 70000 Brasilia, D. F. Brazil

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic institution)

NAME: Prata, Aluizio, M.D.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Radke, M. G. LTC

NAME:

21. GENERAL USE

Foreign inteiligence not considered

22. KEYWORDS (Provide EACH with Security Classification Code)

(U) Brazil; (U) Drug Development; (U) Schistosomiasis; (U) Chemotherapy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Provide test of each with Security Classification Code.)

23. (U) Screen compounds for prophylactic activity against Schistosoma mansoni infections in albino mice by the mortality test system. The lack of prophylactic drugs to prevent schistosomiasis in man is of great medical importance should military personnel be deployed in endemic schistosomiasis areas such as South America, Africa, Asia, and Middle East Countries.

24. (U) WRAIR submitts selected compounds for prophylactic testing in mice that are exposed to 3,000 or more S. mansoni cercariae. These chemical compounds are tested at 640 and 1920 mgs/kg using 5 mice per test dosage. If drugs are found toxic, they are retested at 40 and 160 mgs/kg. Drugs are prepared in peanut oil and administered subcutaneously two days after mice were exposed to a lethal infective dose of cercariae.

25. (U) 74 07 - 75 06 This research is complemeptary to studies being conducted under DAOB 7294, Work Unit 086, entitled Chemotherapeutic Studies on Schistosomiasis. A successful laboratory Biomphalaria glabrata (Paulista Strain) snail colony with concurrent S. mansoni snail infections was established. Since October, an infected snail population of more than 500 was maintained. The infected snails provided weekly collections of 1.5 to 2 million cercariae which are needed for each test group of 20 compounds. The program began to prophylactic test anti-schistosoma' drugs in October 1974. As of 30 June 75, 670 selected drugs were tested for anti-schistosomal activity. Using the mortality test system, a total of 17 drugs were found active against schistosomiasis. However, most of the active compounds are classified as commercially discreet. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon ed/director's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 070 Anti-schistosomal Drug Development

Investigators.
Principal: Aluizio Prata, M.D. (University of Brasilia)
Associate: LTC Myron G. Radke, MSC

Description.

A drug screening program was established to test twenty selected compounds weekly for prophylactic activity against schistosomiasis mansoni by the mouse mortality test system (Radke, et al 1971). The prophylactic anti-schistosomal drug testing program became operational in October 1974 after a three year interruption in testing occurred while the WRAIR Drug Screening Facility was relocated from Japan to Brazil. Each year schistosomiasis becomes a greater medical problem in that new agricultural irrigation systems are developed and/or extended to meet food demands of expanding populations. Furthermore, there is a substantial economic loss of manpower resulting from the nonproductive schistosomiasis infected workers. Also, many of the schistosomiasis endemic areas are high risk areas (Carribbean, South America, Africa, Middle East, and Asia) that may call for deployment of military forces. Presently no known drugs are available to prevent individuals from acquiring schistosomiasis. Schistosomiasis will be controlled when prophylactic drugs are available to treat the non-infected inhabitants while curative drugs are administered to the infected population and molluscicides are used to kill the vector snails. Our research program is directed towards finding an acceptable prophylactic anti-schistosomal drug (s) that can be administered to uninfected people to prevent acquiring the disease.

Progress.

a. Laboratory Facility. The final equipment installation phase was completed in August 1974 with the cage washer and the 500 liter hot water heater operational. However, the 80 gallon air compressor has not been installed due to the 220 volt, 3 phase requirement. An autotransformer is on order and delivery is expected by the end of June.

b. Animal Facility. The University's Bioterio expanded the mouse colony with a June delivery of 600, 18-23 gram mice instead of the 400 mice received weekly.

c. Snail Colony. The laboratory Biomphalaria glabrata snail colony yields weekly, 400 (5-8 mm) snails for the individual exposures to 8 - 12 S. mansoni miracidia. Eighty percent of the miracidia exposed snails are surviving at 42 days, and the snail infection rate is above

50 percent. Consequently, the number of cercariae shedding snails maintained has increased from 572 (October 74) to over 1300 (May 75). With the increased numbers of infected snails, the cercarial production increased from 1.5 to 4 million weekly.

d. Drug Testing. The initial drug testing rate of 20 per week was increased to 30 drugs starting 1 June 75. The concurrent increased cercarial and mouse production enabled us to expand the number of drugs tested weekly without affecting a staff change.

Test Procedure.

All experimental drugs are screened for prophylactic activity by the mouse mortality test system. Mice weighing 18-23 grams (39-43 days old) are exposed 45 minutes by tail immersion to 3,000 or more S. mansoni cercariae. Drugs are formulated in peanut oil, at 640 and 1920 mgs/kg with five mice per test dosage, are administered subcutaneously two days after the cercarial exposure. Any drug found toxic, that is treated mice dying within the first ten days of infection, are retested at 40 and 160 mgs/kg. A routine test group for 20 compounds consists of 320 mice of which 10 mice are normal, and 310 mice are exposed to 3,000 or more cercariae. The two hundred (200) infected mice are used to test 20 candidate drugs, 10 mice are given Niridazole (Reference Drug), and 100 mice are untreated infected controls. An active drug is one in which treated mice survive 49 days after exposure to the 3,000 cercariae. The infected control mice begin dying on the 20th day of infection and by the 30th day there are no survivors. Drug activity is based upon the number of surviving mice and worm burden data as obtained by perfusing the living treated mice on the 49th day of infection.

Results.

Since the anti-schistosome drug testing program was initiated eight months ago (October 74), a total of 670 candidate drugs were tested by the mouse prophylactic mortality test system. Although 670 drugs were under test, final results are available for only 462 drugs (208 drugs are in various stages of testing). The anti-schistosome drug test results for these 462 drugs were: 1) 380 negative, 2) 65 toxic, 3) 61 retest (all negative), and 4) 17 active. The rate of drug actives is high in proportion to the number of drugs tested, but this is a result of the Division of Medicinal Chemistry WRAIR selecting potential active chemical structures for testing. The anti-schistosomal mouse prophylactic mortality test system proved highly efficient in identifying 17 active WRAIR bottle number drugs. The discreet nature of these compounds submitted for testing does not permit disclosure of the compounds. If adequate drug samples exist, the active compounds are tested in the primary and secondary curative test systems.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 070 Anti-schistosomal Drug Development

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6526	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A831	00	071
b. CONTRIBUTING				
c. CARDS 114F				

11. TITLE (Precede with Security Classification Code)

(U) Field Studies of Rickettsiosis and Other Tropical Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 75		259
CURRENT FISCAL YEAR 76		290

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL E.L.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: USAMRU-MALAYSIA
Institute for Medical Research
ADDRESS: Kuala Lumpur, Malaysia

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: de Witt, Dr. G.F.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Huxsoll, LTC D.L.
NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) R. tsutsugamushi; (U) Human infections; (U) Laboratory diagnosis; (U) Epidemiology; (U) Ecology; (U) Immunity; (U) Leptotrombidium spp

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To investigate the incidence of scrub typhus infection in selected local and foreign populations, to relate this to the prevalence of vector chiggers and their common rodent hosts, to develop improved serological methods, to study the basis for immunity, to define vector-rickettsia relationships, and to produce laboratory animals in support of the mission. Scrub typhus is an incapacitating disease of military importance.
24.(U) Coordinated studies are being conducted at selected field sites in an effort to relate the incidence of scrub typhus in humans to numbers of vector chiggers, rodent hosts, and the strains of R. tsutsugamushi prevalent in each. Standard techniques developed here and in other laboratories are employed in other aspects.
25.(U) 74 07 - 75 06 Local and foreign populations have been found which are sustaining significant infections with R. tsutsugamushi. Rodent hosts of the common vector chiggers have been trapped in the areas where several of the populations are exposed to the disease. Rickettsial isolations have been achieved from humans, rodents, and chiggers; and the strains are being identified. A micro-IFA test which produces equivalent data in a shorter period of time with a saving in reagents has been developed. Passively transferred convalescent sera do not completely protect mice against challenge with homologous scrub typhus strains and is ineffective against heterologous strains. L. L. fletcheri and L. L. arenicola chigger colonies infected with R. tsutsugamushi have been employed to study the effects of the duration of feeding and ambient temperature on transmission of the organism. Samples have been shipped to WRAIR to examine the ultrastructure of organism in the vector and the fate of the rickettsia following attempted infections. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 74 - 30 Jun 75.

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Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 071, Field Studies of Rickettsiosis and Other Tropical Diseases

Investigators:
Principal: Dr. George F. de Witt
Associate: LTC David L. Huxsoll, VC; LTC David M. Robinson, VC; MAJ Clifford R. Roberts, VC; MAJ Grahaem W. Brown, RAMC; MAJ Alexander L. Dohany, MSC; CPT Lyman W. Roberts, MSC; Miss Elsie Gan, B.S.; Mr. Sim Ah Bah, B.S.

1. Epidemiology and Ecology of Rickettsia tsutsugamushi.

Studies in this unit in the past have added significantly to knowledge of vector chiggers and their rodent hosts. However, with few exceptions, studies on human infections have not been pursued. To completely accomplish the goal of providing sites and means whereby emerging concepts of rickettsial disease can be tested, it became imperative to add studies on the human disease to the research program. Investigations during the past year have included a number of sites and populations which fall into three broad categories: (1) normal populations, (2) febrile patients, and (3) military populations. Although studies at some of the sites have been fully operational for only 2-3 months, our results show that studies on the human disease are feasible.

In-depth studies on the epidemiology and ecology of the disease have been initiated at the various study sites. Studies described below under "Normal Populations" and "Febrile Patients" involve every department in the unit. Further, having identified sites and populations in which studies of the human disease are feasible, field studies on the epidemiology and ecology of the disease have been confined, for the most part to these areas. In addition to yielding important clinical, epidemiological, and ecological information these studies are providing valuable specimens, i.e. sera and isolates, for other studies.

The primary objective of our first year's study was to identify populations in which the incidence of scrub typhus was sufficiently high to warrant detailed studies.

In order to identify infections in which clinical effects may vary from asymptomatic to severe, studies were conducted in three broad population categories:

(1) Normal populations
(2) Febrile patients
(3) Military populations

(1) Normal Populations were selected in areas of high endemicity. The groups resided in:

(a) Bukit Lanjan is an Aboriginal village on the jungle fringe near Kuala Lumpur. A population of 250 people live there, and pursue a variety of occupations, both urban and in the jungle.

Capillary blood was collected on filter paper from 186 people in a preliminary survey and screened for antibody at a dilution of 1/50. A total of 81% of the males over 25 and 34% of the total population were positive. In young children, 9% of those up to the age of 9 years were positive; but antibody was detected in several infants under 1 year of age.

The unit has surveyed the small rodents and chiggers in this area over the past several years. See Annual Reports for 1970-1974. As these data were available no additional sub-human work was conducted in the area this year.

(b) Pos Iskandar is the 'administrative center' of a group of Aboriginal villages in a large area of secondary jungle in southern Pahang. It is approximately 80 miles from Kuala Lumpur on the edge of Tasek (Lake) Bera. About 1000 people live in the area, practicing mostly 'slash and burn' shifting cultivation in the surrounding jungle.

Monthly visits are made to the area and samples and data collected. A medical orderly who staffs a small clinic in the area cooperates by keeping a log-book of patients. Multiple sera have been collected from about 350 people, with no difficulty. The sero-positivity rate is 50%, but no sequential results are yet available.

Rodents and chiggers are being collected from the same area, but rickettsial isolation results are not yet complete.

(c) Estate Workers. Elmina Estate is a mixed rubber and oil-palm plantation, about 20 miles west of Kuala Lumpur. There are over 100 workers in each type of plantation, and little interchange takes place. Also, the workers generally have well defined jobs: weeders, harvesters, tappers etc.

A monthly clinico-serological study on the oil-palm group has been started. The hospital assistant who staffs the hospital on the estate assists the collection of clinical data by maintaining a record of all illnesses.

Filter paper spots were collected from rubber workers, and oil-palm workers in a preliminary survey. Fourteen (15%) of the 93 rubber

workers, and 54 (40%) of the 135 oil-palm workers were positive for antibody. None of the rubber workers had worked previously in oil-palm. A decision was made to continue the survey on only the oil-palm workers and to broaden the scope of the study to include the small rodent and chigger populations.

The trapping of rodents was confined to an area containing fully mature oil-palm trees of about 12 years old. The area, covering 12 800 sq. meters, was divided into a grid system incorporating 200 trapping points spaced out at intervals of 8 meters on an east-west axis and 8 meters on a north-south axis. Trapping of field rats began in December 1974 and will continue for a full 12-month period.

Trapping is being carried out for a full 7 days the first week of each month. On the first 5 days, the captured rats are marked and released the following day to the original point of capture. Rats captured on the last two days are sacrificed. Before the marked rat is released it is examined for ectoparasites, a blood sample drawn to detect rickettsemia, and a filter paper spot prepared for assay of R. tsutsugamushi antibody. The sacrificed animals are processed similarly with the addition of examination of blood and spleen/kidney pools for the presence of rickettsia.

The fluctuation in monthly catches (Table 1) are probably due to physical factors such as rainfall and temperature in the study area. An estimation of the population of the rats can be computed by using the "Lincoln Index" based on the monthly mark-release and recapture. (See Table 1).

Table 1. Estimated population of R. tiomanicus at the Elmina Estate study site.

Month	No. of rats marked	No. of rats recaptured	Total	Estimated population
December 1974	68	4	72	455
January 1975	65	8	73	112
February	37	4	41	132
March	26	8	34	78
April	49		51	223
May	68	12	80	373

Chiggers have been collected from these rodents, as well as at 22 established black plating sites within the trapping area. Ten black plates are checked at each site, giving a total of 220 plates from which a relative index of the chigger population can be determined.

only 2 species have been identified in large numbers. The scrub typhus vector chigger L. (L.) deliense represented 68.5 percent of the total 6394 chiggers identified from Rattus tiomanicus jalorensis, while Ascoschongastia (L.) indica totaled 26.6 pecent. The other four species identified to date, in exceedingly small numbers, were: Gahrliepia (Walchia) lewthwaiti (3.9), A. (L.) lorius (0.6), Walchiella impar (0.1%) and L. (L.) fletcheri (0.3%).

R. tsutsugamushi has been isolated from whole blood, tissue pools, or both from several rats collected early in the experiment. Other specimens have been stored and will be processed as time permits.

(2) Febrile Patients

(a) Bukit Mendi Clinic

Bukit Mendi is a FELDA (Federal Land Development Authority), government managed, worker's cooperative. It is located in central Pahang, lying in the middle of a triangle formed by Karak, Temerloh and Kuala Pilah, just south of Kemasul Forest Reserve. The jungle is cleared in stages, and oil-palm is planted. All stages currently co-exist, from mature plantation, to newly-felled but not burned jungle, to undisturbed jungle itself. The total population is approximately 10,000.

Of the 46 cases of scrub typhus reported to the Ministry of Health in 1974, 6 were in settlers on this scheme, and a further 5 came from neighboring oil-palm schemes. (Personal communication, Director of Medical and Health Services, Pahang).

Medical care is furnished to the settlers by a clinic located on the FELDA scheme and staffed by a hospital assistant and nurses and visited weekly by a doctor. Cases requiring hospitalization are sent to the 2 District Hospitals, at Kuala Pilah and Mentakab.

All patients attending the clinic with a history of fever, cough, headache, or any general malaise (deliberately loosely defined), are entered in the study. Venous blood is drawn, mice immediately inoculated intraperitoneally, and a further whole blood specimen is placed in liquid nitrogen as a back up sample for the isolation attempts. The serum is decanted from the remainder of the blood sample, divided into two aliquots, and frozen.

The patients then receive treatment, as usual, from the health center staff and are asked to return in 2 weeks for a further specimen of blood to be taken. If they do not appear, the technician visits the house to obtain the specimen. Only serum is collected on the second occasion. Cooperation is excellent.

We have collected 93 pairs of sera in the first 9 weeks of this study. When screened at a 1/25 dilution both sera of a pair were negative in 20 individuals (21.5%); 51 (54.8%) were positive without a significant

rise in titer. In 16 (17.2%) of the pairs, there was a 4-fold or greater rise in titer. In a further 6 (6.5%) there was a rise to positive at 1/50 from negative at the screening dilution. None of these people had eschars or noticeable rashes.

From the 22 patients whose titers rose significantly, we have 8 presumptive isolates.

Recently a field team was sent to Bukit Mendi to collect small rodents and chiggers in areas where humans had been working prior to becoming ill. A total of 47 small rodents were collected and isolation, serological, and chigger infestation results are pending.

(b) Hospitals

Clinical data, plus pairs of sera are collected from febrile patients in a number of hospitals. As far as is possible, the patients are unselected i.e. they are not excluded by a diagnosis such as malaria.

(i) Mentakab Hospital is 80 miles from Kuala Lumpur and 40 miles from our study site at Bukit Mendi. It is in an area containing many oil-palm development schemes. One of our technicians collects the sera, and inoculates mice from all febrile patients. Of the first 50 patients studied 28% had a four-fold or greater rise in antibody, and we have 5 presumptive isolates to date.

(ii) Kuala Pilah Hospital is about 60 miles from Kuala Lumpur, and lies south of Bukit Mendi. Together with Mentakab, it provides the hospital care for a large area of central Malaysia, and for all of our potential patients at Bukit Mendi. The hospital staff collect the sera for us, but are only able (due to pressure of work) to inoculate mice from selected patients.

Data have been collected from 195 patients in the first 10 weeks of the study at Kuala Pilah Hospital. In 8 (4.1%) pairs of sera, there is a 4-fold or greater rise in titer. One of these patients had "classical" scrub typhus, with an eschar, rash and adenopathy. In a further 17 (8.7%) pairs, there was a rise from negative at 1/25 to positive at 1/50.

(3) Military Personnel

(a) Non-indigenous soldiers

We are investigating the incidence of infection with R. tsutsugamushi in soldiers operating in Malaysia, as opposed to the incidence of "classical" scrub typhus.

British and New Zealand soldiers training in jungle areas in Johore for relatively short periods comprise the study groups. Eacl man completes a questionnaire with personal details and history of previous exposure. Blood is drawn for serology prior to and after

exposure. Blood from selected groups of soldiers are also inoculated into mice for attempted isolation of rickettsia. Medical records of all positives are examined, and most positives are recalled for interview about any symptoms.

Multiple samples at various time intervals have been obtained from about 900 troops. These were screened by the F.A. technique for evidence of infection with R. tsutsugamushi.

In the first group of 375 men, sera were collected from them on arrival in Singapore and at the end of a 6 week period of training in Johore. Twenty-three (6.1%) of the second sera were positive, and only 1 of the corresponding first sera was positive. Thus 22 (5.9%) developed antibody during their training. The presence of antibody in the first specimen from the other man is unexplained as he denied any previous service in endemic areas. Both his sera had antibody at 1/50 dilution to both Karp and Gilliam antigens.

In the second group, of 50 men, three sera were obtained from each man prior to exposure, immediately after 5 weeks of jungle training, and again 4 weeks after the second bleeding. At the time of the second bleeding, pairs of mice were inoculated with each man's blood. A total of 5 (10%) of this group developed antibody during training. Results of the mouse inoculation are not yet available.

A total of 28 (6.6%) of the whole 425 thus developed antibody. 9 (32.1%) of these men admitted some form of illness - cough, fever, malaise etc. Forty-seven (12%) of the 397 with no antibody admitted similar illnesses.

Pairs of sera were also collected from 264 British and New Zealand soldiers stationed in Singapore, before and after a particular jungle exercise. These men had all been exposed several times in the previous year. A total of 32 (12.1%) of the soldiers had positive sera. Of these, 4 (1.5%) had 4-fold rise over the period of observation. An additional 14 (5.3%) showed a rise from less than 1/25 to 1/50. None of the 32 men had a history resembling "classical" scrub typhus from the questionnaire, medical documents or interview data.

(b) Indigenous soldiers

Sera and clinical data have also been obtained from 280 Malaysian soldiers, at the beginning and end of their recruit training (6 months).

Two battalions of Malaysian soldiers, consisting of 1000 men were bled prior to 3-4 months of jungle operations on the Thai border. A second specimen will be collected following the operation.

No results will be available until the second sera are drawn, and the sera pairs studied by the FA test.

Trans-placental Passage of Antibody/Infection

R. tsutsugamushi can persist in tissues for years following infection; and transplacental infection of foetuses, as measured by raised IgM levels, occurs in Q fever. Therefore we are collecting mother/cord pairs of sera from a group with a high incidence of scrub typhus antibody - Aborigines at Gombak Hospital.

The sera will be examined for IgG and IgM levels using commercial Mancini plates (Behring-Co). Sera will be tested for IgM and IgG specific fluorescent antibody. We thus hope to demonstrate the presence or absence of transplacental infection or passage of maternal antibody. Preliminary results show that a number of the cord specimens are positive when screened against polyvalent antigens, using labeled anti whole human globulin.

From results obtained during the past year we feel that several populations have been identified in which the incidence of scrub typhus is sufficiently large to warrant detailed studies.

2. Adaption of a Micro-indirect Immunofluorescence Test to the Detection of R. tsutsugamushi Antibody.

Indirect immunofluorescence (IFA) has been employed to detect antibody conversions (1) and to study the maximum response to the Karp, Kato and Gilliam strains as well as the temporal relationships in the development and persistence of the antibodies (2). In both cases the reaction was specific, reproducible, and more sensitive than the CF tests. In those cases where the infecting strain was known the homologous antibody appeared more rapidly, attained a higher titer, and persisted for a much longer period of time.

A microimmunofluorescence (micro-IF) test has been applied to the study of antibody responses to Chlamydia trachomatis infections in humans (3). This test proved to be specific and in general the infecting strain antigen could be precisely determined (4). We have adapted the micro-IF test to a study of antibody produced as a result of natural human R. tsutsugamushi infection to determine its feasibility and reproducibility in detecting the response to the major and minor antigens present in R. tsutsugamushi strains.

Antigens were prepared by inoculating eggs by the yolk sac route with 0.1 ml of a 10^{-1} or 10^{-2} dilution of the seed which was stored as a 20% yolk sac suspension in sucrose phosphate glutamate buffer (SPG) (5). Yolk sacs were harvested from the live eggs when about 20% of the inoculated eggs were dead. Following freezing and thawing the yolk sacs were diluted to a 20% suspension with 0.01N phosphate buffered saline (PBS) pH7.6 and disrupted by treating the chilled suspension in an ice bath with an Omni-mixer (Ivan Sorvall, Inc., Newton, Connecticut) for 4 intervals of 15 sec each at 25,000 rpm. These intervals were interspersed with 30 sec cooling periods. The

suspension was centrifuged at 450 xg for 10 min. The middle layer was removed with a syringe and needle, and serial 2 fold dilutions of this preparation were examined by means of the micro-IF test employing high titer human sera. The proper concentration was determined and the suspension was diluted with PBS, dispensed in 0.1 ml amounts and stored at -169C.

The slides were prepared with 3 rows of antigen spots. Each row contained 6 groups of spots, and each group was composed of 9 individual spots. Thus each slide contained 162 separate antigen spots. The spots were applied by positioning the slide over the template and touching the spot with a pen nib (Kingsley 2788; Hinks, Wells and Co., England) filled with antigen. The volume of the spot delivered was approximately 0.1 lambda. When the spots were complete the slides were dried at least 30 min at room temperature prior to a 10 min fixation in acetone. The fixed slides were allowed to dry at room temperature and stored at -20C for not longer than two weeks.

The antigen slides were warmed to room temperature in circulating air to remove condensate. Each group of 9 microspots was covered with 5 lambda of the appropriate dilution of a serum. The slides were incubated in a humidified cabinet at 35C for 30 min. Following removal of the bulk of the sera by washing with PBS, the slides were immersed in PBS on a magnetic mixer for 10 min. They were then blotted dry and 5 lambda of the appropriate dilution of conjugated rabbit origin anti-human globulin was placed over each group of spots. During the application of both the test sera and the conjugated antiserum care was taken to ensure no mixing occurred between the groups of spots. Following incubation, washing, and drying as described for the test sera the slides were mounted with 25 lambda of 90% glycerin with 10% 0.1N PBS pH 9.4 and a 22 x 50 mm cover slip. The slides were usually read within 24 hr although they could be stored several days at 4C without affecting the results. The criteria of Gan et al (6) were employed to determine endpoints.

Table 2 presents the data derived when a number of sera were simultaneously studied at a 1/50 dilution with the IFA and the micro-IF. While the two methods were in agreement on 151 of the 179 samples (85%) there were obvious discrepancies. Although they detected approximately equal numbers of positive sera these did not completely coincide. The IFA detected antibody in 12 sera which were negative in the micro-IF screen while the micro-IF detected antibody in 16 sera which were negative in the IFA. When these 28 sera were retested many results were at variance with the original test. To examine a possible reason for these discrepancies the sera were titrated beginning at a 1/25 dilution. All 28 of the sera were positive at the low dilution, but the reaction at a 1/50 dilution was variable and none of the sera were positive at the higher dilutions.

Table 2. Correlation between the indirect microimmunofluorescence test and the indirect immunofluorescence test when employed as screening tests.

	IFA		
Micro-IFA	Positive	Negative	Total
Positive	68 (38)	16 (9)	84 (47)
Negative	12 (7)	83 (47)	95 (53)
Total	80 (45)	99 (56)	179

Comparison of titers

Figure 1 compares the titers obtained from representative sera by the two techniques. This figure combines both IFA data derived from 2 fold dilutions as well as 4 fold dilutions recommended by Bozeman and Elisberg (1). Only 8 of 126 sera (7%) had titers that differed $\geq$ 4-fold in the two systems. Repeat titrations of these sera in the micro-IFA system yielded titers within 1 dilution of the previous micro-IFA titer rather than the commonly lower value seen with the IFA technique. The proportion of the sera which differed $\leq$ 1 dilution in the two systems was 78% (98/126).

A comparison of the values indicated that while 58% of the titers were higher in the micro-IF test only 21% were lower. The remaining 21% of the sera gave equivalent titers ir the two systems. A serum which titered 1/40 in the old system commonly titered 1/50 in the micro-IF; a serum which titered 1/160 in the old commonly 1/200 in the micro-IF. If these sera are not included in the totals as being higher in the micro-IF 29% of the sera gave higher titers in the micro-IF test and 21% gave lower titers ($P > 0.05$).

A series of 149 sera were examined by the micro-IF on two occasions two months apart. The titer was the same in 61% (91/149) of the tests, varied from $\geq$ 1 to $\leq$ 2 fold in 36% (53/149), and was 4 fold different in 3% (5/149). In no case were the results greater than 4 fold different.

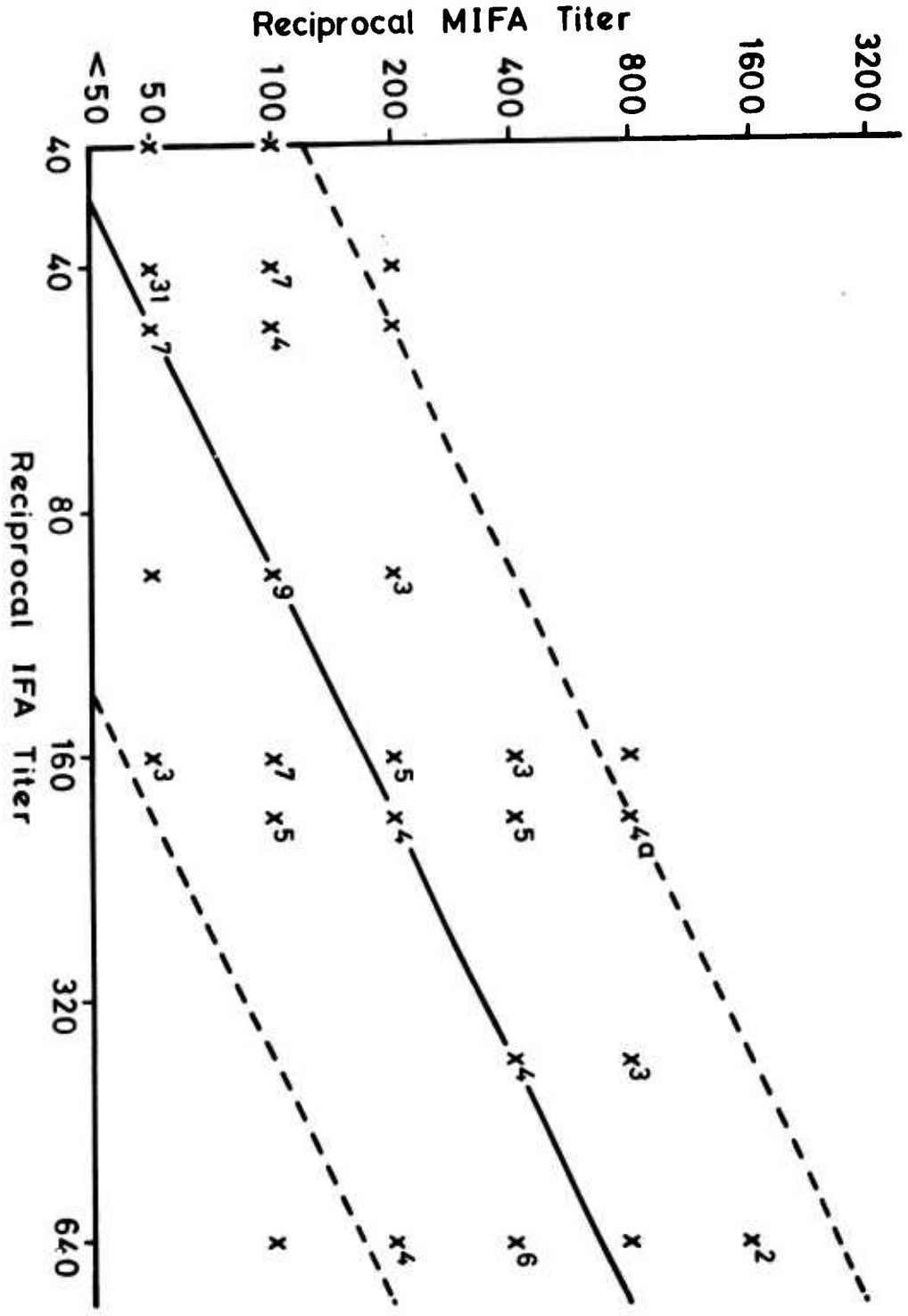
The variation between the procedures occurred in sera containing low titers of antibody; and while endpoints seldom varied more than 1 dilution, the presence of a high proportion of sera with low titers led to greater variation in screening tests. The variation was as prevalent in sera tested repeated by the same system, as when the repeat assay employed the other system. This difference was, therefore, inherent in some groups of sera and little could be done to minimize it. In any case, the variation tended to even itself out yielding an equal proportion of positives in each test. In sera collected from older individuals who lived in hyperendemic areas in which the positive sera generally titered $\geq$ 1/100 no discrepancies were noted between the systems.

Fig. 1 - Comparison of the titers obtained when sera were tested by the microimmunofluorescence test and the immunofluorescence test.

——— line of equivalent titers; ----- line ± a 4 fold titer from equivalence.

a; 3 sera titered 1/800, one serum titered 1/400 on retest with the micro IF test.

Figure 1


Reciprocal MIFA Titer
<50
50
100
200
400
800
1600
3200
Reciprocal IFA Titer
40
40
80
160
320
640

Titers appear somewhat higher in the micro-IF test than in the original system, but since the old system yielded titers of 1/40, 1/160, 1/640, etc. and the new system 1/50, 1/100, 1/200, etc. the number of sera which gave higher titers in the micro-IF test did not differ significantly ($P > 0.05$) from the number which gave lower titers.

Titers derived from repeat titrations in the micro-IF test corresponded with each other more closely than the 93% correspondence within the $\pm < 4$ fold limits which was found when the micro-IF test was compared to the regular IF test. This indicated that the results from the new system were at least as reliable as those from the old system. Although tedious it was possible for a technician to prepare 40 slides a day which was enough to screen 680 sera with controls at a single dilution. This was also sufficient to titer 240 sera through 1/200 or 120 sera through 1/1,600 against any 9 strains. Since sera from certain areas and populations had varying peak titers which were a relatively constant property we were able to chose dilutions to encompass these endpoints without wasting reagents.

3. Protection Against Scrub Typhus Infection Engendered by the Passive Transfer of Immune Sera.

The inability of passive antibodies to protect mice has led us to initiate a series of investigations into the mechanisms of protection operable in the resistance to challenge following recovery from infection. Since R. tsutsugamushi represents a mosaic of strains with varying antigens and virulences, we decided to initially define the contribution of humoral antibody to resistance to challenge with both homologous and heterologous strains.

Sera were prepared by the intraperitoneal (IP) inoculation of 100-1,000 median infectious doses for mice (MID_{50}) of the specific strain. The mice were bled by cardiacpuncture from 30-60 days following inoculation. Infection was controlled in those strains lethal for mice by adding 2.5 mg of chloramphenicol per ml of drinking water from day 3 to day 24 post inoculation. Sera pools represented no fewer than 50 mice and were inactivated at 56C for 30 min prior to inoculation. All sera were from mice convalescent from 1^{o} infections, and no attempt was made to produce hyperimmune sera.

Since all strains of R. tsutsugamushi were not equally efficacious in stimulating antibody production we titrated the antisera against the several antigens present on the selected strains. The results in Table 1 show that the highest homologous titer was produced by the Karp strain at 1/1,280 while the TA 763 and TC 586 strain were 2 fold lower. A significant reciprocal cross reaction was seen with the Karp and TA 763 strains. The TC 586 strain did not cross with either of the other two strains.

Table 3. Titers of antisera employed

Antigen	Antisera		
	Karp	TA 763	TC 586
Karp	1280[a]	160	< 10
TA 763	320	640	< 10
TC 586	< 10	< 10	640

[a]Reciprocal indirect fluorescent antibody titer.

Figure 2 presents the results obtained when predetermined volumes of convalescent Karp strain sera were inoculated subcutaneously (SQ) 1 hr prior to intraperitoneal (IP) challenge with the homologous strain. Although significant protection ($P < 0.025$) was conferred by the administration of the sera, significant differences ($P > 0.05$) were not detected among the doses within the 4 fold range of volumes of immune sera inoculated. This was true even though the higher volumes of sera prolonged the survival times of the mice. The largest dose, 1 ml of whole immune sera, was capable of protecting only 18% (7 of 40) of the inoculated mice.

To investigate the result of varying the challenge dose a group of mice were inoculated with graded doses of the Karp strain following the inoculation of 0.5 ml of homologous convalescent sera. The results of this experiment are shown in Figure 3. An inoculated dose of $10^{3.4}$ median lethal doses (MLD_{50}) was also employed, but since the values fell between the other two doses they are not shown. The number of survivors was not significantly different ($P > 0.05$) with the three doses employed in the challenge. The smallest challenge dose (250 MLD_{50}) did not kill 100% of the non-passively immunized controls.

To investigate the temporal relationships involved in the transfer of immune sera we inoculated 0.5 ml of Karp immune sera SQ into groups of mice from 2 days before challenge with the Karp strain until 8 day post challenge at 2 day intervals. In those groups which received sera on days -2, 0, +2, and +4 from 15 to 26% of the mice survived and no significant differences were detected ($P > 0.05$), but in the groups given sera on days 6 and 8 no mice survived and no differences in death times were noted from the control group given normal sera.

Since we had been successful in protecting a portion of the mice against challenge with a mouse virulent strain by the transfer of immune sera we investigated the response of passively immunized mice

Captions

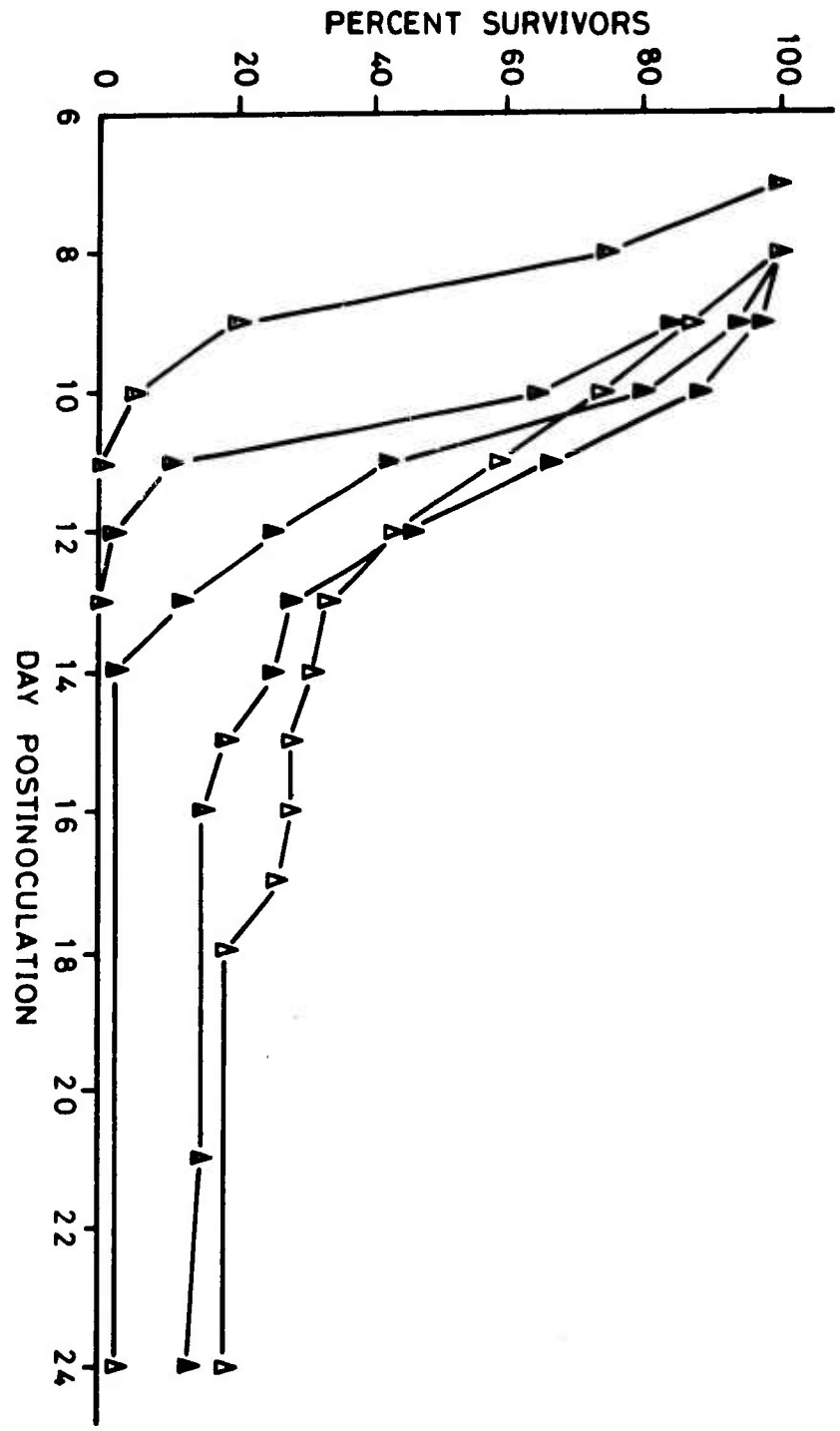
Fig. 2 - Response of mice to Karp challenge following the administration of graded doses of homologous convalescent sera.

△ no sera, ◺ 0.5 ml normal sera, ▲ 0.25 ml convalescent sera, ◭ 0.5 ml convalescent sera, 1.0 ml convalescent sera.

Fig. 3 - The effect of varying the challenge level on the survival of mice following the administration of normal and convalescent sera.

△ ---△ normal sera following by $10^{4.4}$ MID_{50} challenge, ▲ ---▲ immune sera with $10^{4.4}$ MID_{50} challenge, △ ——△ normal sera with $10^{2.4}$ MID_{50} challenge, ▲ ——▲ immune sera with $10^{2.4}$ MID_{50} challenge.

Figure 2


PERCENT SURVIVORS
0
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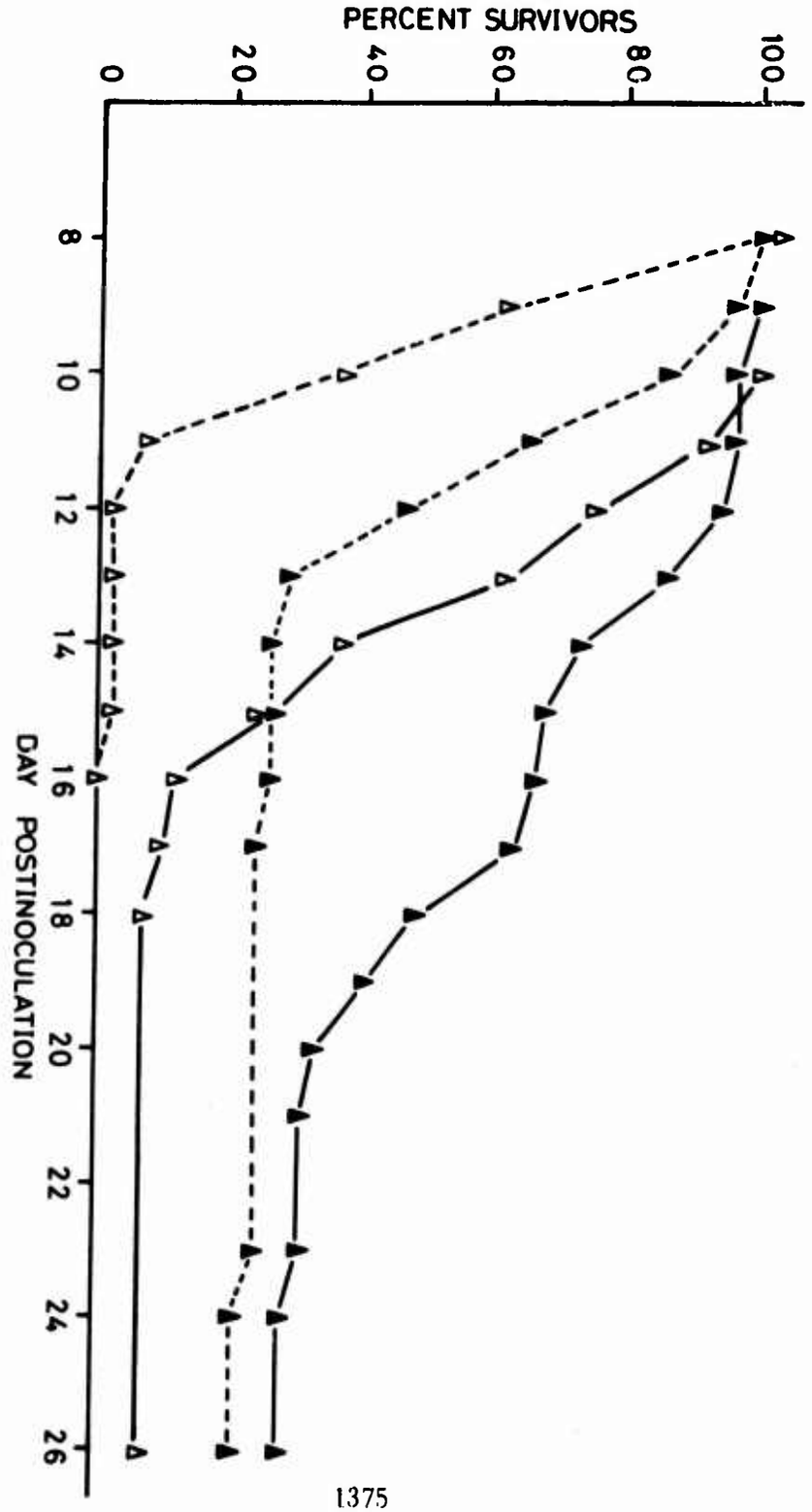
22
24
DAY POSTINOCULATION

Figure 3


PERCENT SURVIVORS
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DAY POSTINOCULATION

to heterologous as well as homologous challenges. The results of these experiments are presented in Table 4. Earlier a challenge dose of 63,000 MID_{50} of the TC 586 strain had killed 85% of the control mice which had been given 0.5 ml of normal mouse sera. A challenge dose of 630 MID_{50} killed 76% of the mice, and survival times were 3-4 days longer. However, the death rate of mice given normal sera again paralleled that of mice given immune sera and no significant differences ($P > 0.05$) could be detected in the survival rates or times. For this reason the data presented were derived from challenge doses of $10^{2.4}$ to $10^{2.8}$ MID_{50} depending on the experiment. Other serial 10 fold doses were employed, but since the data were comparable they are not shown.

Table 4. Response of passively immunized mice to homologous and heterologous challenges.

Challenge strain	Convalescent Sera			
	Karp	TA 763	TC 586	Normal
Karp	5/40[a]	4/40	0/40	0/40
TA 763	1/40	6/40	2/40	1/40
TC 586	4/40	6/40	4/40	6/40

[a]Survivors/total

Karp and TA 763 immune sera were equally effective against Karp challenge. This could be expected because of their close relationship in the fluorescent antibody test (Table 3). TC 586 sera was not effective against either Karp or TA 763 challenge. Again this could be proposed from the lack of relationship in the FA test. However, the complete lack of protection when compared to control values in the homologous TC 586 challenge was surprising. While 10% of the individuals did not die following administration of TC 586 immune sera and homologous challenge this was no different than the proportions following administration of normal sera followed by challenge or challenge alone. The failure of Karp immune sera to protect against TA 763 challenge also remains unexplained.

A group of mice were inoculated with 0.2 ml containing $10^{2.9}$ LD_{50} of the Karp strain which had been incubated for 30 min at room temperature with 0.5 ml of immune sera. Controls were reacted under the same conditions with normal sera. All of the control mice were dead by day 11 postinoculation, but 85% of the mice inoculated with rickettsia treated with immune sera *in vitro* were alive at the termination of the experiment on day 28 postinoculation.

Rickets (7) reported that immunity to spotted fever infections could be transferred by cell free sera. Gambril and Wisseman (8) found

that the passive transfer of immune sera from a hyperimmune human was capable of protecting mice from a normally lethal dose of R. mooseri; but neutralization of R. rickettsia or R. australis by convalescent sera could not be demonstrated in a cell culture system (9). These conflicting results were explained by the requirement of coating of the rickettsia with specific antibody as a prerequisite for destruction of the organism following phagocytosis.

In our system the antibody was not capable of preventing death in the majority of individual mice when it was administered passively. However, when the sera were preincubated with the rickettsia the majority of the mice survived inoculation of the sera/organism mixture. The existence of antigen-antibody complexes in vivo can be demonstrated in scrub typhus infections by specific staining of rickettsia in mouse peritoneal fluid with fluorescein isothiocyanate labelled antimouse globulin. The presence of such complexes, persistent rickettsemias in the presence of circulating antibody, and the inability of passively transferred antibody to protect the majority of mice indicates that humoral antibody may not be important in determining the fate of R. tsutsugamushi in vivo. The transfer of peritoneal fluid containing organisms which are coated with antibody results in a course of illness in the recipient indistinguishable from the course produced by the inoculation of equivalent doses of rickettsia of egg yolk sac or cell culture origin. Additionally, sera administered as late as 4 days after challenge was as effective in protecting mice as sera administered simultaneously. These two observations would seem to diminish the importance of coating with antibody to the in vivo fate of R. tsutsugamushi.

The increased protection found when the immune sera was incubated with the microorganism prior to inoculation is unexplained. Several possibilities exist. Kenyon and McManus (9) were not able to demonstrate neutralization of R. rickettsii or R. australis in cell culture employing immune sera alone. However, when a standard amount of anti-globulin was added a decrease in the number of plaques was found with increasing immune sera concentrations. It would appear that we detected neutralization by immune sera alone, but we did not conduct parallel titrations in egg yolk sacs. Therefore, we can't comment on the physical state of the organisms. It is possible that immune sera produces aggregation of the rickettsia and that each aggregate is handled as an infectious unit which would effectively decrease the titer. Rickettsial agglutination tests have been employed to quantify antibody for several years (10). It is also possible that inoculated rickettsia promptly enter cells and escape the complete effects of being coated with immune sera.

The immune mechanisms operable in the recovery from R. tsutsugamushi infections appear to be analogous to those in intracellular bacteria e.g. tuberculosis, leprosy, tularemia, etc. rather than in typhus organisms where passively administered immune sera is protective and immunity is complete and long lasting. Whether this points to

different evolutionary processes within the rickettsia is a matter of speculation.

4. Colonization of Trombiculid Mites.

The Acarology Section is continuing to maintain colonies of vector and nonvector mites. The infected colony of L. (L.) fletcheri is currently in the 17th and 18th generations, while the colony of infected L. (L.) arenicola is in the 6th generation.

Rapmund (11) presented a detailed study of the first 5 generations of the L. (L.) fletcheri colony, and its progress has been reported in previous USAMRU-M Annual Reports. Much of the rearing after the 5th generation was conducted in pools, with individuals being studied only in the 9th and 10th generations. In the 11th generation, three lines (11158, 11203 and 11408) were established from a single adult (10885) of the 10th generation. Pools are obtained from a common pool of larvae collected from all the adults of the preceding generation. Of 153 pools tested through the 16th generation, 94.8% were positive for Rickettsia tsutsugamushi. In the 17th and 18th generations, negative pools became apparent. Currently, comprehensive data is available only on line 11203, although, to date, tests from line 11408 have produced only negative results. In addition to the change in the rate of infection within the colony, the sex ratio (previously 100% females) has also changed. Table 5 presents the infectiveness and sex ratio data for the 18th generation of line 11203. Of 27 pools obtained from a common egg pool, 26 were positive and one was negative. Of 46 pools derived from single adults, 10 were positive and 36 were negative. The sex ratio of 16 of the 26 positive pools from the common egg pool was determined, as well as the one negative from the same pool. The positive pools contained 163 females and 36 males (81.9% female), while the negative pool contained 8 females and 9 males (47.1% female). Of the pools derived from single adults, two positive and 18 negative pools were available for sex determination. The two positive pools produced 14 females and no males (100% females), while the 18 negative pools produced 85 females and 87 males (49.4% females). The reason for the change within this colony is not known at the present time; however, intensive studies have been initiated in an effort to determine the cause.

Table 5. The infectivity and sex ratio of the 18th generation (Line 11203) of a Rickettsia tsutsugamushi-infected colony of L. (L.) fletcheri.

Transmission during feeding	Total No. of Pools	No. of Pools Sexed	Sex Ratio (♀/♂)	Percent (♀/♂)
		Common Egg Pool		
+	26	16	163/36	81.9/18.1
-	1	1	8/9	47.1/52.9
		Individual Adults		
+	10	2	14/0	100.0/0.0
-	36	18	85/87	49.4/50.6

The filial infection and transovarial rates of the fifth generation of the infected L. (L.) arenicola colony were similar to those reported in the past. 23 F_4 adults were separated for study of their progeny. Approximately 20 larvae from each line were studied. Of 21 lines in which information is currently available, 353 of a total 366 were positive, giving a filial transmission rate of 96.4 percent. All 21 adults transmitted Rickettsia tsutsugamushi transovarially.

Part of the objective of the Acarology section is to gather comprehensive data on the life histories and rickettsial transmission of the three proven vectors in Malaysia; therefore it is important that an infected L. (L.) deliense colony be obtained. As was reported in the USAMRU-M 1974 Annual Report, infection rates of specific rodents were high at Bukit Lanjan, Selangor, and the only vector collected from that area during previous studies was L. (L.) deliense. Thus, it seemed likely that the infection rates of collections of L. (L.) deliense would also be high. However, this has not been the case. In addition to the negative results reported in the previous annual report, an additional 38 pools, totalling 1,423 chiggers, were fed on white mice. Of these, only 5 were positive. However, due to loss during pool feeding and cannibalism within the post-larval stages, a positive colony was not established from any of these pools.

As reported in the USAMRU-M 1974 Annual Report, over 2,500 specimens of L. (L.) scutellare were collected from a banana grove in the Cameron Highlands, Pahang, in June, 1974. The final mouse passage results showed that, of the 44 pools having a total of 2,200 chiggers that were fed on white mice, no positive pools were obtained. This data corresponds with collections of L. (L.) scutellare previously made by this unit.

5. Duration of Vector Attachment and Transmission of Rickettsia tsutsugamushi to Mice.

Although chiggers usually feed to repletion on their animal hosts, they do not normally do this on humans. Abrasive action of clothing and personal hygiene tend to interrupt their feeding. Even so, humans do contract scrub typhus. Recently, this was demonstrated when two members of the British Army contracted scrub typhus after investigating a helicopter crash in Mersing, Johore, West Malaysia. Both individuals left the crash site each night, giving a hypothetical maximum time between attachment and an evening's bath of 12 hours. It seems apparent that full engorgement is not necessary for transmission.

In an effort to determine the length of attachment necessary for a chigger to transmit scrub typhus, a series of interrupted feedings was conducted with varying lengths of feeding time. Chiggers were allowed to attach to white mice and then were disengaged by gently manipulating them with a sharpened applicator stick. Table 6 presents the data of interrupted feedings of infected chiggers of both L. (L.) fletcheri and L. (L.) arenicola.

Table 6. Duration of attachment and rate of transmission of Rickettsia tsutsugamushi to mites by two species of Leptotrombidium larvae.

Hours	L. (L.) fletcheri		L. (L.) arenicola	
	Mouse Feeding		Mouse Feeding	
	Positive / Total	Percent Transmission	Positive / Total	Percent Transmission
1	0/5	0	0/5	0
6	0/5	0	0/5	0
12	10/15	67	1/15	7
16	5/10	50	3/10	30
18	15/15	100	-	-
20	7/10	70	6/10	60
24	19/30	63	12/15	80
28	6/10	60	8/10	80
30	13/15	87	-	-
36	10/15	67	-	-
Repletion	7/10	70	10/10	100

6. Effects of Temperature on Rickettsia tsutsugamushi Infections in Naturally Infected Chiggers.

Positive larvae of L. (L.) fletcheri when treated at 40°C for 72 hours did not transmit rickettsia to white mice during feeding (USAMRU-M 1974 Annual Report). Extreme temperatures are known to have both a direct and an indirect effect upon the transmission of numerous disease organisms. Temperatures play a direct role in the incubation

times of arbovirus and in the interaction of blood clotting and infection with the plague bacillus in fleas. Temperatures are indirectly involved in the transmission of diseases by affecting the physiology of vectors, either by extending or decreasing the life cycle or by causing estivation or hibernation to occur. Jameson (12, 13) has studied the temperature-development relationships of several Japanese trombiculids, L. (L.) deliense from Malaysia, and Eutrombicula belkini from California. In general, within normal limits, developmental times decreased with an increase in temperature.

Infected pools of L. (L.) fletcheri adults were maintained in controlled environmental chambers of constant temperatures of 22° and 40°C for a period of 30 days or more. Thirty larvae from each temperature were fed singly on mice. At both temperatures, the larvae, when fed on mice, were negative for R. tsutsugamushi.

Larvae of L. (L.) arenicola were maintained at both 22° and 40°C for 24 and 72 hours after which they were fed singly on laboratory mice. In the 22°C experiment, 16 larvae were held for 24 hours and 10 for 72 hours, while in the 40°C experiment 15 larvae were held 24 hours and 13 were held for 72 hours. In all cases, the larvae transmitted the infection during feeding.

As negatives had begun appearing in the infected L. (L.) fletcheri colony, adults of both L. (L.) fletcheri and L. (L.) arenicola were separated from positive pools and were tested for their infectivity. Only known infected adults were used to initiate these temperature experiments. Several temperature treatments have been started; but, as of this report, results are not available. The treatments for both L. (L.) fletcheri and L. (L.) arenicola include: egg-laying adults treated at both 22° and 40°C for over one month; eggs and egg-deutoval stages treated at both 22° and 40°C for the duration of these stages; and additional larval treatments at each temperature. Treatment of post larval stages is planned. Bionomic studies, including developmental rates and egg production, of the temperature-treated mites are also being undertaken.

7. Ultrastructure of Rickettsia tsutsugamushi in Chigger Cells and the Fate of Rickettsiae Taken Up by Previously Uninfected Chiggers.

In an effort to study the ultrastructure of Rickettsia tsutsugamushi in chigger cells and the fate of rickettsiae taken up by previously uninfected chiggers, a cooperative study between the USAMRU-M Acarology, Rickettsia Sections and the WRAIR Division of Pathology has been instigated. USAMRU-M will supply both whole and dissected specimens of variously treated and untreated chiggers, and known positive and known negative specimens of both L. (L.) fletcheri and L. (L.) arenicola. WRAIR Division of Pathology will be responsible for studies utilizing the electron transmission microscope.

The specimens listed below have been fixed and shipped in gluteraldhyde to WRAIR for embedding and study. Additional specimens are being prepared by fixation and embedding at USAMRU-M and will be shipped to WRAIR.

	unfed larvae	engorged larvae	protonymphs	deutonymphs	tritonymphs	adults
L. (L.) fletcheri known positives	20	--	20	20	20	20
L. (L.) fletcheri known negatives	10	8	--	10	10	10
L. (L.) fletcheri Attempted Infections	--	30	20	20	--	--
L. (L.) arenicola Attempted Infections	--	20	22	20	20	11

In addition to the whole specimens listed, specimens of L. (L.) arenicola were dissected and organs and tissues were sent to WRAIR for embedding and study. Twenty specimens, all of larval and post-larval stages, were dissected. The material included:

(a) unfed larvae -- salivary glands, mid-gut
(b) engorged larvae -- salivary glands, mid-gut
(c) protonymph, deutonymphs, tritonymphs, adult -- salivary glands, mid-gut, epidermis, ovaries, excretory tubules.

8. Management of Tropical Laboratory Animal Resources.

The major effort of the Department of Laboratory Animal Medicine is the production and care of ICR outbred mice used in scrub typhus research. Production is a joint project with the IMR, and personnel, equipment, and supervisory responsibility are shared. Approximately 90,000 four week old mice were issued to investigators in FY 75, of which 75,000 were issued to USAMRU for scrub typhus related projects. The average USAMRU experimental mouse is kept 30 days, thus, our average experimental mouse population was in excess of 6,000.

Although all of the old mouse cages in the production colony have now been replaced with new cages, the experimental colony still requires approximately 600 new cages.

Serum samples from the mouse production colony were sent to Microbiological Asso. in the United States in August, 1974, for testing for antibody to 14 indigenous murine viruses. REO III antibody was found in 10% of the samples; all other tests were negative.

Dogs are now being used in small numbers (20-30). They are purchased as puppies to reduce the possibility of natural exposure to selected infectious agents. No spontaneous disease problems have occurred, but some difficulty in getting the dogs to eat ordinary dog food has been encountered. We now require that the dogs be accustomed to dry dog food before purchase.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 071 Field Studies of Rickettsiosis and Other Tropical Diseases

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6527	75 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	H. Term.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A831	00	072
b. CONTRIBUTING				
c. ~~XXXXXXXX~~	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Ecological Surveys of Tropical Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 01	75 06	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	2.0	82
FISCAL YEAR CURRENT 75	2.0	100

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

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ADDRESS: Institute for Medical Research
Kuala Lumpur, Malaysia

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: de Witt, G.F.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Huxsoll, LTC D.L.

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Malaysia; (U) Leptospirosis; (U) Malaria; (U) Anopheles; (U) Arboviruses

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To conduct bacteriological, ecological, entomological and epidemiological surveys in the transmission and incidence of tropical diseases of military importance in the Peninsular Malaysia.

24. (U) Surveys were conducted in Peninsular Malaysia at Oil Palm and Rubber Estates, and Rural Villages and in various ecologic habitats, including a unique forest transect site at Bukit Lanjan.

25. This work unit has been consolidated with work unit 071, Field Studies of Rickettsiosis and Other Tropical Diseases, Project 3A762759A831, Tropical Medicine. Progress is reported therein.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6528	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SUM'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM / A. WORK UNIT
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A831	00	073
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Provide with Security Classification Code)

(U) Disease Transmission in Tropical Populations

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 002600 Biology 005900 Environmental Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 11	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	5	275
FISCAL YEAR SUMMARY 76	6	220

19a. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20a. PERFORMING ORGANIZATION

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TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Dixon, K. MAJ, MC

NAME: Roberts, R. MAJ, MSC

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide KWIC and Security Classification Code) (U) Brazil; (U) Infectious disease; (U) Epidemiology; (U) Entomology; (U) Parasitology; (U) Virology; (U) Serology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Conduct epidemiologic studies of infectious disease transmitted among populations along the Trans-Amazon Highway in collaboration with the Institute Evandro Chagas. Information regarding the health hazards are of importance to military personnel transiting or stationed in this geographic area.

24. (U) Routine diagnostic, epidemiological, entomological, microbiological, serological and virological procedures are being employed. Emphasis is placed on field studies covering 800 Km of the Transamazon Highway and epidemic investigations with appropriate laboratory support.

25. (U) 74 07 - 75 06 Pilot studies and acquisition of disease data from area health agencies were completed. A stratified random sample of 2300 colonists along 800 Km of the highway is under biweekly epidemiologic surveillance as are all local health care facilities. Serologic studies revealed a small proportion of the sample to have arbovirus antibodies and suggested moderate acute toxoplasmosis. Scorpion and snake bites caused considerable morbidity. Malaria, predominately falciparum, was the principal disease encountered with significant extradomiciliary transmission. Infection occurred in the absence of reported primary and secondary vectors and varied greatly with season and area. Simulium biology and bite-induced reactions including thrombocytopenic purpura were investigated. An outbreak of oropouche virus was investigated in which transmission by culicoides was suspected. Investigation of 400 imported cases of schistosomiasis was begun. Two imported cases of Chagas disease were identified. Suitable vectors for each disease have been found in the study area. A new focus of onchocerciasis was investigated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1974-30 June 1975.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

PROJECT 3A762759A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 073 Disease transmission in tropical populations

Investigators

Principal: LTC Craig H. Llewellyn, MC; MAJ Kenneth E. Dixon, MC; CPT James K. Lovelace, MSC; Mr. Norman Petersen, M.S.; MAJ Donald R. Roberts, MSC.

Associate: Miguel Azevedo, M.D.; Amelia Andrade, B.S.; Gilberta Bensabath, M.D.; SFC Ignacio Bocanegra; Mario Moraes, M.D.; Francisco Pinheiro, M.D.; MSG Henry Radcliffe.

INITIAL REPORT USAMRU-BELEM (TRANSAMAZON)

INTRODUCTION:

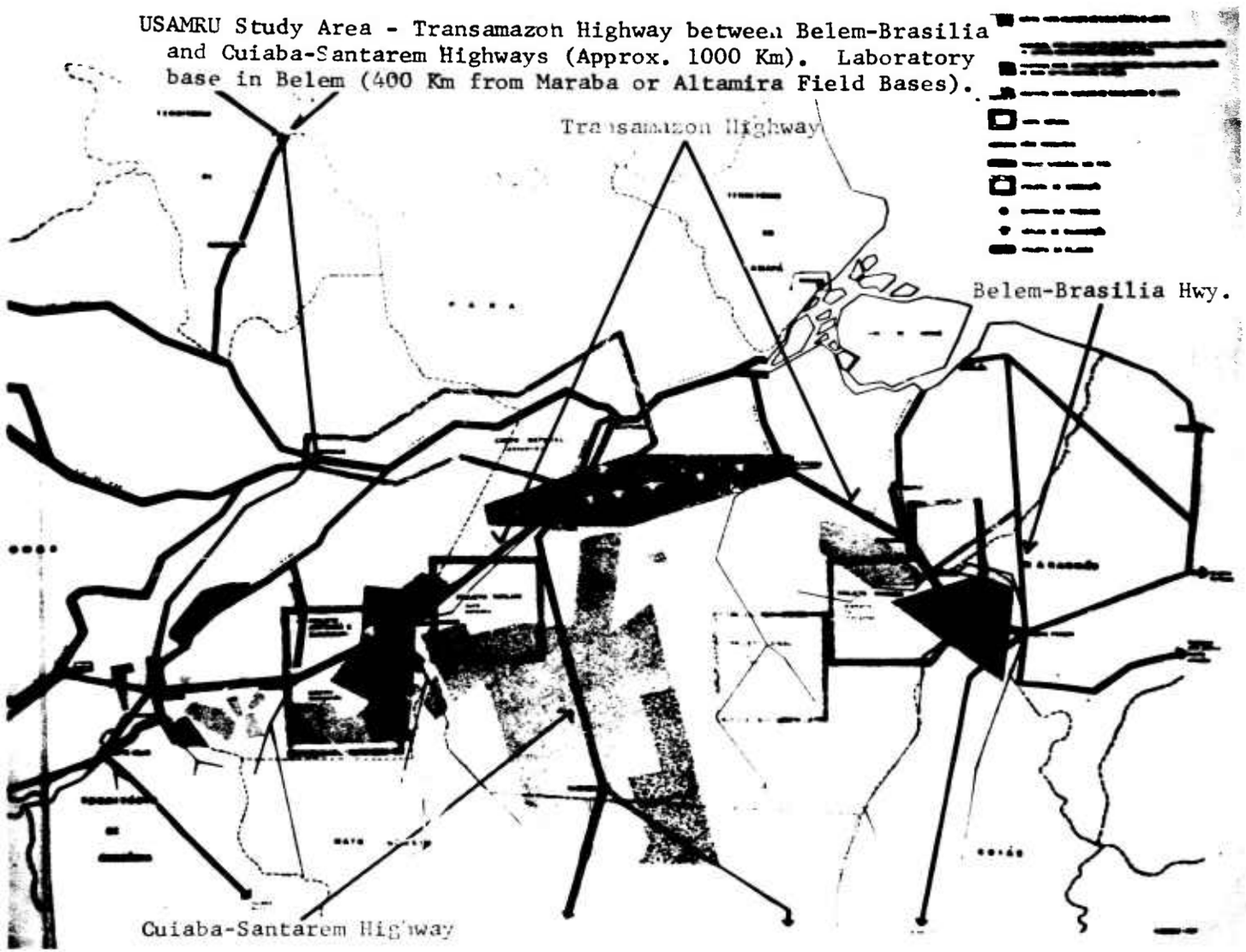
In 1970 the Brazilian government began construction of the Transamazon Highway (see map) on an east-west axis from the Belem-Brasilia Highway to Peruvian border (3,000 kilometers). A government-planned and sponsored program of colonization along the route began in 1971. At the time of official opening of the first section of the road (Maraba-Altamira-Itaituba) in September 1973, 18,000 colonists had entered and settled along the recently completed 1000 kilometers of unsurfaced roadway.

Prior to the opening of the highway, tropical disease studies in the Amazon basin had been generally confined to riverine ecology. No studies have been conducted in the newly penetrated virgin forest. The human populations available for study in these areas now include colonists from every state in Brazil who will undoubtedly be encountering some disease agents for the first time and who also may be carrying with them organisms foreign to this huge region. Ecologic change induced by man's entrance may augment or diminish the potential for transmission of various disease agents.

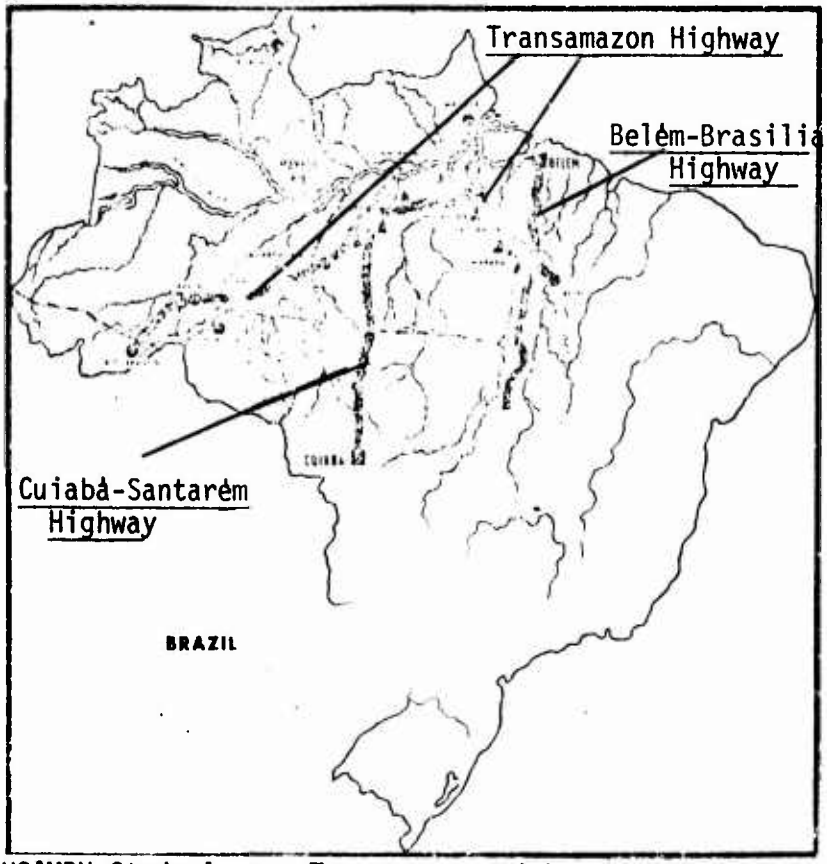
While many disease agents have been identified in other parts of the Amazon basin, only malaria (falciparum and vivax) was a documented disease hazard of high incidence along the Transamazon Highway. The scarcity of medical diagnostic and treatment facilities in the newly colonized areas, the vast distances and low density of human population, and the communication problems all made it highly unlikely that other emerging disease threats would be rapidly identified unless a broad-based program of epidemiologic studies incorporating several diseases of military medical importance would be implemented. The similarity between the ingress of the colonists (100,000 expected prior to 1980) and the deployment of troops in a medically unstudied area led to the establishment of USAMRU-Belem (Transamazon) in late 1973.

Ground reconnaissance and collection of all available demographic, disease and biomedical data pertaining to the region began in early 1974. Based upon this information and experience, pilot studies were begun in the Maraba area in July 1974. Epidemiology, entomology and wildlife ecology teams operated simultaneously along a 280 kilometer stretch of the highway for six months and demonstrated the feasibility of and necessity for this basic approach to identifying present and potential health hazards. A decision was then made to:

1. Continue the program in the Maraba-Altamira jurisdiction;



USAMRU Study Area - Transamazon Highway between Belem-Brasilia and Cuiaba-Santarem Highways (Approx. 1000 Km). Laboratory base in Belem (400 Km from Maraba or Altamira Field Bases).



USAMRU Study Area - Transamazon Highway between Belém Brasilia and Cuiabá- Santarém Highways (approx 1000 Km). Laboratory base in Belém (400 Km)from Marabá or Altamira Field bases).

2. Extend the program to the Al mira-Itaituba sector;

3. Develop the field and base laboratory support facilities for this program;

4. Initiate a program of malaria studies near both Maraba and Altamira.

By March 1975, the field surveillance programs and malaria studies had been established and during April, laboratory operations began at the USAMRU base in Belem, the Evandro Chagas Institute and WRAIR.

The present program serves to:

1. Gather baseline epidemiologic (incidence and prevalence), entomologic, and ecological information simultaneously from multiple sites in previously unstudied areas;

2. Rapidly identify and investigate outbreaks of disease;

3. Identify specific research targets for more detailed investigation.

Investigations have also been conducted in other parts of the Brazilian Amazon, an area corresponding to approximately 2/3 of the continental United States. Specifically, the investigations have included the Federal Territories of Amapa and Roiraima, the Solimoes River in the state of Amazonas from Manaus west to Benjamin Constant, areas in the state of Para east of the Belem-Brasilia highway and the riverine border between Para and Goias (see map).

Significant progress to date includes:

1. Data showing malaria transmission in the absence of the putative primary vector and suggesting the importance of extra-domiciliary transmission. Clinical observations suggest varying degrees of chloroquine resistance in P. falciparum;

2. The identification of disease outbreaks of an incapacitating dengue-like febrile illness with high attack rates caused by Oropouche virus. No vector has been identified and the sylvatic cycle is completely unknown.

3. The recognition of Simulidae as a significant health hazard due to human reactions to bites which vary from local reactions which can be incapacitating to life-threatening episodes of thrombocytopenic purpura.

The taxonomy and biology of these ubiquitous and voracious Amazonian species which are present in high densities six months per year is virtually unknown. Available repellents appear to be without effect. Each of these findings indicates a problem of military medical importance requiring further study.

I. EPIDEMIOLOGY

A. PRE-PILOT STUDY - INFECTIOUS DISEASE SURVEILLANCE

PROGRAM OBJECTIVES: The primary task of the epidemiology section is the implementation of a comprehensive communicable disease surveillance program along the Transamazon Highway. A pre-pilot study was designed to:

1. Provide first-hand observational data on the areas and population to be studied;

2. Collect data from Brazilian agencies working in these areas; (FSESP - providing medical care and disease notification; SUCAM - malaria surveillance and control programs; INCRA - managing the colonization program.)

3. Use the information obtained to design a surveillance program and develop appropriate instruments for data gathering (i.e. questionnaires).

WORK ACCOMPLISHED: Multiple visits were made to the Maraba and Altamira jurisdictions (see map). In each jurisdiction each colonist family has been assigned its own lote (size (1000 m x 500 m). These lotes are grouped into glebas (Fig. 1) each with 10 road front lotes (5 Km) and 0-63 interior lotes of equal size. Families owning interior lotes usually live in agrovilas (small planned settlements of 60 families) near their lotes. Colonists contacted seemed cooperative, suggesting the possibility of regular surveillance visits.

In Maraba, the chief physician of the 50 bed FSESP Hospital employing three physicians agreed to provide laboratory space in the hospital and gave permission for conducting a hospital surveillance program. SUCAM provided access to malaria data from the area. From INCRA, demographic information pertaining to the colonists was obtained.

In Altamira the FSESP Hospital is twice as large as that in Maraba and has a more active outpatient service. Data was obtained on a 50% sample of all 1973 admissions. SUCAM provided malaria data of questionable quality. INCRA provided detailed maps of the colonization areas and access to demographic data.

A case/control survey was conducted between Gleba 1 and 40 in Maraba in which all persons who had malaria diagnosed by SUCAM during one month were interviewed.

DATA OBTAINED:

1. INCRA data - maps were obtained showing the location of each lote, gleba and agrovila and the location of all health posts

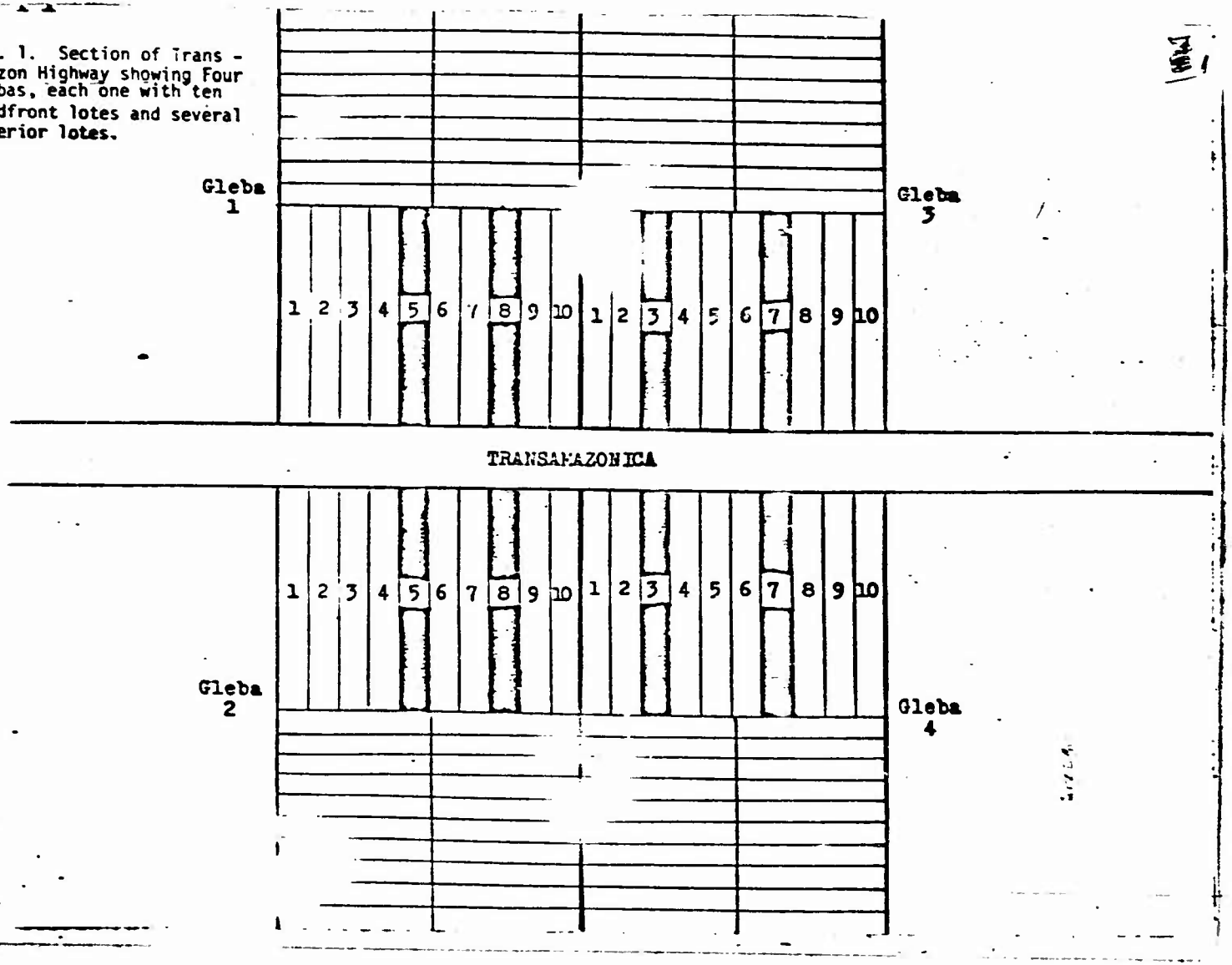


Fig. 1. Section of Trans - Amazon Highway showing Four Glebas, each one with ten Roadfront lotes and several interior lotes.

was ascertained. INCRA data provided: the number of colonists in each area (Table 1); by state of origin (Table 2); the distribution of population by gleba (Tables 3,4,5); and by age and sex (Fig. 2).

A sample of INCRA files showed that 42 of 325 persons sampled had a last name different from the lote owner. Thus, 13% of the people living along the highway can be located only by a painstaking and time-consuming search of these files.

2. SUCAM data - see Epidemiology F. - Malaria.

3. FSESP data - see Epidemiology C. - Passive Surveillance and Epidemiology F. - Malaria.

4. Malaria case/control survey - see Epidemiology F. - Malaria.

RESULTS:

1. Five questionnaire instruments were designed for use in the surveillance program (Figs. 3,4,5,6,7).

2. A decision was made to choose a study population by random selection of two road front lotes from each gleba (Fig. 1), and to include entire representative agrovilas at a later date.

3. Based upon these observations and discussions with local physicians, it was apparent that malaria was the only readily identifiable infectious disease problem in these areas of the Transamazon.

4. Although small numbers were involved, the case/control survey of persons experiencing malaria suggested extra-domiciliary transmission due to the increased risk to adult males who occasionally sleep outside their houses.

Table 1. Distribution of INCRA Colonists by Stretch of Highway (Jan 1974)

AREA	LOT OWNERS	TOTAL POPULATION
Maraba - Altamira	644	3,782
Altamira - Maraba	532	3,128
Altamira - Itaituba	2,455	13,893

Table 2. Distribution of INCRA Colonists by State of Origin for Each Stretch of Highway

STATE	MARABA-ALTAMIRA	ALTAMIRA-MARABA	ALTAMIRA-ITAITUBA
Acre	0	0 (0)	2 (.08)
Amazonas	0	1 (0.2)	2 (.08)
Para	187 (29.1)	144 (27.1)	485 (19.8)
Maranhao	181 (28.2)	69 (13.0)	249 (10.14)
Piaui	24 (3.7)	42 (7.9)	73 (2.32)
Ceara	25 (3.9)	65 (12.2)	269 (10.96)
R.G. do Norte	2 (0.3)	46 (8.6)	253 (10.30)
Paraiba	4 (0.6)	8 (1.5)	28 (1.12)
Pernambuco	5 (0.8)	41 (7.7)	74 (2.96)
Alagoes	1 (0.2)	15 (2.8)	24 (0.97)
Sergipe	0 (0)	1 (0.2)	2 (.08)
Bahia	24 (3.7)	11 (2.1)	76 (3.04)
Minas Gerais	17 (2.6)	14 (2.6)	94 (3.76)
Espirito Santo	20 (3.1)	5 (0.9)	15 (.60)
Rio de Janeiro	0 (0)	1 (0.2)	5 (.20)
Guanabara	4 (0.6)	0 (0)	3 (.12)
Goias	120 (18.7)	30 (5.6)	77 (3.08)
Distrito Federal	13 (2.0)	2 (0.4)	31 (1.24)
Mato Grosso	3 (0.5)	2 (0.4)	25 (1.0)
Sao Paulo	3 (0.5)	6 (1.1)	60 (2.4)
Parana	6 (0.9)	21 (3.9)	396 (16.13)
Santa Catarina	2 (0.3)	1 (0.2)	58 (2.38)
R.G. do Sul	0 (0)	6 (1.1)	151 (6.14)
Roraima	0 (0)	0 (0)	0 (0)
Rondonia	0 (0)	1 (0.2)	1 (.04)
Amapa	0 (0)	0 (0)	2 (.08)
Fernando Noronha	0 (0)	0 (0)	0 (0)
Foreigners	1 (0.2)	0 (0)	0 (0)

Table 3. Number of INCRA Colonists by Gleba: Altamira - Itaituba (Jan. 1974).

GLEBA	NO. OF LOTS	POPULATION	GLEBA	NO. OF LOTS	POPULATION
1	6	32	40	24	141
2	5	43	41	75	105
3	43	268	42	0	0
4	35	237	43	0	0
5	65	394	44	0	0
6	57	350	45	0	0
7	44	239	46	0	0
8	53	318	47	0	0
9	39	203	48	0	0
10	45	184	49	0	0
11	56	357	50	0	0
12	59	345	51	10	42
13	60	395	52	10	56
14	55	368	53	9	55
15	60	315	54	10	66
16	66	350	55	8	46
17	57	288	56	9	62
18	62	363	57	8	50
19	58	352	58	8	35
20	53	308	59	8	45
21	59	281	60	8	57
22	55	332	61	11	66
23	60	284	62	33	178
24	50	296	63	23	133
25	49	264	64	32	135
26	50	258	65	32	168
27	44	280	66	30	124
28	51	287	67	30	140
29	43	249	68	23	108
30	51	283	69	29	141
31	41	203	70	20	108
32	55	295	71	18	120
33	33	189	72	9	51
34	53	324	73	10	64
35	31	183	74	10	68
36	44	162	75	9	57
37	38	191	76	10	69
38	40	234	77	10	84
39	42	213	78	10	67

Table 3. (cont.) Number of INCRA Colonists by Gleba: Altamira - Itaituba (Jan. 1974).

GLEBA	NO. OF LOTS	POPULATION	GLEBA	NO. OF LOTS	POPULATION
79	10	65	85	10	60
80	9	60	86	10	80
81	10	66	87	10	66
82	9	70	88	9	60
83	10	63	89	8	49
84	9	58	90	8	48

Table 4. Number of INCRA Colonists by Gleba: Altamira - Maraba (Jan. 1974).

GLEBA	NO. OF LOTS	POPULATION	GLEBA	NO. OF LOTS	POPULATION
1	4	16	38	9	44
2	5	22	39	9	62
3	10	74	40	10	39
4	10	48	41	9	73
5	11	66	42	9	54
6	9	38	43	7	35
7	10	47	44	7	47
8	9	65	45	8	65
9	6	35	46	7	35
10	8	54	47	9	47
11	8	57	48	8	40
12	10	69	49	8	48
13	4	21	50	7	37
14	3	31	51	8	41
15	8	44	52	8	56
16	10	56	53	8	29
17	0	0	54	8	51
18	10	49	55	9	73
19	0	0	56	7	43
20	8	49	57	9	53
21	0	0	58	6	28
22	0	0	59	9	53
23	10	64	60	9	48
24	0	0	61	7	39
25	8	57	62	7	31
26	0	0	63	9	63
27	9	57	64	6	36
28	8	56	65	9	58
29	9	43	66	8	41
30	9	52	67	8	45
31	9	51	68	10	58
32	8	46	69	5	18
33	7	54	70	7	48
34	9	49	71	1	12
35	10	60	72	3	12
36	10	65	73	3	17
37	9	50	74	1	3

Table 5. Number of INCRA Colonists by Gleba: Maraba - Altamira (Jan. 1974).

GLEBA NO.	INCRA COLONISTS	GLEBA NO.	INCRA COLONISTS
1	12	36	54
2	25	38	41
3	157	40	68
4	62	42	58
5	178	43	23
6	45	44	30
7	191	45	39
8	93	47	39
9	110	49	44
10	105	51	57
11	58	53	76
12	65	55	67
13	58	56	26
14	37	57	52
15	58	58	79
16	53	59	57
17	58	60	57
18	52	61	48
19	41	62	47
20	49	63	61
21	53	64	32
22	49	65	49
23	53	66	54
24	36	67	39
25	67	68	51
26	74	69	50
27	58	70	38
28	50	71	48
29	50	72	54
30	74	73	13
31	39	74	19
32	39	75	54
34	70	76	62

Gleba Nos. 33,35,37,39,41,46,48,50,52 and 54 are vacant.

Figure 2. Age-Sex Distribution of INCRA Colonists on Transamazon between Maraba and KM 274 (Municipios of Itupiranga, Jacunda and Tucurui)

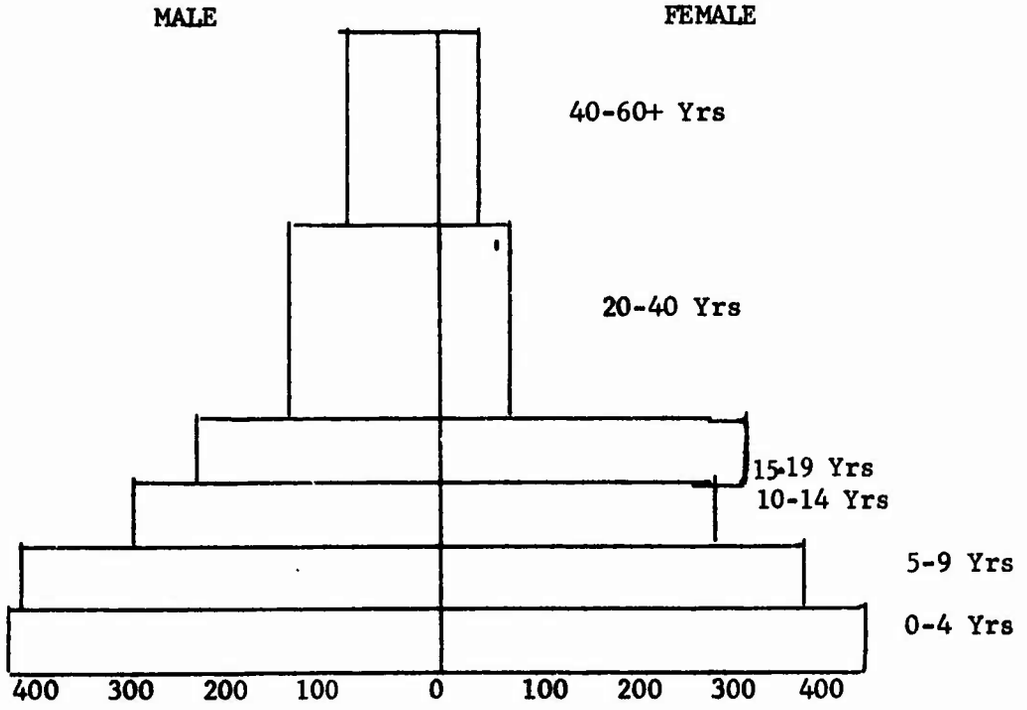


FIGURE 3

TRANSLATION

Date of interview ..... Name of interv. ver .....

[illegible] (01 + 02)

1) Name.....

2) Identification Number 3) Family Identification Number

Maraba .....Maraba

4) Gleba ..... Lote ..... Km .....From ..... Altamira to ..... Altamira

Itaituba ..... Itaituba

5) Age ..... 6) Date.of.Birth..... 7) Sex ..... Male

Female

8) Jobs .....

9) Place of Birth (the State) .....

10) Where did you live before coming to the Transamazon (the State)

11) Date of entrance into the Transamazon .....

12) Do you live a)..... on a lot or b).... in an agrovila (village).....

13) When did you move to this lot? .....

14) Where does the water that the family drinks come from?

a).....well b).....s eam c)....tap water d).....other. Describe....

15) Is the water boiled before drinking? a).....yes b).....no

16) Where does the water for washing clothes come from? a).....well b).....stream

c).....tap water d).....other. Describe.....

17) Where does the water for bathing come from? a).....well b).....stream

c).....tap water d).....other. Describe.....

18) How far away is the nearest standing water?

a).....less than 100 meters b).....100-200 meters c).....more than

200 meters.

Fig. 3 Cont.

19) How far away is the nearest stream? a).....less than 100 meters
b).....100-200 meters c).....more than 200 meters.

20) What is the terrain of the lot? a).....rolling hills b).....steep hills
c).....flat land

21) How far away is the forest?.....

22) What are the principle crops? .....

23) What animals do you Keep? .....

24) Where does the family urinate?.....

25) Where does the family defecate?.....

26) Do you have mosquitos in the house? a).....yes b).....no

27) If so, when do they bite? a).....morning b).....afternoon c).....night

28) Do you have mosquitos in the open areas around the house? a).....yes b)....no

29) If so, when do they bite? a)....morning b).....afternoon c)....night

30) Is the family bitten by sandflies? a)....yes b).....no

31) Is so, when? a).....morning b).....afternoon c).....night

32) Where? a).....open areas b).....forest c).....house d).....other.
Describe.....

33) Are you bitten by black flies? a).....yes b).....no

34) When? a).....morning b).....afternoon c).....night

35) Where? a).....open areas b).....forest c).....house
d).....other.. Describe.....

36) Are you bitten by triatomid bugs? a).....yes b).....no

37) Are you bitten by ticks? a).....yes b).....no

38) When does the family go to bed? .....

39) Does the family occasionally sleep outside the house? a).....yes b).....no/

Fig. 3 Cont.

OBSERVATIONS BY THE INTERVIEWER

Area of Defecation

40) Type.....

41) Distance from the house a).....less than 100 meters b).....100-200 meters

c) .....more than 200 meters

42) Distance from drinking water a).....less than 100 meters

b).....100-200 meters c).....more than 200 meters

43) Other observations.....

44) Type of House a).....wood (INCRA) a).....straw..... c).....mud

d).....other

45) When someone is sick, whom do you consult? a).....private doctor

b).....F-SESP Hospital c).....SUCAM d).....INCRA Health Post

e).....other

46) Does the family belong to the Sindicate? a).....yes b).....no

47 Serum number

48 Slide number

Fig. 4

Date of interview..... Name of interviewer.....

QUESTIONNAIRE 2 REGISTRATION DATA (INDIVIDUAL)

1) Name.....

2) Identification Number..... 3) Family Identification Number.....

4) Gleba.....Lot.....Km..... From Maraba / Altamira / Itaituba To .....Maraba / .....Altamira / ..... Itaituba

5) Age.... 6) Date of birth.....7) Sex.....Male / .....Female

8) Occupation.....

9) Place of birth (the State).....

10) How long have you lived here?.....

11) Did tou have malaria before coming to the Transamazon? a)....Yes..b)..No

12) Was it proven? a)...Yes b)...No

13) Have you had malaria since arriving in the Transamazon? a)...Yes b)..No

14) Was it proven? a)....Yes b)....No

15) How many times have you had malaria (proven) in the Transamazon?
a)....0 b)....1 c)....2 d)....3 e).... More than 3 times

16) Serum number.....

17) Slide number.....

Fig. 5

TRANSLATION

Date of Interview ..... Name of interviewer.....

QUESTIONNAIRE 2 - [illegible]

1) Name.....

2) Identification Number.....

3) Gleba..... Lote .... Km..... from Maraba Altamira to Maraba Altamira Itaituba Itaituba

4) Age..... 5) Date of Birth.....6) Sex .....Male .....Female

7) Were you hurt or did you have an accident in the past two weeks? a).....no b).....burn . c).....car accident d).....work accident e).....other. Describe.....

8) Were you bitten or stung by an animal during the past two weeks? a).....no b).....bee c).....snake d).....scorpion e).....spider f).....other. Describe.....

9) Did you have any illness during the past two weeks? a).....yes b)....no

10) While you were sick, did you have

11) .....fever 12).....chills 13).....diarrhea 14).....nausea

15) .....dizzyness 16).....cough 17)..... headache 18)...muscle aches

19) .....sore throat 20).....rash 21)..... other skin lesion

22) .....Stomach ache 23).....runny nose 24)..... bleeding or bruissing

25) .....vomiting 26).....dark urine 27)..... light-colored feces

28) .....pain when urinating 29).....Bloody sputum 30).....shortness of breath 31).....other symptoms. Describe.....

Fig. 5 Cont.

32) How long did the illness last? .....

33) Were you treated by anyone? a).....no b).....doctor
c).....nurse d).....other. Describe.....

34) Was the illness diagnosed? a).....yes b).....no

35) What was the diagnosis? .....

36) The individual is a).....present b).....absent-working
c).....absent-sick d).....absent-travelling e).....absent-dead
f).....absent for another reason

37) Only for adult females: During the last two weeks:
did you a)....have a child b).....have a miscarriage c).....neither

ONLY FOR THE INTERVIEWER

38) Did you draw blood a).....yes b).....no

39) Serum number ..... 40) Slide number .....

41) Other comments .....

Fig. 6

FORM A - BIWEEKLY VISITS

NAME	ADDRESS	Rm	INDIVIDUAL I.D. No.	FAMILY I.D. No.	Date of Birth	Age	Sex	STATE A- absent PD - present-sick PND- present-not sick D - dead	BLOOD DRAWN Serum no - yes N -No.	Reason I-relative of case V-survey O-original R-repeat	DATE OF VISIT	Int

1409

Fig. 7

ANNEX 7

FORM NUMBER 5 CONTROL ROSTER OF FIELD SURVEILLANCE VISITS

SEMESTER..... YEAR:..... LOCALITY.....

ER	NAMES	AGE	GLEBA LOT	Individual Number	Original Serum	1 visit	2 visit	1 visit	2 visit	1 visit	2 visit	1 visit	2 visit	1 visit	2 visit	1 visit	2 visit	Semestral Serum	

1410

B. PILOT STUDY - INFECTIOUS DISEASE SURVEILLANCE PROGRAM

The purpose of this study was to implement and evaluate a program of active field surveillance including the following objectives:

1. To train field workers;
2. To use and evaluate questionnaires;
3. To develop procedures for obtaining, labelling, handling and shipping various biological specimens;
4. To define the refusal/loss rate in the study population;
5. To assess the probability of maintaining a visit schedule;
6. To develop a firm communication system between the Belem base (400 Kms away) and the study area;
7. To observe the number of specimens for laboratory examination generated by this program.

WORK ACCOMPLISHED: In 60 days field workers in Maraba enrolled the stratified random sample of 125 families (801 individuals) according to the scheme developed in the pre-pilot study. Questionnaires were completed on each family and individual. Malaria smears and blood samples were obtained from over 600 of those enrolled, the delinquents being children under two years of age and older children not living at home in order to attend school.

For six months each family was visited every two weeks. When persons with a history of febrile illness since the previous visit were encountered, blood for virus isolation, serology and malaria smears was drawn and a form was completed recording symptoms, duration of illness, and diagnosis and treatment, if any.

After six months, an attempt was made to rebleed each person in the sample and revalidate questionnaire data obtained at time of enrollment.

DATA OBTAINED.

1. Fig. 1 shows the age/sex distribution of the sample enrolled and compares favorably with that obtained for the entire colonist population. As planned, the 125 families and 801 individuals enrolled comprised a greater than 20% sample of the colonist population in the Maraba-Altamira jurisdiction.
2. Table 1 shows number of illnesses identified during each bi-weekly visit during this six month period.

Fig. 1 Age-Sex Distribution of INCRA Colonists in USAMRU Sample between Maraba and Km 274.

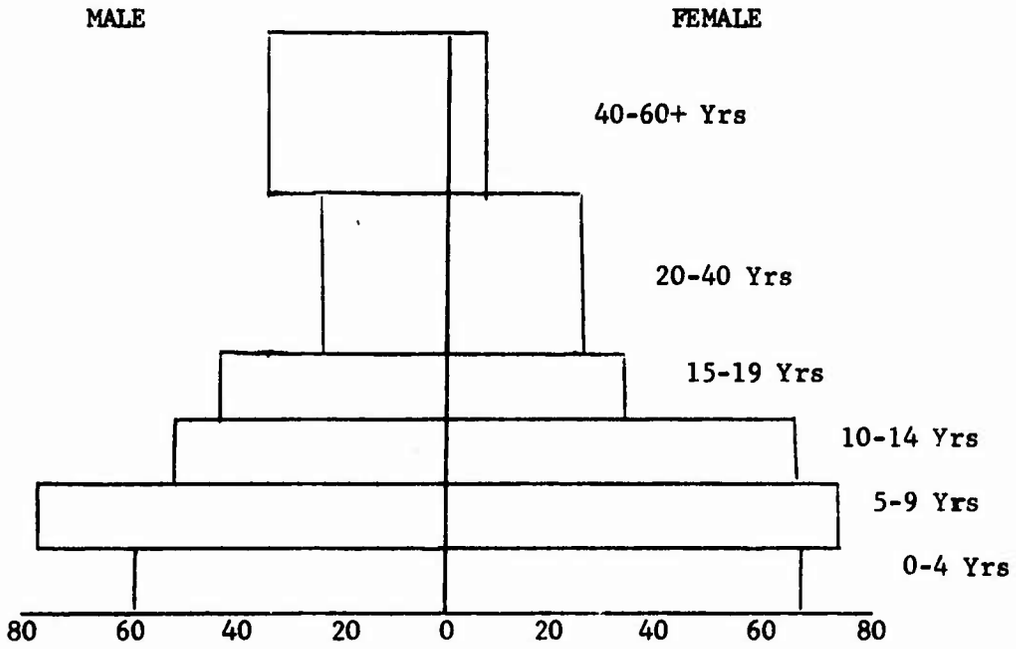


Table 1. Number of Ill Persons Seen on Each Visit Before the Six Month Collection in Maraba.

VISIT	DATE ENDING	NUMBER DAYS COVERED	CASES FEVER	TOTAL ILLNESS
1	27 Aug	28	14	24
2	11 Sep	15	12	23
3	24 Sep	13	13	17
4	18 Oct	24	7	12
5	30 Oct	12	9	11
6	20 Nov	20	6	11
7	7 Dec	17	9	16
8	20 Dec	13	16	30
9	14 Jan	25	8	12

3. At the time of the six month visit, blood was obtained from 495 (83%) of the 600 persons originally bled. There were 52 refusals and 53 persons had either moved away or were absent from their lotes on three consecutive visits. Blood specimens were obtained from an additional 196 individuals not previously bled. When a family drops out of the study for any reason, a new family from the same gleba is enrolled.

4. For malaria data see Epidemiology F. - Malaria.

OBSERVATIONS: The questionnaires were found to be satisfactory. Modification and changes in wording were made and procedures for review of all forms were developed.

The system for labelling and handling specimens worked well using 2 cc tubes in the field which obviates making aliquots in the laboratory. The liquid N_2 system functioned well.

Schedules were virtually unaffected by road conditions and/or vehicle breakdown.

The need for more detailed screening, training and supervision of field workers was recognized.

In March 1975 aliquots of the enrollment and six month rebleed specimens plus acute illness specimens were sent to Walter Reed Army Institute of Research and to the Institute Evandro Chagas for virus isolation studies and various serological tests. The USAMRU-Belem laboratory became operational and began testing these specimens in May 1975. Collation of test results with epidemiologic and demographic data is in process.

C. PASSIVE SURVEILLANCE PROGRAM

DESCRIPTION OF PROGRAM AND OBJECTIVES: The passive surveillance program is designed to collect disease information from Brazilian organizations active in the study areas. These include SUCAM, the FSESP Hospital, health posts and the town death registries. Private physicians were not included because of the poor quality of their records.

WORK ACCOMPLISHED: In Maraba and Altamira data was obtained from SUCAM showing the number of slides examined each month from January 1973 to the present, and the number found positive. We also obtained a list of all cases of schistosomiasis diagnosed in the area.

Information collected from the FSESP Hospital included the number of admissions by diagnosis and month of admission, the number of deaths and the number of reportable diseases seen.

Information from the death registries is collected each week.

ANALYSIS OF DATA:

1. The malaria data collected from SUCAM is summarized in Epidemiology F. - Malaria. Figures 1 and 2 and Tables 1 and 2 are included here to show that within the Maraba and Altamira jurisdictions, the incidence of malaria varies temporally and geographically. In addition, a list of all registered cases of schistosomiasis (400 since 1971) was made. There were 67 cases diagnosed in the Altamira jurisdiction in 1974, all of which were thought to be imported.

2. A 50% sample of all admissions to the FSESP Hospital in Altamira in 1973 was recorded and analyzed. The number of admissions and deaths per month is shown in Table 3. The most common admission diagnoses were normal gestation, malaria and trauma (Table 4). The major causes of death were enteritis, gastroenteritis and dehydration (Table 5). The only diseases showing a seasonal variation were malaria (see Epidemiology F. - Malaria), enteritis, asthma and the hemorrhagic syndrome of Altamira (see Figure 3).

3. A similar sample of admissions to the Maraba Hospital was not available. Data obtained showed seasonal variation in respiratory diseases and gastroenteritis in Maraba during 1974 (Figure 4).

4. All officially reportable diseases seen at the FSESP Hospital are recorded by our field supervisor each month. The results by disease and month in Maraba, 1974 are shown in Table 6.

5. Data collected at the Altamira death registry was analyzed by diagnosis (Table 7). Data from Maraba were reported by month (Table 8).

OBSERVATIONS: The data collected to date reflect a low admission rate for infectious/febrile disease, thus emphasizing the need for active surveillance outside established health care facilities. Limited laboratory facilities permit diagnoses of disease only by examination of blood smears (malaria) and direct fecal preparations (intestinal parasites).

Less than 50% of malaria cases reported by the Maraba and Altamira hospitals were confirmed by a positive blood film. No liver function tests are available in these hospitals and jaundice is difficult to detect due to pigmentation of most patients making the diagnosis of hepatitis difficult.

Fig. 1. CASES OF MALARIA REPORTED BY SUCAM AND THE FSESP HOSPITAL IN MARABA 1973-1974

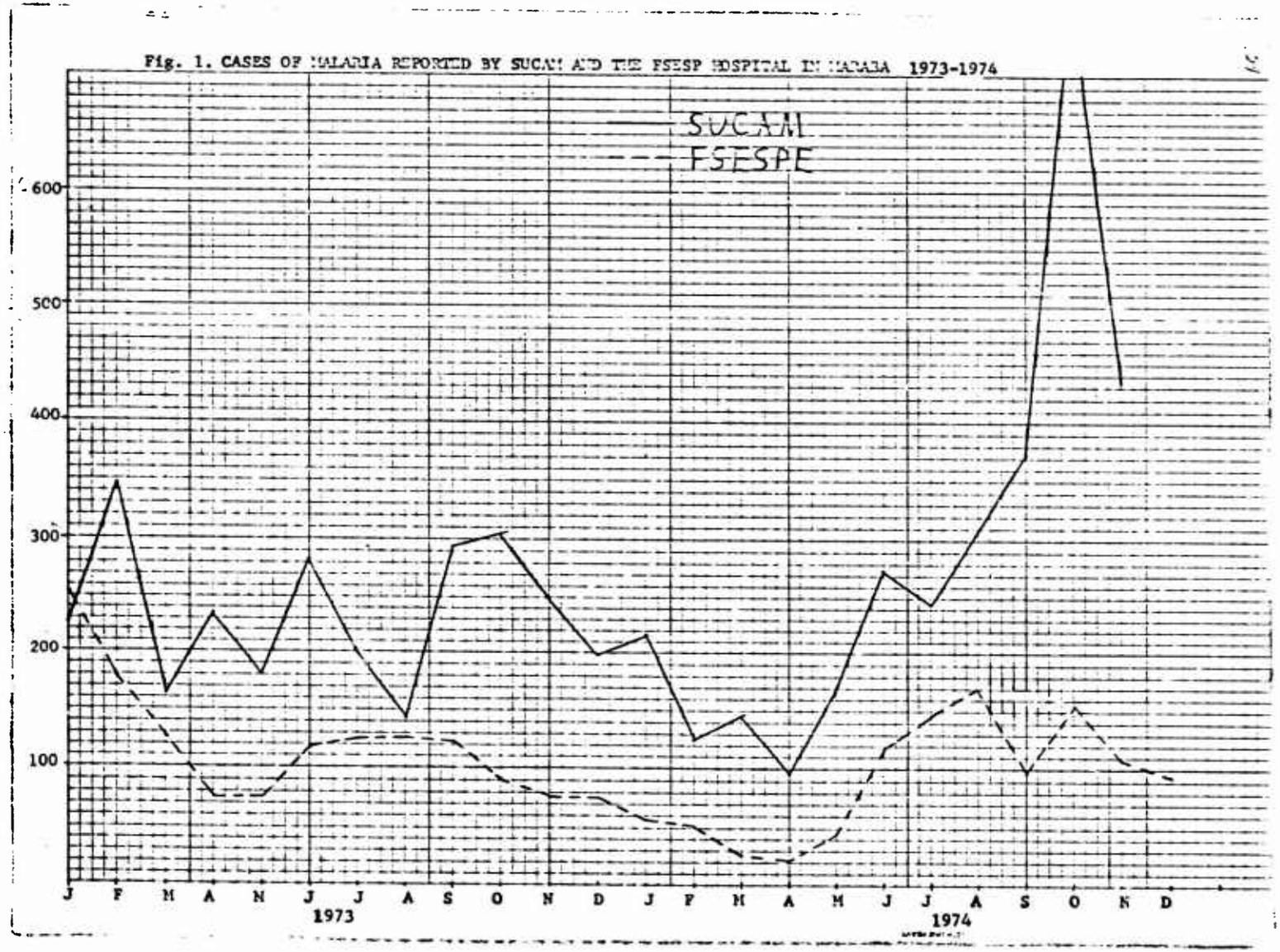


Fig. 2. CASES OF MALARIA REPORTED BY SUCAM AND THE FSESP HOSPITAL IN ALTAMIRA 1973-1974

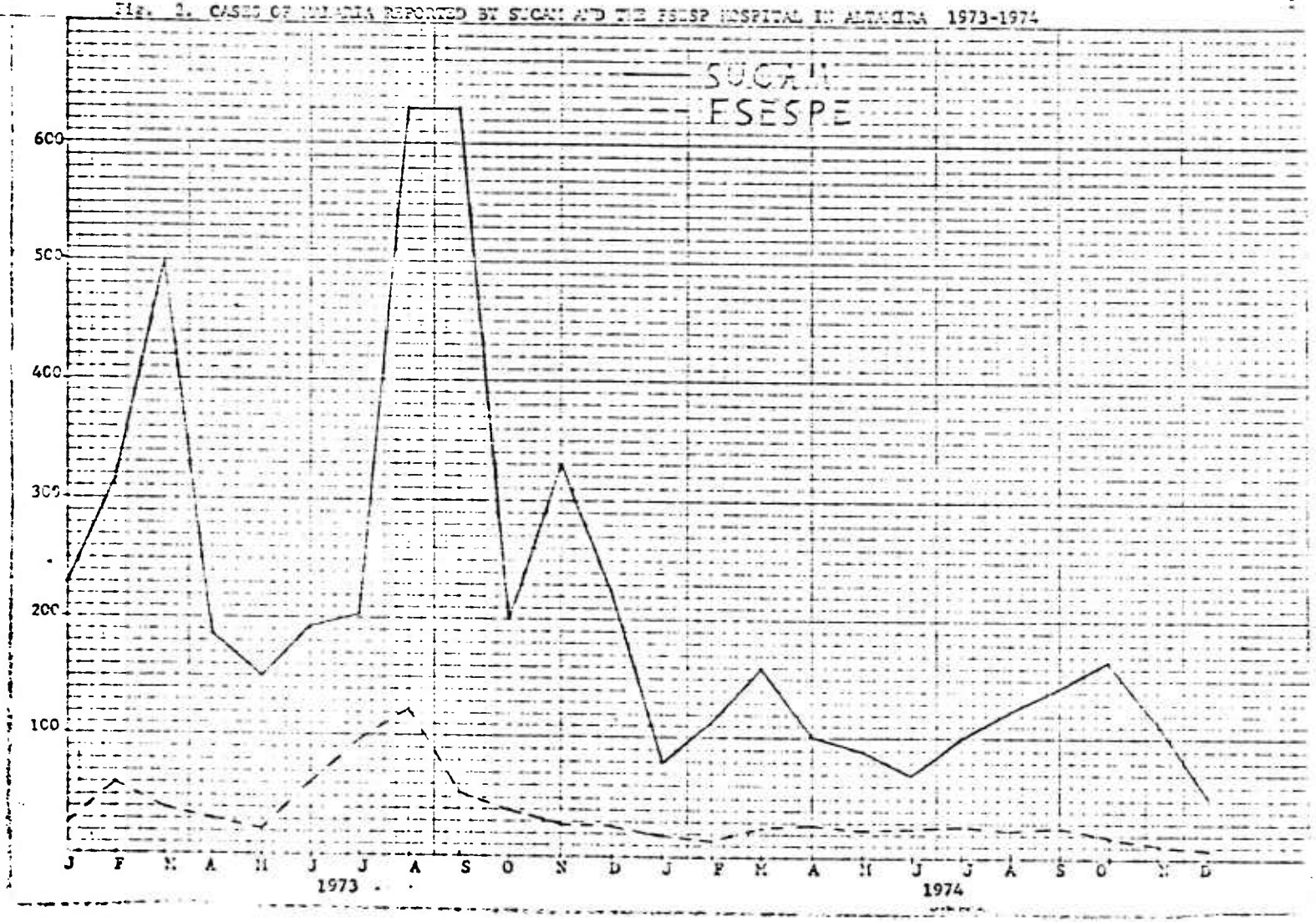


Table 1. Positive Malaria Slides Found by SUCAM in 1974 in Each of the Municipios (Counties) in the Maraba Jurisdiction.

	CONCEI	ITUP	JACUNDA	MARABA	S. JOAO	SANTANA	TUCURUI
Jan	1	15	29	94	40	0	41
Feb	12	10	19	66	22	81	11
Mar	5	19	5	95	25	0	0
Apr	7	2	2	88	12	5	0
May	26	7	13	118	25	162	9
Jun	18	15	18	185	55	3	19
Jul	3	7	10	173	63	1	0
Aug	3	14	13	197	76	0	12
Sep	10	13	16	261	56	2	32
Oct	17	17	24	385	328	9	47
Nov	4	9	4	340	93	1	2
Dec	13	28	16	372		5	26
TOTAL	119	156	169	2374		269	199

Table 2. Positive Malaria Slides Found by SUCAM in 1974 in Each of the Municipios (Counties) in the Altamira Jurisdiction.

	ALTAMIRA	SEN JOSE PORFIRIO	PORTEL	PRAIHNA
Jan	63	8	2	9
Feb	26	31	21	25
Mar	73	51	22	15
Apr	51	20	21	6
May	71	17	1	3
Jun	67	2	0	0
Jul	66	10	16	11
Aug	102	15	8	2
Sep	126	10	7	2
Oct	110	28	21	4
Nov	76	13	17	9
Dec	22	14	14	2

Table 3. Number of Admissions and Deaths by Month at the FSESP Hospital in Altamira, 1973.

	ADULTS AND CHILDREN		NEWBORN	
	ADMITTED	DIED	ADMITTED	DIED
January	291	22	63	2
February	275	18	58	3
March	288	18	65	2
April	223	7	74	0
May	257	12	81	2
June	320	11	74	4
July	326	15	73	2
August	340	17	76	1
September	222	3	73	1
October	200	5	68	0
November	210	4	67	3
December	223	13	71	1
TOTAL	3175	145	843	21
DEATH RATE	45.66/1000		24.91/1000	

Table 4. Diagnoses of a 50% Sample of Persons Admitted to the FSESP Hospital in Altamira in 1973.

DIAGNOSES	NUMBER OF CASES	%
Normal Gestation	403	25.6
Malaria	317	20.2
Miscellaneous	273	17.4
Trauma	108	6.9
Enteritis	56	3.6
Abortion	52	3.3
URI	46	2.9
Pneumonia	32	2.0
Syndrome Pleuri-C	32	2.0
Snakebite (of Idismo)	28	1.8
Bronchitis	29	1.8
Gall Bladder Disease	28	1.8
Toxic Hepatitis	27	1.7
Schistosomiasis	25	1.6
Asthma	23	1.5
Gastro-enteritis	22	1.4
Hemorrhagic Syndrome	22	1.4
Anemia	20	1.3
Dehydration	12	0.7
Parasitosis	9	0.6
Infectious Hepatitis	4	0.3
Burns	5	0.3
TOTAL	1573	100.1%

Table 5. Causes of Death at the FSESP Hospital in Altamira, 1973.

DIAGNOSIS	NO. DEATHS	NO. CASES	CASE MORTALITY RATE (%)
Enteritis	13	56	23.2
Gastro-Enteritis	4	22	18.2
Dehydration	1	12	8.3
Toxic Hepatitis	2	27	7.4
Pneumonia	2	32	6.3
Miscellaneous	12	273	4.4
Malaria	13	317	4.1
Schistosomiasis	1	25	4.0
Syndrome Pleuri-C	1	32	3.1
URI	1	46	2.2
Trauma	2	108	1.9
Normal Gestation	2	403	0.5

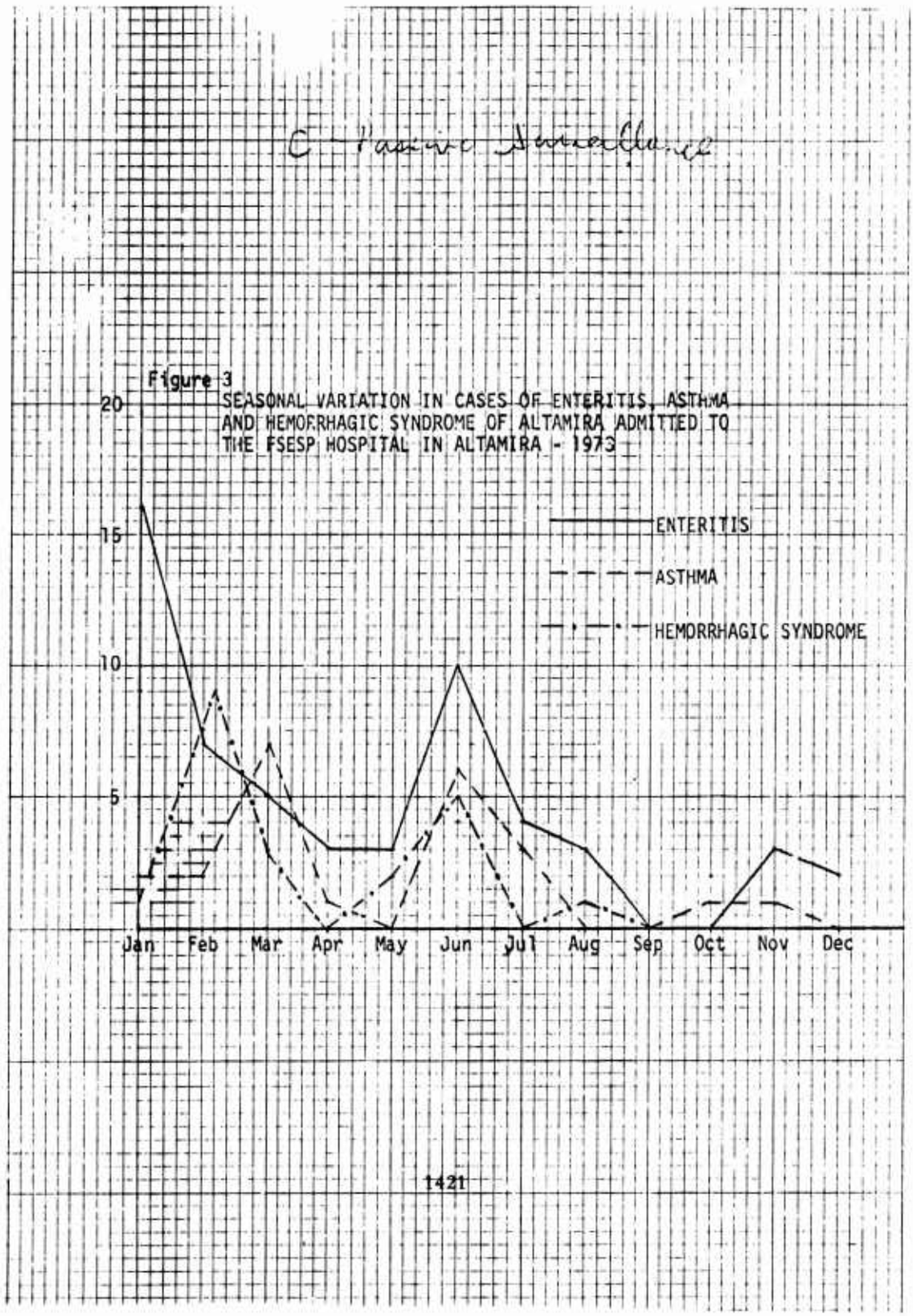


Figure 3
SEASONAL VARIATION IN CASES OF ENTERITIS, ASTHMA AND HEMORRHAGIC SYNDROME OF ALTAMIRA ADMITTED TO THE FSESP HOSPITAL IN ALTAMIRA - 1973

Fig. 4 NUMBER OF CASES ADMITTED TO FSESP HOSPITAL IN MARIB IN 1974 BY DIAGNOSIS AND MONTH

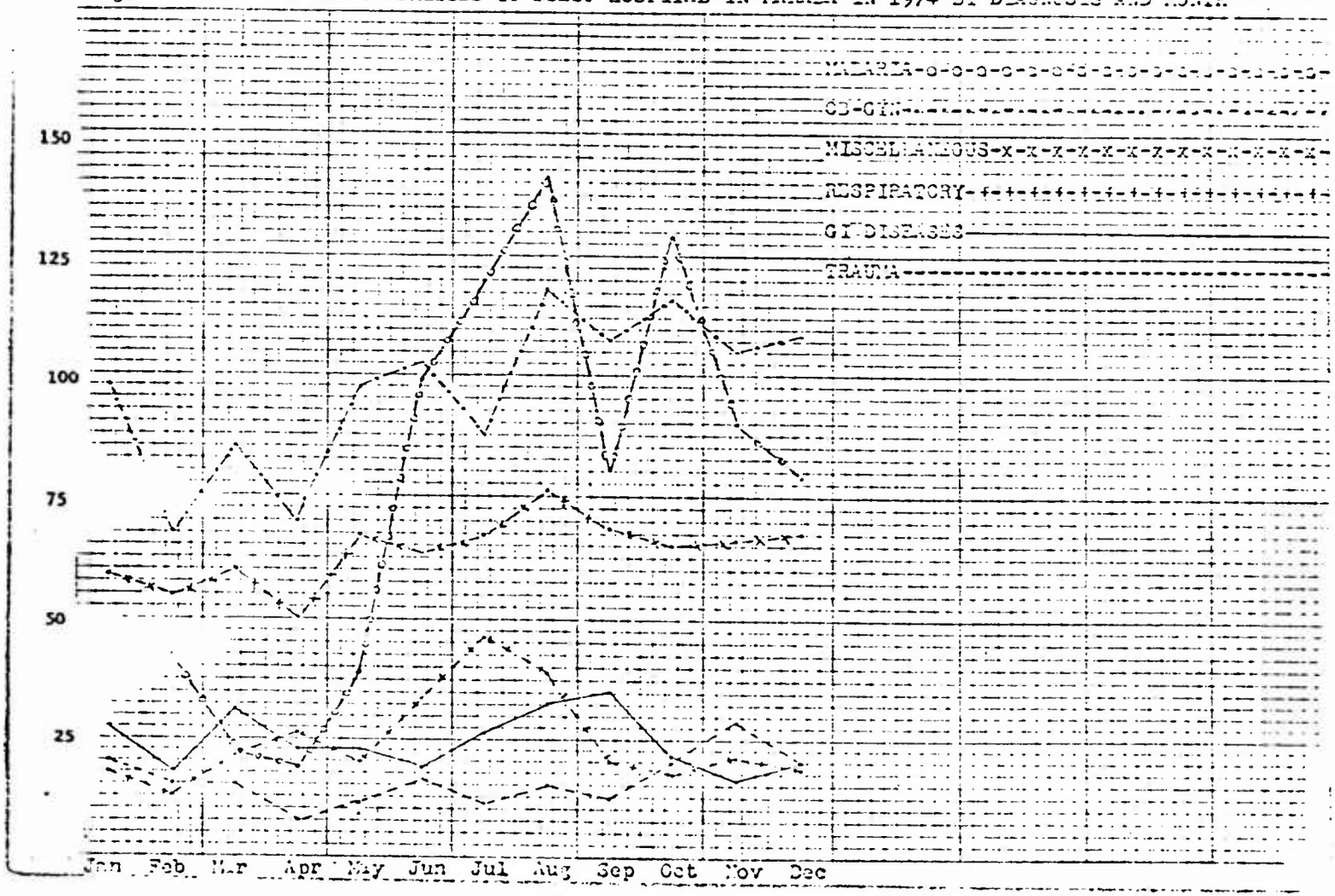


Table 6. Reportable Diseases Seen at the FSESP Hospital in Maraba, 1974 by Diagnosis and Month.

MONTH	MALARIA	HEPATITIS	SYPHILIS	TETANUS	TB	MEASLES	POLIO	LEPROSY	LEISH-MANIASIS	YELLOW FEVER
January	48	4	5	2	4	1	--	--	--	--
February	28	2	--	2	3	--	--	1	--	--
March	30	1	1	--	4	1	1	--	1	--
April	17	5	4	--	3	1	--	1	--	--
May	44	5	1	--	3	--	--	--	1	--
June	86	1	--	--	5	2	1	--	1	--
July	149	12	1	1	3	1	--	--	2	--
August	143	4	3	1	4	2	--	--	--	--
September	87	13	--	--	4	1	--	--	--	--
October	118	12	16	1	4	2	--	1	--	--
November	66	9	3	--	2	--	--	--	--	--
December	112	12	--	1	2	1	--	--	--	--
TOTAL	928	80	34	8	41	12	2	3	5	--

Table 7. Number of Deaths in the Altamira Jurisdiction in 1974, by Diagnosis.

DIAGNOSIS	NUMBER OF DEATHS
Anemia	3
Anoxia	3
Hook Worm	1
Appendicitis	1
Bronchial Pneumonia	16
Hepatic Cirrhosis	2
Cardiac Disease	1
Toxic Shock	1
Dehydration 3°	23
Epilepsy	1
Eclampsia	2
Gastro-Enteritis	14
Hypertension	5
Toxic Hepatitis	9
Renal Insufficiency	7
Respiratory Insufficiency	20
Infection	1
Malaria	17
Muscular Paralysis	1
Pneumonia	5
Prematurity	2
Bites	1
Burns	1
Fetal Distress	8
No Medical Assistance	186
Tetanus	2
Tuberculosis	3
Traumas	29
Poison	2
FUO	6
TOTAL	373

Table 8. Number of Deaths in the Maraba Jurisdiction in 1974, by Diagnosis and Month.

MONTH	MALARIA	HEPATITIS	DISEASES OF THE GI TRACT	DISEASES OF THE RESPIRATORY TRACT	NO MEDICAL ASSISTANCE	OTHERS
January	1	1	3	4	2	14
February	--	1	2	2	3	4
March	--	--	7	1	12	8
April	1	--	3	5	2	7
May	1	--	--	5	5	9
June	1	3	--	6	2	13
July	5	--	2	3	4	8
August	7	1	2	7	3	5
September	2	2	6	7	8	3
October	8	--	4	9	7	11
November	1	3	3	8	7	13
December	1	1	10	4	6	7
TOTAL	28	12	42	61	61	102

Specimens for histopathology examination are rarely obtained. Tissue examinations and routine culture can only be performed in Belem.

The majority of patients from whom the above statistics were derived came either from the town itself or from close-in colonization areas, therefore not providing a valid picture of disease experience along the Transamazon Highway.

D. ACTIVE HOSPITAL SURVEILLANCE PROGRAM

DESCRIPTION OF PROGRAM AND OBJECTIVES: An active hospital surveillance program has been in progress in Maraba since September 1974, and in Altamira since April 1975. In each location, a member of the field team visits the hospital each day to check for new medical admissions. A history is taken from each new patient and the information is recorded on a copy of Questionnaire 3 (see Epidemiology A - Pre-Pilot Study). From each patient with a fever or a history of recent fever, blood is drawn for a malaria smear, virus isolation and serology, the specimens then being shipped to Belem for examination in the laboratories of WRAIR, the I.E.C. and USAMRU-Belem.

DATA OBTAINED: Only work conducted in Maraba will be discussed, since the program in Altamira did not start until April 1975. Between September 1, 1974 and April 24, 1975, forms were completed on 443 patients. Blood was obtained from 294 (66%) of these patients. Specimens collected before March 1975 were sent to WRAIR for virus isolation and serology. One isolate of Guaroa virus and several high complement fixation titers against the same agent have been identified. Examinations for toxoplasmosis, leptospirosis and other agents are in process.

With the base laboratory in Belem operational, emphasis is now being directed to the establishment of basic clinical pathology support for the hospital surveillance program in Maraba and Altamira to include hematology, clinical chemistry and clinical microbiology. Outpatients seen at the FSESP hospitals and health posts and presenting at the local SUCAM offices will be included and more detailed clinical data will be obtained.

Clinical observations in a small number of falciparum malaria patients have indicated some degree of chloroquine resistance. Each of these patients required intravenous quinine in high doses for more than 48 hours before the parasitemia was markedly reduced. In vitro chloroquine sensitivity testing is now being carried out in falciparum malaria cases.

Selected disease data from the Altamira hospital for 1974-75 show the constant morbidity caused by snake and scorpion bite in this area, problems seldom seen in the Maraba hospital. The reactions

vary from incapacitating to fatal. The patients are usually colonists or recent arrivals who are living in rural areas recently cleared or crowded with secondary growth. (See Table 9.)

The apparent decrease in malaria during 1975 may be due to either more stringent diagnostic criteria (laboratory confirmation), a true reduction in transmission or both of these factors.

The hemorrhagic syndrome of Altamira continues to be seen during periods of simulium abundance.

E. EPIDEMIOLOGY SURVEILLANCE

DESCRIPTION OF PROGRAM AND OBJECTIVES: The surveillance program along the Transamazon Highway is designed to provide:

1. Prevalence data on the sample population every six months;

2. Incidence data on the same population;

3. Rapid identification of disease outbreaks so that intensive investigations may be started at the earliest possible moment.

The study population is composed of persons living along the road and persons living away from the road. The former were chosen by randomly selecting a 20% sample of roadfront lotes (2 in each gleba) along one side of the highway and then including an equal number of lotes directly across the road. This was done from Maraba west to Aratau (approximately 270 Km) and from the city of Altamira west to the end of the Altamira jurisdiction (about 250 Km). (See maps Introduction). In the Altamira area, many people live off the roadfront, in agrovilas (small villages of approximately 60 houses). Six of these agrovilas were chosen for study, and all families living in them were included in our sample.

On the first visit to a family, blood is drawn from each family member for serological studies, a malaria smear is made and copies of questionnaires 1 and 2 (see Epidemiology A - Pre-Pilot Study) are filled out. Every six months thereafter, blood is drawn from each person and the data on the questionnaires is updated. Every two weeks after the original survey, each family is visited by field teams. If any member of the family has had an illness episode during the previous two weeks, the details are noted on questionnaire 3 (see Epidemiology A - Pre-Pilot Study). At this time, blood is drawn for virus isolation, serology and a malaria smear. Liquid nitrogen is used for holding and transport of specimens.

Table 9. SELECTED DISEASE DATA - ALTAMIRA FSESP HOSPITAL

	ADM	DISCH	DEATHS	MALARIA	HEMORR SYN of ALTAMIRA	SCORPION BITE	SNAKE BITE	GASTRO-INTES	ASTHMA	PNUE
1974										
Jan	197	202	9	20		1	2	3	3	13
Feb	187	166	12	16	3	1	2	10		11
Mar	222	219	7	25	1	1	3	7	2	25
Apr	202	192	8	27	3	2	2	3	7	14
May	238	208	7	25	9	2	6	3	2	19
Jun	212	223	7	24	9	4		4	1	18
Jul	232	216	12	27		4	4	3	1	9
Aug	241	217	20	25		4	1	3	2	10
Sep	213	212	10	25		3	5	5	3	9
Oct	187	184	16	18	1	3	7	5		6
Nov	210	108	3	10	1	1	3	3	1	5
Dec	216	209	16	8		3	3	6	5	16
1975										
Jan	209	206	8	14		5	5	7	2	8
Feb	166	155	11	11		4	7	13		8
Mar	191	188	1	11		6	4	4	4	14
Apr	112	205	9	10	5	5	3	4	3	21
May*	99	101	3		8	1	1	1		4

*May dates are from 1 May 1975 to 15 May 1975.

Information on the number of illnesses encountred, types of illness and results of malaria slides is summarized on a spot-map (Table 1) each week by the field supervisor and transmitted to Belem. If it appears that a disease outbreak is in progress, supplementary data from the records of FSESP, SUCAM and the death registry, together with more detailed information from the colonists, may be requested. If these data support the original data, an investigating team is sent from Belem.

Each of the two field sites has a field supervisor and a simple laboratory equipped with laboratory benches, stools, a desk, a file cabinet, equipment for separating serum and staining slides, and enough supplies to last three months. In Maraba there are three field workers and one vehicle; in Altamira there are four field workers and two vehicles.

WORK ACCOMPLISHED: The Pre-Pilot and Pilot Studies have been described (Epidemiology A - Pre-Pilot Study and B - Pilot Study) and the results in Maraba through the six month collection in January covered. Since then, there has been a great improvement in the regularity of visits, the completeness of the forms, the numbering of serum specimens and collection and reporting of data. Lessons learned in the pilot project were essential to the improvement of the surveillance program. The number of illness episodes seen in each two week period in Maraba is shown in Table 2. This surveillance program will continue for a total of 18 months.

The original visit to roadfront lotes in Altamira was completed in November 1974, with the enrollment of 802 persons from 113 families. Between January and March 1975, 694 persons from 127 families living in agrovilas were enrolled (Table 3). The number of persons who were ill in each two week period is shown in Table 4.

Four outbreaks of disease were identified, two at each study site. Two were malaria outbreaks, and are discussed in Epidemiology F - Malaria. The other two were limited outbreaks of a URI, and were not intensively investigated.

DATA OBTAINED: Environmental and demographic data were recorded for each of the 2300 individuals in the study sample. Descriptive data was recorded for each illness episode, and blood was obtained from most cases of febrile illness for agent isolation, serology and a malaria smear.

Laboratory results are being collated with epidemiological data at present. An expanded program of laboratory examinations will soon be available closer to the field study areas. The combination of active home, hospital and health post surveillance with better diagnostic

Table 1. Spot Map used by USAMRU Field Teams in Maraba and Altamira to Report the Number of Acute Diseases in Each Two (2) Week Period.

				F					
76-74	72-70	68-66	64-62	60-58	56-54	52-50	48-46	44-42	40-38
75-73	71-69	67-65	63-61	59-57	55-53	51-49	47-45	43-41	39-37
F			F F		F		F		
F			M		F				
F									
F									
F									

F = Fever; M = Malaria; B = Bites; A = Accidents; O = Others

Table 2. Number of Ill Persons seen on Each Visit in Maraba from the End of the Six Month Collection to the Present.

VISIT	DATE ENDING	NO. DAYS COVERED	CASES FEVER	TOTAL ILLNESSES
A	29 Jan	15	22	36
A-1	4 Feb	5	1	5
A-2	10 Mar	34	30	44
A-3	17 Mar	7	11	13
A-4	4 Apr	18	23	24
A-5	19 Apr	15	13	21

Table 3. Number of Persons Living in Agrovilas Who are Enrolled in the Altamira Surveillance Program.

AGROVILA	NO. OF FAMILIES	POPULATION
Da Uniao	42	297
Pouso Alegre	20	131
Km 40	20	144
Km 70	29	175
Km 100	11	61
TOTAL	128	737

Table 4. Number of Ill Persons Living on Road Front Lotes Seen on Each Visit Before the Six Month Collection in Altamira.

VISIT	DATE ENDING	NO. DAYS COVERED	CASES FEVER	TOTAL ILLNESS
1	02/12/74	12	6	26
2	17/12/74	15	11	18
3	15/01/75	28	11	34
4	27/01/75	12	8	12
5	19/02/75	23	21	41
6	27/02/75	8	10	20
7	12/03/75	15	14	18
8	31/03/75	19	14	19
9	10/04/75	10	13	22
10	27/04/75	17	10	13
11	15/05/75	18	6	8

facilities and passive surveillance through official Brazilian agencies provides both early warning of disease outbreaks and a wealth of baseline data acquired prior to a local outbreak. The disease incidence data with temporal and geographic distribution when collated with the entomological and wildlife ecology data simultaneously collected from the same areas should provide insights into disease transmission and suggest productive avenues for in-depth study.

RELEVANCE TO MILITARY MEDICINE: The study population, composed of recent immigrants to the Transamazon, is somewhat analogous to troops entering a new area. The region itself is tropical forest within which many diseases of proven military medical importance have been recognized. Colonists entering the area from all other states of Brazil are carrying agents such as Schistosoma mansoni and Trypanosoma cruzi.

Malaria is a major problem in the area, and is discussed in detail in Epidemiology F. Yellow fever is present, and an outbreak was investigated by USAMRU personnel in March 1974. Diarrheal disease of unknown etiology is a major cause of death in infants and may incapacitate adults. Skin diseases occur in up to 50-60% of the colonists and may be caused by bacteria, fungi or parasites. Insect bites are annoying and may become serious enough to limit the activities of soldier or colonist. Simulium and phlebotomines are two particularly vicious pests common to this area, the bites of which frequently cause morbidity in persons working in the field. Snakes and venomous insects cause considerable morbidity. Other diseases of possible military medical importance found in the Transamazon include arboviral diseases, (e.g., Oropouche) Hemorrhagic Syndrome of Altamira and Labrea Fever (Febre Negre), a virulent hepatitis/encephalitis with a high mortality rate.

In addition, the surveillance program itself is of military medical importance when viewed as an experimental tool to monitor disease in a field situation. It is the only reliable source of disease information in the study areas at this time. The program is currently measuring disease incidence on a bi-weekly basis in a well-described population of 2300 persons living along 750 Km of jungle highway. The routine work in the field is being done by indigenous personnel with no more than a high school education. If the program is successful in accurately measuring disease incidence and rapidly identifying outbreaks, it may be applicable in other areas of the world where large populations are entering new environments.

F. MALARIA

BACKGROUND: Malaria, a disease of documented military medical importance, has long been recognized as one of Brazil's most important health problems. Official efforts to mount extensive campaigns against the disease include the formation of CEM in 1965 and SUCAM in 1969, autonomous organizations reporting directly to the Minister of Health. Of the malarious areas in Brazil, the Transamazon has been thought to be the most refractory region because of the mobile elements in the population, the scarcity of health facilities, periodic flooding, difficulties in transportation, low population density, inadequate housing and the opening of large new areas to colonization. In 1973, the Ministry of Health noted that the principal malaria problems with the Transamazon Region were found along the highway between Maraba and Altamira. It was in this area that the USAMRU surveillance program was begun in 1974.

PRE-PILOT STUDY: In January-March 1974, data collected by SUCAM and the FSESP hospitals in Maraba and Altamira were reviewed and evaluated. The incidence of malaria appeared to be higher in the Maraba jurisdiction, and in both areas the peak incidence occurred in June - September (dry season) with a smaller peak in January-February (wet season) (Figures 1 and 2). Certain deficiencies in the collection of data became apparent, and these are listed below for each organization.

SUCAM Data

1. Irregular intervals between visits due to inaccessability during the rainy season;

2. Insufficient manpower;

3. Lack of field personnel with statistical training;

4. Self-administration of non-curative doses of chloroquine by the colonists, leading to false-negative results due to chronic, low-level parasitemia. Very limited availability and use of primaquine. Incomplete treatment outside of hospitals.

FSESP Hospital Data

1. Unknown cachement area;

2. Diagnosis of malaria despite negative results on slides examination.

Fig. 1. CASES OF MALARIA REPORTED BY SUCAM AND THE FSESP HOSPITAL IN MARABA 1973-1974

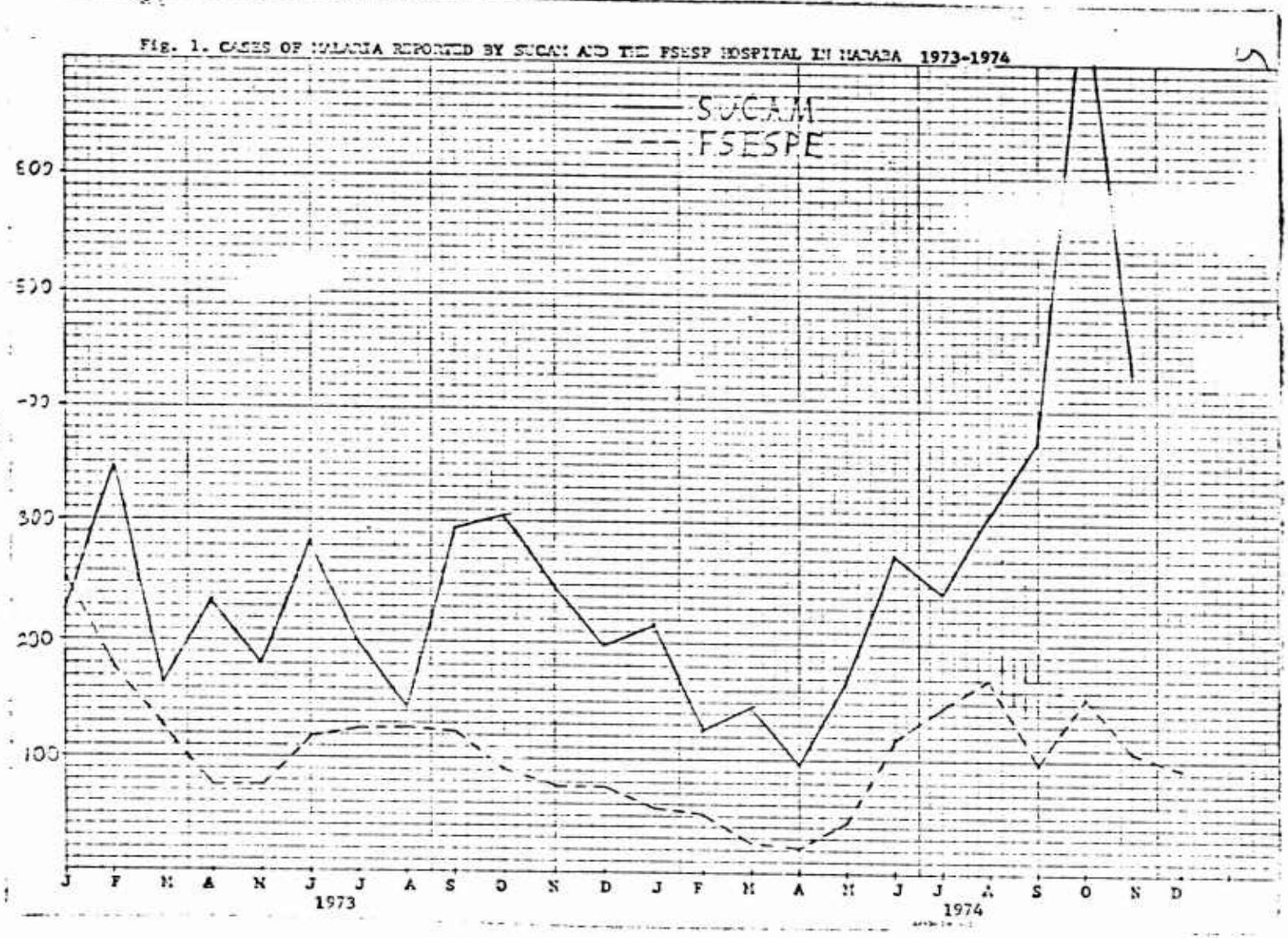
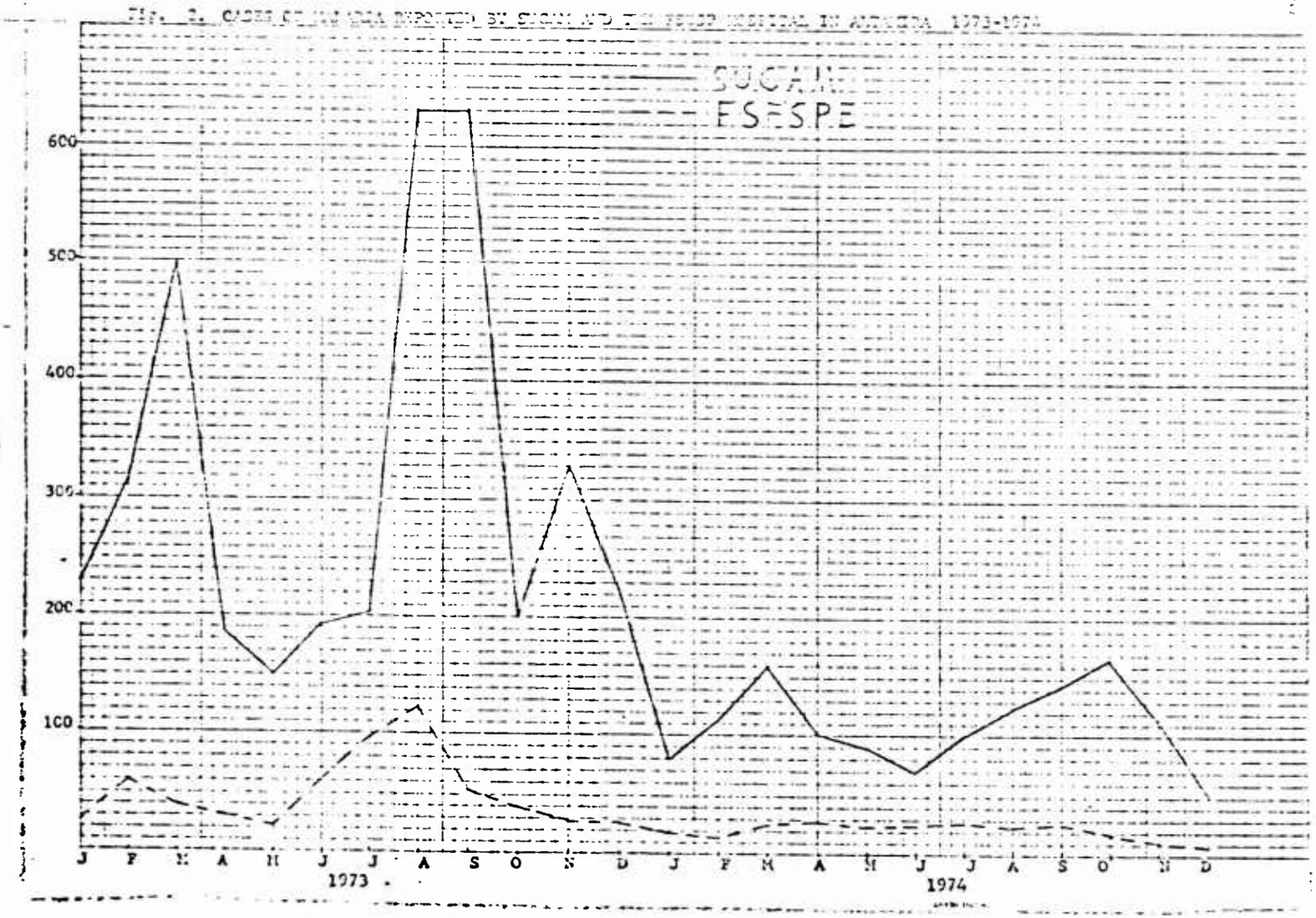


Fig. 2. CASES OF MALARIA REPORTED BY SUCAM AND THE PUBLIC HOSPITAL IN ALTAMIRA 1973-1974



Despite these deficiencies, malaria data collected by SUCAM and by FSESP hospitals is a valuable adjunct to the USAMRU surveillance program and our field teams continue to collect and report data from these sources on a monthly basis.

A short trip to Maraba was made in January 1974 to gather first-hand information about the area and its suitability for pilot stu[illegible].

1. An age-sex analysis of all malaria cases identified by SUCAM in 1973 revealed an over-representation of males 5-4[illegible] years old.

2. All persons living in Glebas 1-40 who had malaria between 12 December 1973 and 12 January 1974 were interviewed. An association was found between working in the forest and having a history of malaria, although the numbers were too small to be statistically significant.

This association, in view of the high male-female ratio of malaria cases, suggested an exophilic vector, a possibility thought worthy of further investigation. All these data were considered when the questionnaires were designed for use in the Maraba pilot study.

EPIDEMIOLOGY SURVEILLANCE PROGRAM - MALARIA DATA:

Maraba

When the history of malaria in the area was analyzed, the highest incidence was found in males aged 30-49 and females aged 20-39 (Table 1). By area, the highest incidence rate was found between Gleba 33 and Gleba 40 (Table 2). The cases found by history were tested against environmental and behavioral variables and a positive correlation was found for type of terrain and sleeping outside the house (Table 3). No correlation was found for distance of house from forest, type of house or presence of mosquitoes in or around the house.

During this original visit,an attempt was made to collect a malaria smear from every person in the sample. There were some refusals and some slides were poorly prepared or lost in shipment, but 508 were finally read by the laboratory. Of these, 32 were positive (13 falciparum and 20 vivax). The highest incidence was found in children and adult males (Table 4). The highest percentage of positive slides came from Gleba 33-56 (Table 5). Of the five landowners who had malaria, four occasionally slept outside the house at night.

Visits were made to each of the families in our sample every two weeks. Only one case of malaria was diagnosed over a six month period, despite the fact that SUCAM and FSESP continued to report high

Table 1. Incidence of Malaria Found by History in the USAMRU Survey in Maraba, June 1974, by Age and Sex.

AGE	CASES/PERSONS/YEAR	
	MALE	FEMALE
1	0.00	0.14
1-4	0.17	0.08
5-9	0.14	0.08
10-14	0.17	0.13
15-19	0.07	0.07
20-29	0.10	0.20
30-39	0.28	0.32
40-49	0.23	0.03
50-59	0.07	0.00
60+	0.11	0.13

Table 2. Geographical Distribution of Malaria Cases Found by History in the USAMRU Survey in Maraba, June 1974.

GLEBA	CASES/PERSONS/YEAR
1-6	0.145
1-8	0.036
9-16	0.166
17-24	0.154
25-32	0.136
33-40	0.386
41-48	0.147
49-56	0.124
57-64	0.167
65-72	0.128
73-76	0.078

Table 3. Relation of the Number of Malaria Cases Found by History in the USAMRU Survey in Maraba, June 1974, to Sleeping Outside the House and Type of Terrain.

WHAT IS THE TERRAIN OF THE LOTE?

	ROLLING HILLS	STEEP HILLS	PLAIN
MALARIA	100	13	42
NO MALARIA	406	25	193

$X^2 = 5.47$ $P = .10 < P < .05$

DOES THE FAMILY SOMETIMES SLEEP OUTSIDE THE HOUSE?

	YES	NO
MALARIA	22	116
NO MALARIA	58	564

$X^2 = 5.25$ $P = .05 < P < .02$

Table 4. Prevalence by Age and Sex of Slide-Proven Malaria Found by USAMRU Survey in Maraba, June 1974.

	CASES	POPULATION	PREVALENCE
Males ≥15 Years Old	9	139	.06
Females ≥15 Years Old	4	118	.03
Children < 15 Years Old	19	215	.09
TOTAL	32	472	

Table 5. Positive Malaria Slides from Original USAMRU Survey Data in Maraba July-August 1974.

GLEBA	POPULATION	SLIDES READ	% READ	SLIDES POSITIVE	SLIDES POSITIVE SLIDES READ
1-8	95	16	16	0	0
9-16	97	25	26	0	0
17-24	76	52	68	0	0
25-32	102	69	68	1	1%
33-40	68	51	75	9	18%
41-48	61	50	82	7	14%
49-56	62	43	69	8	19%
57-64	84	72	86	2	3%
65-72	113	84	74	2	2%
73-76	54	46	85	3	7%
TOTAL	812	508		32	

rates of malaria from the general area until November. This discrepancy could be due to a number of factors including:

1. Prompt and adequate treatment of the cases identified in our original survey might have lead to decreased transmission along the highway;

2. The actual incidence of malaria along the highway might have been decreasing with the onset of the rainy season;

3. Personnel problems and changes may have lead to inaccurate reporting.

The six-month follow-up visit occurred in January, during the height of the rainy season. An attempt was made to get a malaria smear from each of the 801 persons in our sample. Only two persons with parasitemia (both vivax) were identified out of 624 slides examined.

Altamira

The surveillance program in Altamira started in November 1974, with the enrollment of 802 persons living on roadfront lotes west of the city. They reported a very low incidence of malaria, about .02 cases/persons/year, distributed evenly along the highway. Most of the cases occurred in adult males (Table 6). No significant association was found with any of the previously-mentioned environmental variables. Since the original visit, two cases of malaria have been found along the highway.

In addition, agrovilas were enrolled in the Altamira jurisdiction. In Agrovila da Uniao, between Gleba 3 and Gleba 5 and close to the Xingu River (10 Km to south) the incidence of malaria was .32 cases/persons/year. Those who owned lotes closer to the river and farther from the highway had the highest incidence of malaria. Those who regularly went to their lote and slept there overnight had a higher incidence than those who stayed in the agrovila (Table 7). Males were much more frequently affected than females (Table 8).

In Agrovila Pouso Alegre, situated to the north, between the Agrovila da Uniao and the highway, the incidence was .04 cases/persons/year and all three cases of malaria occurred in a 24 year old male whose lote was well removed from the highway.

At Agrovila Miguel Gustavo, situated near the highway at Km 100, there was only one case of malaria by history out of 60 people interviewed. This occurred in a 52 year old male.

At Agrovila Carlos Pena Filho, on the highway at Km 40, there was no malaria by history in the 133 persons interviewed.

Table 6. Age-Sex Distribution of Malaria Cases Found by History in USAMRU Survey in Altamira, November 1974.

AGE	MALE	FEMALE
0-5	1	1
5-9	3	0
10-14	2	2
15-19	1	1
20-29	8	1
30-39	9	2
40-49	2	0
50-59	1	1
60+	0	0
TOTAL	27	8

Table 7. Relation of Regular Visits to Lote to Incidence of Malaria found by History in the USAMRU Survey of Agrovila da Uniao in January 1975.

	GO TO LOTE	DON'T KNOW	DON'T GO TO LOTE
Cases	22	56	6
Persons-Years	56	163	49.5
Attack Rate	.39	.34	.12

Table 8. Age-Sex Incidence of Malaria Found by History in USAMRU Survey of Agrovila da Uniao in January 1975.

AGE	SEX	CASES	PERSON-YEARS	ATTACK RATE
0-4	M&F	4	41	.097
5-14	M	27	61.5	.439
5-14	F	1	28	.035
≥ 15	M	38	66.25	.573
≥ 15	F	16	66	.242

SPECIAL PROJECTS: In order to take advantage of the smaller malaria peak that sometimes occurs in January-February, two special projects were carried out in January 1975.

The first of these took place in the Maraba area. A review of the records of SUCAM and the FSESP hospital showed a high frequency of malaria cases being reported outside Maraba along highway PA-70 and in a small town near the River Araguaia called Palestina. After visits to each locale, Palestina was chosen for study. Headquarters was set up in the school serving this town of 2000 inhabitants and initial work involved:

1. Making a map of the town;

2. Identifying active malaria cases and obtaining epidemiclodical information on them and their families;

3. Mosquito collections/equipment for drawing blood, making, staining and reading smears and drugs for treatment were shipped to the school.

Our plan called for moving the mosquito collection sites to the areas of highest malaria incidence. Unfortunately most of the cases identified seemed to have acquired their malaria in a nearby area called Grota Vermelha, which was inaccessible at that time. One hundred eighty-nine people were examined for malaria, based upon recent or present illness. From these, 13 cases of falciparum, 14 cases of vivax and one mixed infection were identified. The main thrust of the project was vector collection and identification and these results are reported in the Entomology section.

The second project was done in the Agrovila da Uniao. The Epidemiology results have been reported above and the vector studies are covered in the Entomology section. No cases were identified during this study, but A. darlingi was captured for the first time.

CONCLUSIONS AND PLANS FOR FUTURE: Malaria continues to be a serious problem in the Transamazon, although its distribution is spotty, temporally and geographically. Many colonized areas along the highway have a low incidence, but in others, the disease is a major threat, causing much morbidity and mortality. In Gleba 33-40, west of Maraba, the incidence by history was 39 cases per 100 persons per year. In June 1974, the prevalence of slide positivity was 6% for the entire stretch of highway between Gleba 1-76, but between Gleba 33-56 it reached a much higher level, 14-19%. In the town of Palestina, east of Maraba, no prevalence or incidence figures can be given since at this time we do not have accurate denominator data. However, this area accounted for more cases and more deaths in the Maraba FSESP Hospital than any other. The Agrovila da Uniao has a similar high

incidence of malaria. The attack rate in males 15 years and older is 57 cases/100 persons/year.

Malaria is the greatest single reported cause of death in the area. The physicians at the FSESP hospitals are beginning to report cases in which there is little or no clinical response to chloroquine. Evidence is accumulating that the disease may be acquired outside the house from an exophilic, exophagic vector. Relevant factors about which very little is known include the effect of self-administration of chloroquine in non-curative doses and the introduction of malaria by migrant workers. Now that most areas of the world are served by some sort of malaria control program, the situation found in the Amazon in 1975 may be more typical than the classic hyperendemic malaria found in Africa and southeast Asia.

Based upon past experience, malaria cases should start to peak in June or July and in anticipation of this, several measures have been instituted to improve the quality of data collection. Data from SUCAM and FSESP are being collected more regularly and reported more rapidly to Belem. Deaths are being recorded from the local Vital Statistics office and reported weekly to Belem. Information is being collected on a weekly basis from the health posts situated in many of the agrovilas. The results of the USAMRU surveillance program are now being recorded on a spot-map which is sent to Belem at the end of each two week period. In addition, each of the field stations reports at least weekly by telephone and immediately if there is any evidence of a disease outbreak.

All surveillance activities will be used to identify the onset of the summer peak of malaria incidence. At that time, studies will be initiated in Palestina, Agrovila da Uniao, the roadfront lotes west of Maraba and wherever localized outbreaks may occur. The main purpose of these studies will be to clarify the epidemiological picture and to identify vectors. If adequate numbers of untreated falciparum cases can be found, in vitro testing for chloroquine resistance will be attempted. The response of vivax cases to the standard chloroquine regimen will also be observed.

G. INVESTIGATION OF EPIDEMIC OF INFLUENZA IN THE TERRITORY OF RORAIMA, BRAZIL

DESCRIPTION OF PROGRAM AND OBJECTIVES: In April-May 1974, USAMRU and Institute Evandro Chagas personnel investigated an epidemic of a flu-like illness in the territory of Roraima (see maps).

WORK ACCOMPLISHED: In Boa Vista, the capital city of Roraima, the Secretary of the Ministry of Health and various private physicians provided initial information about the outbreak which had occurred in both military and civilian populations, beginning a month before in the center of the city and spreading to the edges of the city and then to surrounding towns. A plan of investigation was set up and carried out that included:

1. Prompt referral of all suspicious cases seen by private physicians to a central location manned by the Institute Evandro Chagas technician.

2. Investigation of all acute cases seen at the military barracks.

3. These investigations of acute cases included history, physical examination, and collection of blood for serology and throat washings for virus isolation.

4. A review of all deaths occurring since January 1974.

5. A review of all hospital admissions since January 1974.

6. A review of school absences in March and April in a large primary school located near the center of the city.

7. Serum survey of 105 out of approximately 400 soldiers quartered in Boa Vista.

8. An investigation of reported cases in the outlying villages of Surumu, Catrimani and Puxa Faca.

DATA OBTAINED: Only four patients were referred for examination in Boa Vista. None had the classic symptoms of influenza, and no virus was isolated from throat washings.

There had been a sharp rise in the number of cases of respiratory disease admitted to the two hospitals in the city. This rise had occurred in March (no figures were available for April in one hospital) and was accompanied by a rise in deaths due to respiratory disease (Table 1). School absences had risen steadily from the first week in March to the second week in April, then had declined

Table 1. Respiratory Diseases in Boa Vista: Number of Hospital Cases and Deaths January-April 1974.

1974
CASES OF RESPIRATORY DISEASE HOSPITALIZED IN BOA VISTA

	JAN	FEB	MAR	APR
Hospital Cel Mota	1	1	20	6
Hospital Fatima	2	3	24	

1974
DEATHS DUE TO RESPIRATORY DISEASE IN BOA VISTA

	JAN	FEB	MAR	APR
Deaths	2	1	5	8

abruptly after a week-long vacation (Fig. 1). Sixty-six of the 105 soldiers examined had HI antibodies to influenza type A-2. There was a higher rate of positivity in those having a history of recent respiratory disease than in those who did not (Table 2).

Many of the residents of Puxa Faca were experiencing flu-like symptoms. Laboratory results supported the clinical impressions gained in the field. The only isolations of influenza virus were obtained in Puxa Faca, where 13 of 18 specimens were positive (Table 3). Serological evidence of a past encounter with influenza were found in Puxa Faca in May, and even higher rates in July.

The people of Surumu had no illness at the time of investigation, but reported many recent cases of a flu-like illness. The rate of positives from Surumu was already 98% in May specimens, so no re-bleeding was done in July. (Table 4.)

The Indians living in Catrimani were in the middle of an epidemic of URI, but it did not appear to be flu. In Catrimani, there was no serological evidence of influenza in either May or July.

ANALYSIS OF DATA: An examination of data and interviews with local residents leads to the conclusions that there was an epidemic of influenza A-2 in Boa Vista in March and the first week of April. The origin of this outbreak is unknown, but it may have been imported from Venezuela. There were outbreaks of influenza in Venezuela earlier in the year and these could have spread to Boa Vista along the road from Venezuela. The disease could have been carried by military or civilian populations. It later spread to outlying villages whose only communication with Boa Vista was by foot, horseback or small plane. It did not apread to the Indian village of Catrimani, which is usually visited only by missionaries travelling by small plane.

MILITARY-MEDICAL IMPORTANCE: Approximately 25% of the military personnel in Roraima became ill, and military operations were curtailed for one month.

Fig. 1.

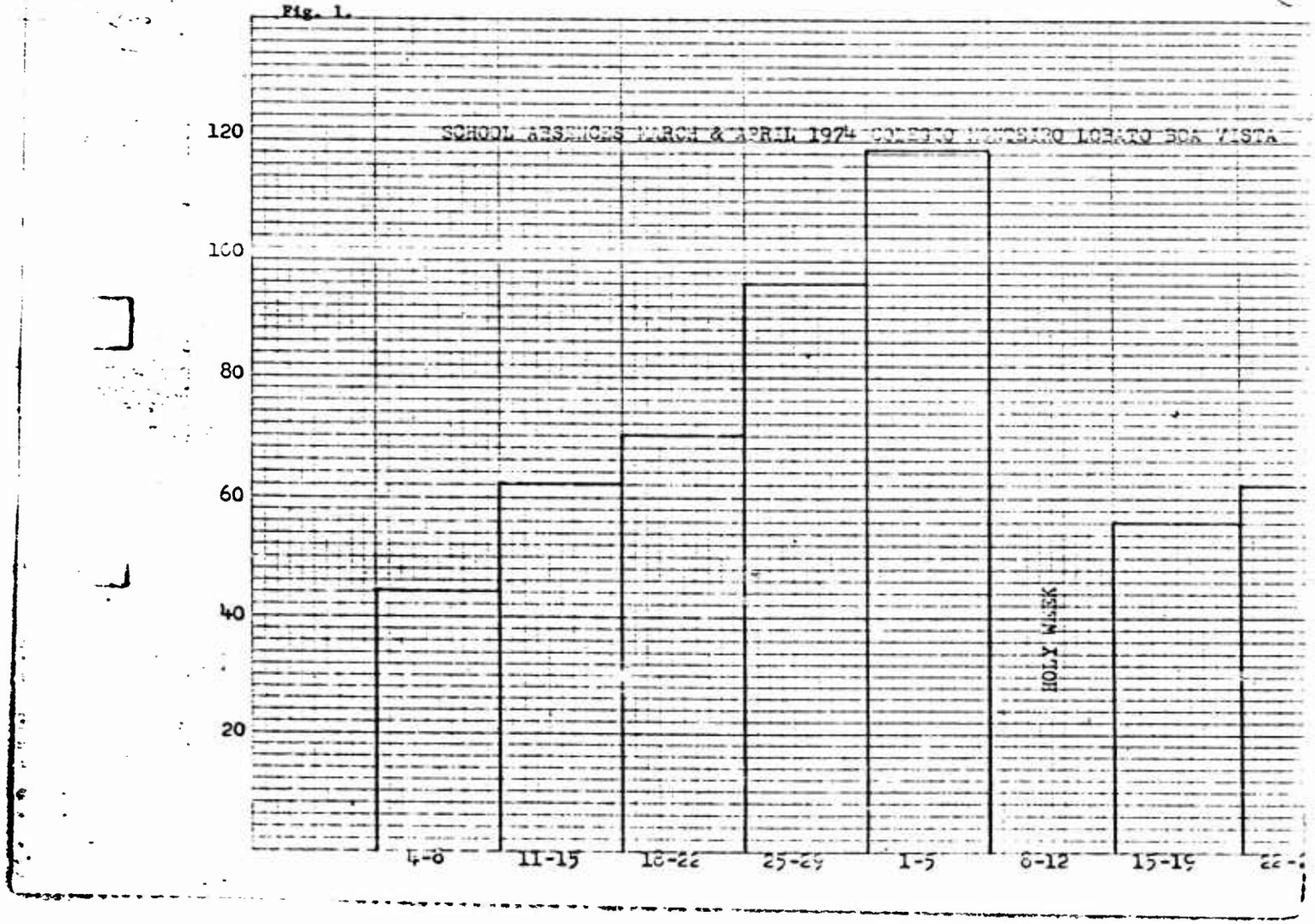

SCHOOL ABSENCES MARCH & APRIL 1974 COLEGIO MONTEIRO LOBATO BOA VISTA
120
100
80
60
40
20
HOLY WEEK
4-8
11-15
18-22
25-29
1-5
8-12
15-19

Table 2. HI Antibodies to the Port Chalmers Strain of Influenza A-2 Virus in Military Personnel of Boa Vista, Territory of Roraima 1974.

CLINICAL HISTORY OF RESPIRATORY DISEASE	DAYS OF ILLNESS	POSITIVE/TESTED	%
Yes	$\leq$ 10 days	6/8	75
	$>$10 days	37/52	71
No		8/21	31
Ignored		15/24	62

Table 3. Isolation of Influenza A-2 Virus by Locality in Territory Roraima, May 1974.

LOCALITY	NUMBER OF SAMPLES TESTED*	NUMBER OF ISOLETES
Boa Vista	4	0
Catrimani	12	0
Surumu	1	0
Puxa Faca	18	13
TOTAL	35	13

* Throat Gargle

Table 4. HI Antibodies to the Port Chalmers Strain of Influenza A-2 Virus in Persons from Three (3) Localities, Territory of Roraima, 1974.

LOCALITIES	CLINICAL HISTORY OF RESPIRATORY DISEASE	MAY POSITIVE/TESTED	%	JUNE POSITIVE/TESTED	%
Catrimani	<10 days or neg	1/13	0	--	--
	>10 days	--	--	0/9	0
	Ignored	--	--	0/6	0
Surumu	<10 days	1/1	--	--	--
	>10 days	17/18	98	--	--
Puxa Faca	<10 days	6/27	22	--	--
	>10 days	2/2	--	9/9*	100
	Ignored	--	--	15/17	88

*Six (6) of these individuals, Influenza Virus was isolated in May.

II. ENTOMOLOGY

A. SURVEILLANCE FOR MEDICALLY IMPORTANT INSECTS ALONG THE TRANSAMAZON HIGHWAY

BACKGROUND: The entomology surveillance program along the Transamazon Highway is an integrated field and laboratory effort. Field work consists of a routine program of collecting medically important insects in the Maraba and Altamira areas. The laboratory work consists of identifying and processing collections, and analyzing resultant data. The full-scale field program was operational in November 1974 and the laboratory program became operational in April 1975. Specific objectives for the entomology program were to provide.

1. Distributional information on medically important insects by habitat, time, season of year and geographical location along the highway.

2. Insect specimens for pathogen identification and/or isolation.

The Transamazon Highway, at present, could be described as a narrow ribbon of cleared land, surrounded by vast areas of unoccupied virgin forest. The land along the highway is colonized in small sections of land known as 'lotes', one family per lote. A minimum of three habitats can be described for each lote; cleared land, the forest "edge" and the tropical forest. Subclassifications can be made due to man's efforts and terrain variation.

Routine surveillance is conducted at selected sites from the Araguaia River, east of Maraba, to Km 160 west of Altamira (Figure 1). After preliminary reconnaissance, individual lotes were selected in order to compare the fauna of low-lying wet areas with more dry highland areas included in the epidemiology and animal ecology surveillance programs.

The collection program was designed to sample a variety of habitats through much of the day and night using several collecting methods (Figure 2) and is staffed with 3 teams of collectors, (2 persons per team). In the Maraba and Altamira areas 4 and 8 road-side lotes are sampled respectively. Three additional sites have been established for malaria studies. Each lote is sampled two days every 3 weeks, with emphasis on man-biting collections. Collections are also made with Shannon traps, light traps and Disney traps.

A field form that is simple, but adequate, for recording meaningful information for each collection was developed (Figure 3). Information recorded includes type of collection, ecological description of collection site and meteorological observations. Space is also provided for recording numbers collected by insect group. Weekly reports are

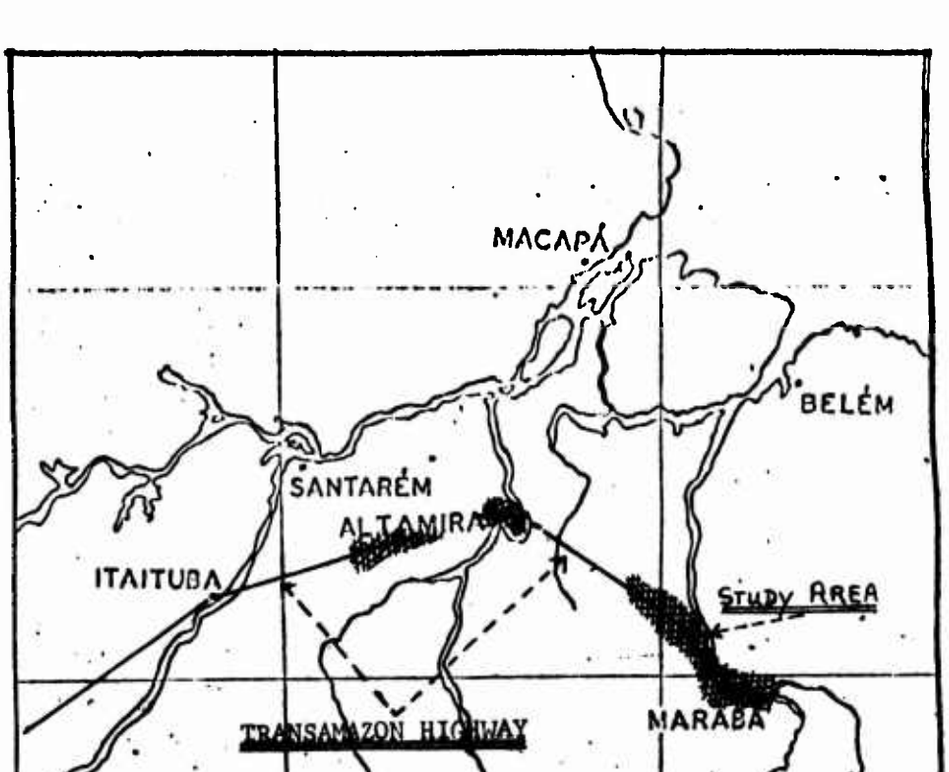


Figure 1. Maraba and Altamira Study Areas along the Transamazon Highway.

Figure 2. Standardized Collecting Program

FIRST DAY AT COLLECTING SITE

1200 - 1215	A man-biting collection near the house (cleared area). At selected sites this collection is made in the shrub vegetation near the forest.
1230 - 1245	Man-biting collection in the forest at a designated location.
1400 - 1500	Man-biting collection in the tree tower.
1530 - 1545	Man-biting collection in the cleared or shrub area.
1600 - 1615	Man-biting collection in the forest.
1815	Connect portable light traps; one each in forest and cleared areas.
1830 - 1845	Man-biting collection in the forest.
1855 - 1910	Man-biting collection in cleared area near house. At selected sites these collections are made in the house.
1925 - 1940	Man-biting collection in the forest.
1955 - 2010	Man-biting collection near or inside the house.
2030 - 2130	Collection with Shannon trap.
2145 - 2200	Man-biting collection in the forest.
2215 - 2230	Man-biting collection in or near the house.

SECOND DAY AT COLLECTING SITE

0630	Collect the light trap captures.
0900 - 0915	Man-biting collection in the forest.
0930 - 0945	Man-biting collection in cleared or shrub areas.
1000 - 1100	Man-biting collection in tree tower.

After 1000 continue the same program outlined for the 1st day.

Figure 2. (cont.) Standardized Collection Program

THIRD DAY AT COLLECTING SITE

0630 Collect the light trap captures.

Prepare materials and move to the next location to begin work at 1200 hours.

Figure 3. Entomology Form I (Form Adult Insects)

1. Collection No. _____ 2. Gleba _____ 3. Lote _____.

4. Jurisdiction _____ 5. Municipality _____ 6. Time collection was initiated _____ 7. Time of termination _____ 8. Type of collection _____ 9. Air temperature (dry bulb)_____ 10. Air temperature (wet bulb)_____ 11. Number of collectors_____.

12. Topography: (1) hill top, (2) hill side, (3) valley, (4) plain.

13. Drainage: (1) dry land, (2) occasionally flooded, (3) usually inundated.

14. Environment: (1) forest, (2) shrubs, (3) cut-area, (4) orchard, (5) cultivated and planted, (6) cleared and clean of plants, (7) village.

15. Type of vegetation: (1) trees, (2) trees and palms, (3) palms, (4) Acaizeiros, (5) secondary shrub, (6) grass, (7) mixed orchard, (8) Orchard_____, (9) cultivated, (10) house, (11) other_____.

16. Distance to house: (1) 10 m, (2) 10-99 m, (3) 100-999 m, (4) 1000 m or more.

17. Sky: (1) clear, (2) slightly cloudy, (3) cloudy, (4) drizzly, (5) raining, (6) heavy rain.

18. Shade: (1) none, (2) partial, (3) complete.

19. Wind: (1) none, (2) slight wind, (3) gusts of wind, (4) strong wind.

20. Height above ground: (1) ground level, (2) 1-4 m, (3) 5-8 m, (4) 9-12 m, (5) 13-16 m, (6) 17-20 m, (7) 20 m or more.

Figure 3. (cont.) Entomology Form I (Form Adult Insects)

21. Number collected: Anopheles _____, Culicines _____,

Sandflies_____, Black flies _____, others _____, Negative.

22. Preserved _____, 23. Comments_____.

sent to Belem indicating numbers and types of insects collected by habitat, time of day and collection method. These statistics are standardized by numbers of collections and man-hr. worked, and provide a system for monitoring work performance in the field, a knowledge of numbers of specimens coming to the laboratory and current data on insect population densities at the various field sites.

PROGRESS: Emphasis in the collection program has been placed on temporal and spatial representation of the insect fauna. Although the program was not designed to collect large numbers of insects for pathogen isolation, the monthly number of insects captured in ground level man-biting collections approaches 5000 specimens. This excludes the quantities captured with other collection methods and those collected at the special malaria study areas, which comprise 1/3 of the entomology field program.

Considerable variation in population structure of hematophagous insects has been found between areas. This is illustrated by comparing man-biting collection data from four sites in Altamira with results from four sites in Maraba during April, 1975 (Table 1). In Altamira, 65% of all insects collected were Culicidae compared to 35% in Maraba. The proportion of sandflies collected was higher in Maraba than Altamira, but total sandflies collected was less. The mean collection size for all groups of insects in all habitats and time intervals was much larger in Altamira than in Maraba. No single cause seems adequate to explain this result. It may be due to a combination of actual differences in population structure, seasonal differences and differences in collector efficiency between the two areas.

Large differences in population structure have also been found between individual lotes in the same study area. Four sites were arbitratily selected from the Altamira area. During the months of April and May, 1 lote gave highest numbers of black flies and mosquitoes, another had the largest populations of biting midges. A 3rd lote yielded the most dense populations of sandflies. The 3rd site also had large populations of biting midges.

Preliminary information has been compiled on the spatial and chronological distribution of the hematophagous insects along the highway, for rough-sorted insect groups (Table 2). The majority of anophelines are collected in the forest at sunset. Culicines are also intensely active at this time, but greater numbers, which probably represent relatively few species, are collected in the extradomicilary environment; sandflies are primarily active after sunset in the forest, whereas, the Simuliidae are diurnal and are predominantly captured in open areas. The Culicoides were active day and night in the forest and active in the cleared areas at night. Although these findings are, more or less, predictable; the results indicate the validity of the broad-based collection program for general sampling of hematophagous insect populations.

Table 1. Population structure of hematophagous Diptera in Maraba and Altamira, Para, Brazil. Numbers based on man-biting collections at 4 sites in both areas for the month of April 1975.

AREA	% of Total Collected by Insect Group					Number Collections	Total No. Collected	Mean No. Per Collection
	Anopheles	Culicini	Sand Flies	Black Flies	Biting Midges			
Maraba	4	32	48	13	2	95	668	7.03
Altamira	13	47	18	9	8	124	2339	18.86

Table 2. Spatial and Temporal Distribution of Man-biting Diptera at Four Collection Sites During April 1975, in the Altamira Area, Para, Brazil.
Numbers Collected Based on 15-minute Man-biting Collections, 2 Collectors/Collection.

		TOTAL NUMBER COLLECTED BY INSECT GROUP					
LOCATION	TIME	MOSQUITOES	SAND FLIES	BLACK FLIES	BITING MIDGES	COLLECTIONS	TOTAL NUMBER COLLECTED
Near House (Clearing)	1200	(24+59)[a] 83	0	24	0	12	107
	1530	(53+31) 84	0	62	0	12	346
	1855	(52+280) 332	3	0	20	12	355
	1955	(7+70) 77	0	0	32	12	109
	2215	(6+113) 119	0	0	8	12	127
Forest	0900	(3+46) 49	7	4	7	5	67
	1230	(15+47) 62	3	14	4	12	83
	1600	(32+81) 113	6	5	12	12	136
	1830	(205+217) 422	71	0	13	11	506
	1925	(16+15) 31	126	0	1	12	158
	2145	(13+46) 59	194	0	92	12	345
TOTAL							2339

[a](Numbers of Anophelini and Culicini Collected).

Results from one site in Maraba, Gleba 5, Lote 5, are used to present a list of mosquito species found and for making a preliminary comparison of collection methods. A total of 27 species of mosquitoes have been tabulated for this site (Table 3), each represented by few numbers in each collection. Man-biting collections have contributed the bulk of these, a total of 23 species, whereas, light traps and Shannon traps have provided only 8 and 3 species respectively (Table 3). The forest has been most productive in numbers of species collected; 22 species compared to 8 species collected in the open areas.

The species list will increase as more collections are identified from different sites, but the general pattern of species occurrence by habitat and type of collection will probably remain much the same. Species representation of Culicoides in Shannon trap collections in unknown at this time. A sample of 210 sandflies from one Shannon trap collection in the Altamira area produced 15 species. However, the majority of the collection was composed of only 5 species. Man-biting collections were also productive for phlebotomines as revealed by the presence of 9 species in a collection of 67 specimens.

The least productive collection method has been the portable light trap. Few species of mosquitoes were obtained and the mean number collected per trap night was low ($\bar{X} = 6.88$). It is noteworthy, however, that 4 species have been collected only by this method (Table 3).

COMMENT: The process of identifying all the field collections is underway. Phlebotomine pools are being processed by Department of Virology I.E.C. for virus isolation attempts in vero cells. All other insect groups will be processed in suckling mice and tissue culture for virus isolation both at WRAIR and the I.E.C.

MILITARY IMPORTANCE: Insect-borne diseases are endemic along the Transamazon Highway, including parasitic and viral agents. Chief among the parasitic diseases is malaria which is found at numerous areas on the road in spite of past efforts at eradication. A variety of arboviral diseases occur sporadically in the Amazon region and may affect the colonists along the highway. The insect problems are not confined to transmission of disease, but also appear in the form of allergic reactions and secondary infections to insect bites, ranging from annoying to incapacitating.

The temporal, geographical and environmental distribution of insects is of military medical value in determining where and when insect problems do or can occur, and providing base-line information for investigating outbreaks of vector-borne disease and for insect-related disease problems. The surveillance program developed has application to military operational field environments where information regarding insect problems is required. Collation of entomological data with epi'emio-

Table 3. Distribution of Mosquito Species by Collection Method and Habitat. Information from Collections at Gleba 5 Lote 5 on the Transamazon Highway, approximately 50 Km west of Maraba, January - April 1975.

	MAN-BITING COLLECTIONS			LIGHT TRAP COLLECTIONS		SHANNON TRAP COLLECTIONS
SPECIES	PERIDOMICILIARY	FOREST	TREE TOWER	PERIDOMICILIARY	FOREST	FOREST
Aedes						
cerratus	--	+	--	--	--	--
argyrothorax	--	+	--	--	--	--
fulvithorax	--	+	--	--	--	--
septemstriatus	--	+	--	--	--	--
Anopheles						
nuneztovari	+	--	--	--	--	+
oswaldoi	--	+	--	--	--	+
trianmulatus	+	+	--	--	--	+
Coquillettidia						
lynchi	--	+	--	--	--	--
venezuelensis	+	+	--	--	+	--
Culex						
coronator	--	--	--	--	+	--
declarator	--	--	+	--	+	--
SP BN^{O} 17	--	--	--	+	--	--
SP BN^{O} 1	--	--	--	+	+	--

Table 3. (cont.) Distribution of Mosquito Species by Collection Method and Habitat. Information from Collections at Gelba 5 Lote 5 on the Transamazon Highway, approximately 50 Km west of Maraba, January - April 1975.

SPECIES	MAN-BITING COLLECTION			LIGHT TRAP COLLECTIONS		SHANNON TRAP COLLECTIONS
	PERIDOMICILIARY	FOREST	TREE TOWER	PERIDOMICILIARY	FOREST	FOREST
Haemagogus leucocelaenus	--	+	+	--	--	--
Limatus durhami	--	+	--	--	--	--
Mansonia titallans	+	--	+	+	+	--
Psorophora albipes	--	--	+	--	--	--
Psorophora cingulata	--	+	--	+	+	--
Psorophora ferox	--	--	+	--	--	--
Sabethes belisarioi	--	--	+	--	--	--
Sabethes chloropterus	--	+	+	--	--	--
Sabethes cyaneus	--	--	+	--	--	--
Sabethes glaucodaemon	--	--	+	--	--	--
Sabethes quasicyaneu	--	--	+	--	--	--

Table 3. (cont.) Distribution of Mosquito Species by Collection Method and Habitat. Information from Collections at Gleba 5 Lote 5 on the Transamazon Highway, approximately 50 Km west of Maraba, January - April 1975.

SPECIES	MAN BITING COLLECTION			LIGHT TRAP COLLECTIONS		SHANNON TRAP COLLECTIONS
	PERIDOMICILIARY	FOREST	TREE TOWER	PERIDOMICILIARY	FOREST	FOREST
Trichoprosopon digitatum	--	_	--	--	--	--
Uranotaenia geometrica	--	--	--	+	+	--
Wyeomyia aporonoma	--	+	+	--	--	--

logical and mammalogy information obtained simultaneously from the same areas should provide insights into infectious disease agent maintenance and transmission.

B. MALARIA

BACKGROUND: Marlaia remains the major health problem in the Amazon basin and in many areas has been found refractory to the national malaria eradication effort. The cause for this remains unknown, but probably relates to vector ecology, repeated introduction by infected migrants, and parasite resistance to the treatment regime. The major vector in the Amazon interior is Anopheles (Nyssorhynchus) darlingi Root (Deanne, et al., 1946). Secondary vectors of malaria are An. (N.) albitarsis Lynch Arribalzaga and perhaps An. (N.) brazilensis (Chagas). The major vector is generally considered to be a riverine mosquito and most studies in the epidemiology of malaria have been conducted in riverine semi-urban habitats.

Opening of the Transamazon Highway has focused attention on malaria transmission in the non-riverine habitats where the circumstances for malaria transmission are different. The USAMRU program for the study of malaria in these areas consisted of:

1. A routine surveillance program for vector species along the highway in the Maraba and Altamira areas;

2. Two sites (Palestine - a village southwest of Maraba and Agrovila Uniao at Km 35 east of Altamira) for studying the entomological aspects of malaria epidemiology and

3. Special short-term efforts to study malaria vectors in areas of active malaria transmission as identified.

PROGRESS: Information obtained from all 3 aspects of the malaria program indicates the presence of an unidentified secondary, exophilic vector which may be highly important in maintaining malaria endemnicity. Observations from the program are as follows:

1. The primary vector, An. (N.) darlingi, has been found in only 1 malaria focus - Agrovila Uniao west of Altamira.

2. Anopheles albitarsis has been found only near the larger towns where little transmission occurs. In rural areas, where malaria is transmitted, it does not seem to exist.

3. The common denominator species throughout the study area are An. (N.) oswaldoi (Peryassu), An. (N.) triannulatus (Neiva and Pinto) and An. (N.) muneztovari Gabaldon (Table 1).

Table 1. Species of Anopheles Collected by Area in the USAMRU Surveillance Program, November 1974 - May 1975.

SPECIES	PALESTINA	PA-70 (Km 66)	MARABA	TRANSAMAZON MARABA-ALTAMIRA	AGROVILA da UNIAO
Anopheles (Nyssorhynchus)					
oswaldoi	X	X	X	X	X
nuneztovari	X	X	X	X	X
triannulatus	X	X	X	X	X
rangeli	-	-	-	X	-
*noroestensis	X	-	-	X	-
darlingi	-	-	-	-	X
albitarsis	X	-	X	-	-
*aquasalis	X	-	-	-	-
brasiliensis	X	-	X	-	-
peryassu	X	-	-	-	-
Anopheles (Anopheles)					
mediopunctatus	X	-	-	X	-

*Not recorded for this geographical area of Brazil and are probably morphological variants of An. (N.) nuneztovari.

4. Dissection of 382 anophelines for oocysts and sporozooites in three areas have been negative (Table 2).

5. The 3 common species (3 above) do not appear to be strongly endophilic; although they are commonly found close to the house and may occasionally enter for a blood meal.

6. Peak biting activity for An. (N.) oswaldoi and An. (N.) nuneztovari occurs in the early evening (Figure 1).

7. Numerous problems have been encountered in the identification of anophelines, Nyssorhynchus subgenus, in the Maraba area. Morphological variation of An. (N.) nuneztovari is considerable and frequently casts doubt on the identification of any single specimen.

COMMENTS: Collections are being made at Agrovila Uniao to elucidate the cycle of biting activity of An. (N.) darlingi and quantify their endophilic behavior. Similar data are collected on the suspect vector species in the Maraba area. Efforts are also being made to obtain taxonomic specimens to resolve the taxonomic difficulties of the Nyssorhynchus.

At present, it appears that considerable malaria transmission occurs in our study areas in the absence of the putative vectors, An. (N.) darlingi and An. (N.) albitarsis. Intensive efforts to identify the malaria vectors in these areas are under way. Specimens of each potential vector are being fed upon patients circulating gametocytes. Salivary gland and stomach dissections are being performed. Exit and entry traps are being used to define the degree of endophily of potential vectors.

The absence of strongly endophilic anophelines in areas of malaria transmission lends support to the epidemiologic data suggesting the importance of extradomiciliary transmission.

C. OROPOUCHE VIRUS

BACKGROUND: Oropouche virus, distantly related to the Simbu group of arboviruses, occurs in epidemic form in the Amazon basin. The vector is unknown, but Culex pipiens quinquefasciatus Say and Culicoides paraensis (Goeldi) are suspect species. The USAMRU-Belem entomology department has worked with 3 epidemics of Oropouche virus, February - July 1975. Observations on the biology of C. p. quinquefasciatus have also been made in the process of conducting malaria investigations. Each of the epidemics will be presented separately.

Table 2. Numbers of Specimens for Each Species Dissected for Malaria Parasites at 3 Locations. All dissections have been negative.

LOCATION	SPECIES	NO. DISSECTED
PA-70 (KM 66) (January 1975)	Anopheles (N.) trianmulatus	6
	Anopheles (N.) oswaldoi	3
	Anopheles (N.) nuneztovari	11
		20
Transamazon Gleba 36 L 06	Anopheles (N.) nuneztovari	21
	Anopheles (N.) oswaldoi	236
	Anopheles (N.) trianmulatus	1
	Anopheles (N.) spp.	37
		295
Transamazon Estreito, Palestina (Jan-Feb 1975)	Anopheles (N.) albitarsis	46
	Anopheles (N.) nuneztovari	18
	Anopheles (N.) oswaldoi	3
		67
	TOTAL	382

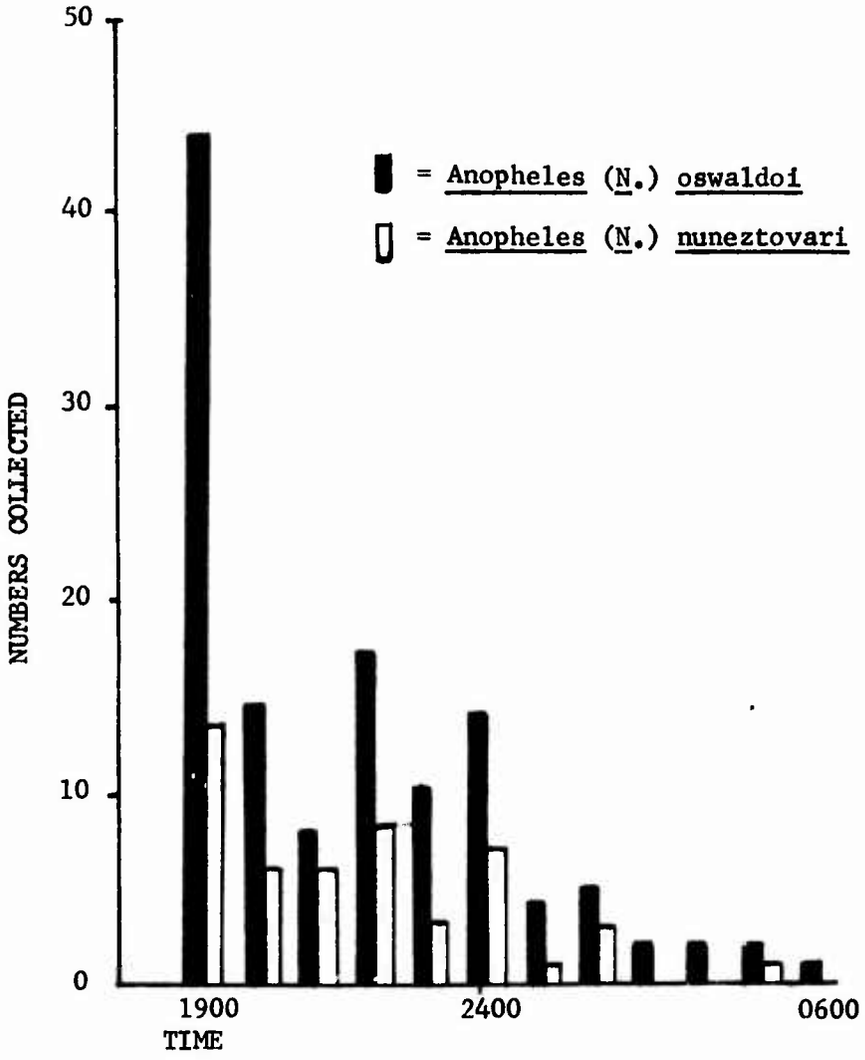


Figure 1. Temporal distribution of host-seeking anophelines in a peridomestic habitat at Km 125 east of Maraba, Para, Brazil. Numbers based on ½ hr. collections each hr. throughout the night with 2 collectors per collection.

WORK ACCOMPLISHED:

Mojui dos Campos, Para, Brazil

USAMRU-Belem was notified the end of February, 1975 that an epidemic of Oropouche virus had occurred January-February 1975 in the village of Mojui dos Campos, Para, Brazil. The village is located 33 Km southeast of Santarem with a population of approximately 2000 people. The epidemic was studied in cooperation with the Evandro Chagas Institute, Belem, Brazil. The work schedule at Mojui dos Campos was as follows:

1. Man-biting collections were made continuously at 1 house, each day, starting before sunrise and continuing until after sunset; employing 2 teams, (1 inside and 1 outside the house) with 2 men per team. Teams were relieved by fresh collectors every 3 hours. Collections were made for 50 min. each hr.

2. The initial impression was that the Culicoides had an early morning and late afternoon peak of biting activity. Thus, additional collections were made in early morning and late afternoon at 3 separate houses, in an attempt to collect as many Culicoides as possible for virus isolation attempts.

3. In-house resting captures of Culex pipiens quinquefasciatus Say were made routinely.

4. Collections were routinely made with light traps and Shannon traps.

These programs of collecting at Mojui dos Campos were conducted 3-14 March 1975.

Itupiranga, Para, Brazil

In June 1975, the entomology section, USAMRU-Belem, started an investigation of the Itupiranga epidemic of Oropouche virus. This investigation was conducted for approximately 2½ weeks. The collecting program included the following procedures:

1. Continuous man-biting collections at selected sites from 0600 to 2000 hours were made by 2-man teams, in 3 hr. shifts. Due to the low densities of Culicoides, collections were made only in the back yard of each house.

2. Shannon trap collections were made for 1 hr. each night within the city. Light trap collections were also made.

3. Man-biting collections were made on the river front to sample the large numbers of Anopheles and Mansonia that were present around 1900 hr.

All specimens were preserved in LN_2 for virus isolation attempts.

Santarem, Para, Brazil

A study of the Oropouche virus epidemic in Santarem, Para, Brazil was begun late in June 1975. The plan for this investigation was to gain information on the distribution of C. paraensis and C. p. quinquefasciatus throughout the city and simultaneously obtain specimens for virus isolation attempt. This approach was adopted for the following reasons:

1. Santarem is a large and densely populated city (located at the junction of the Tapajos and Amazon rivers).

2. No specific information was available on foci of the epidemic within the city.

3. Sufficient background information was available from previous investigations to state the peak biting time of C. parensis and devise a collecting program to include collections of both C. parensis and C. p. quinquefasciatus.

4. It was hoped that a correlation between the densities of one or both species and occurrence of Oropouche virus infections would be found.

The study plan included a number of detailed procedures. The city was divided into 6 areas for entomologic and epidemiologic sampling (Fig. 1). Collections were made at 6 randomly selected houses in each area. Collections were made in the backyard of each house. Twelve teams of collectors, 2 men per team, were employed and 2 areas were covered simultaneously. Each team collected continuously from 1400-2000 hr. each day for a total of 3 days at each house. Each team was visited hourly by trained entomology technicians. During the visits, the hourly collections were gathered and new collection containers were left with the teams. The collections were returned to the laboratory where the C. parensis were killed, identified, enumerated and grouped for virus isolation. Mosquitoes were preserved in LN_2 for later processing. The teams were rotated; thus no team was at one house for more than 1 day. With this collecting scheme, the entire city was covered in 9 collecting days (3 days in each area, 2 areas every 3 days for a total of 6 areas).

Since it was previously speculated that C. paraensis were limited in distribution to human population centers, a study was undertaken to

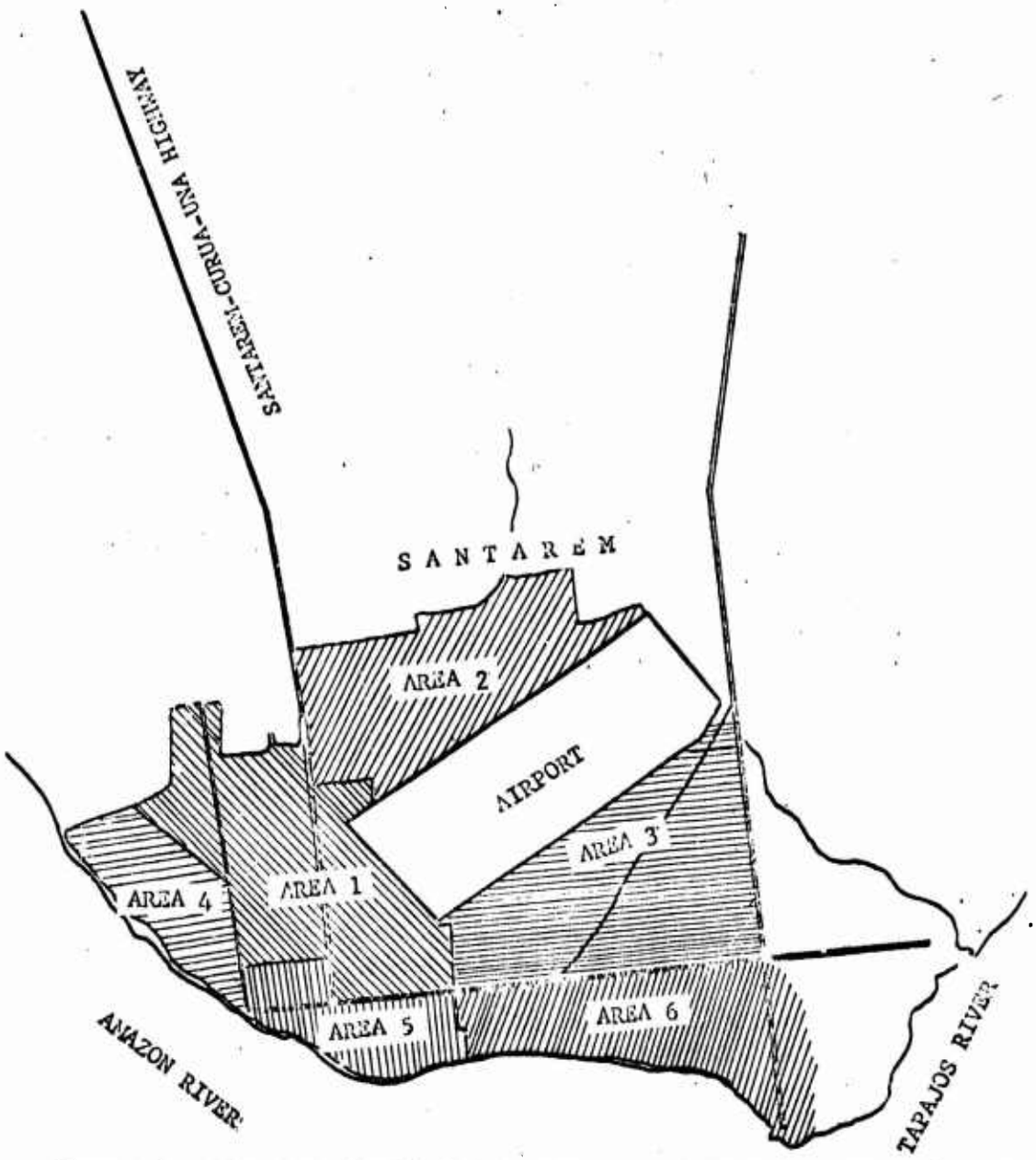


Figure 1. Six study areas within the city of Santarem, Para, Brazil.

define the distribution of this biting-midge at various distances from the city. Four houses within the city, and 8 houses along 21 Km of the Santarem-Curua-una highway were randomly selected as collection sites (Fig. 2). The collecting program was, in other respects, identical to that employed in the city.

Forty-eight sites were sampled during the overall investigation. This large number of collecting sites provided an opportunity to obtain detailed environmental data from which the factors associated with the occurrence of C. paraensis and C. p. quinquefasciatus may be defined. A field form was devised and completed for each site. Data from field forms are being collated and analyzed at present.

In the 3 epidemics it was necessary to offer workers incentive pay for collecting biting midges, since the bite of these insects is generally not felt by local residents.

Culex pipiens quinquefasciatus Say

Culex pipiens quinquefasciatus is found in association with population centers throughout the Amazon basin. The nocturnal nature of this mosquito made it convenient to make observations on its behavior in the process of conducting malaria studies. These observations resulted from systematized man-biting collections, both indoors and outdoors, for ½ hr. intervals each hr. from 1800 until sunrise. In addition, resting captures were made on inside walls for 10 min. each hr. The study site was in Palestina, approximately 100 Km southwest of Maraba, Para, Brazil. These collections are made monthly, but data will be presented only for 1 week's collection in March 1975.

PROGRESS: Much of the material obtained from the 3 epidemics is still being processed by USAMRU-Belem and the I.E.C. Entomological findings, to date are as follows:

Mojui dos Campos, Para, Brazil

1. Two species of hematophagous insects were sufficiently abundant to support an epidemic - C. paraensis (identification verified by Dr. W. W. Wirth, USDA, Smithsonian Institution, Washington, D.C.) and C. p. quinquefasciatus.

2. The procedures employed by the I.E.C. in identifying and recording information negated much of the biological information sought. However, information was obtained on the cycle of biting activity inside and outside of the houses for the biting midges (Fig. 3). This may not represent the true behavior pattern for C. paraensis, although this species composed $< 90\%$ of the population collected.

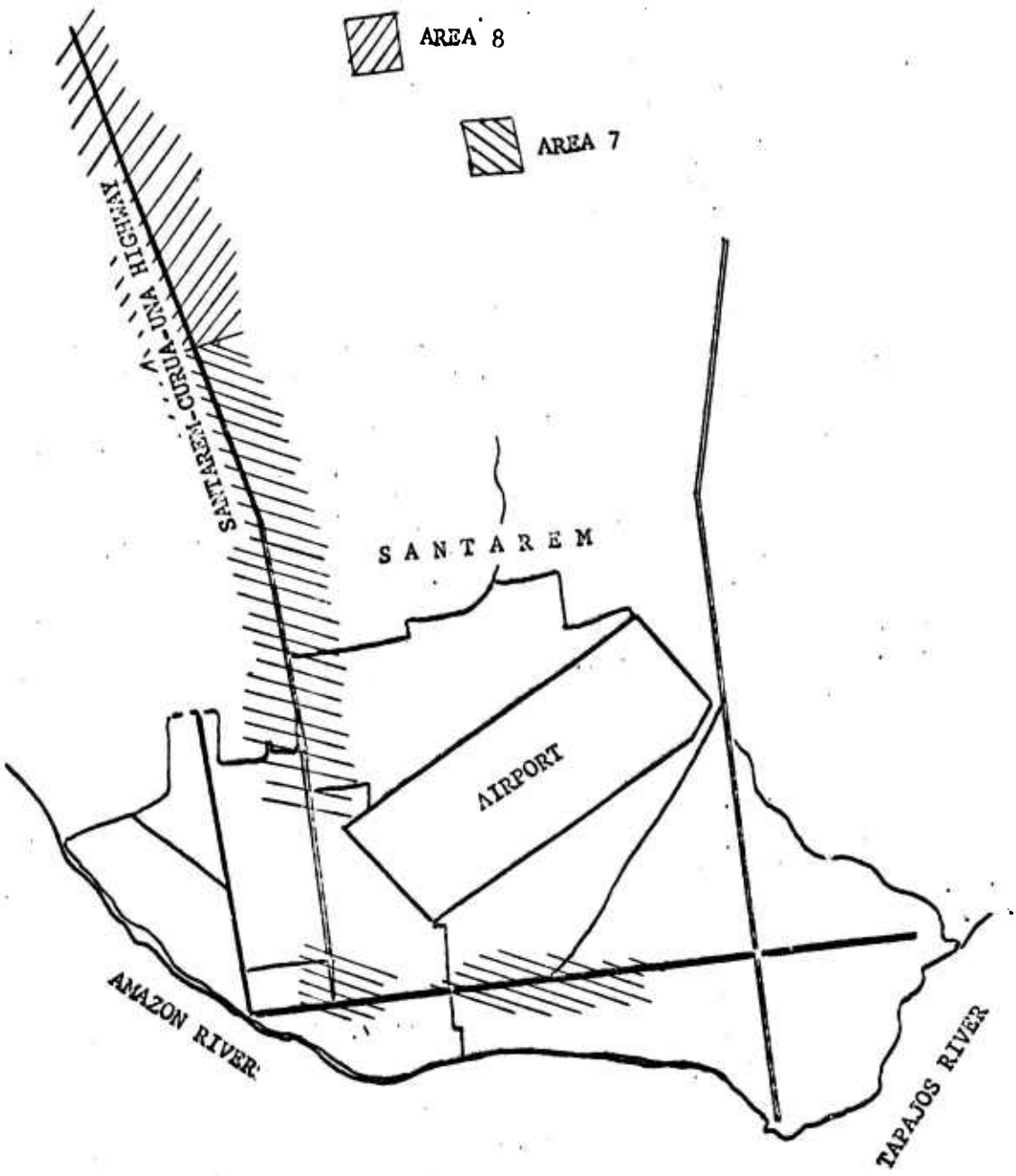


Figure 2. Two study areas encompassing the City of Santarem, Para, Brazil and 21 Km of the Santarem - Curua-una Highway.

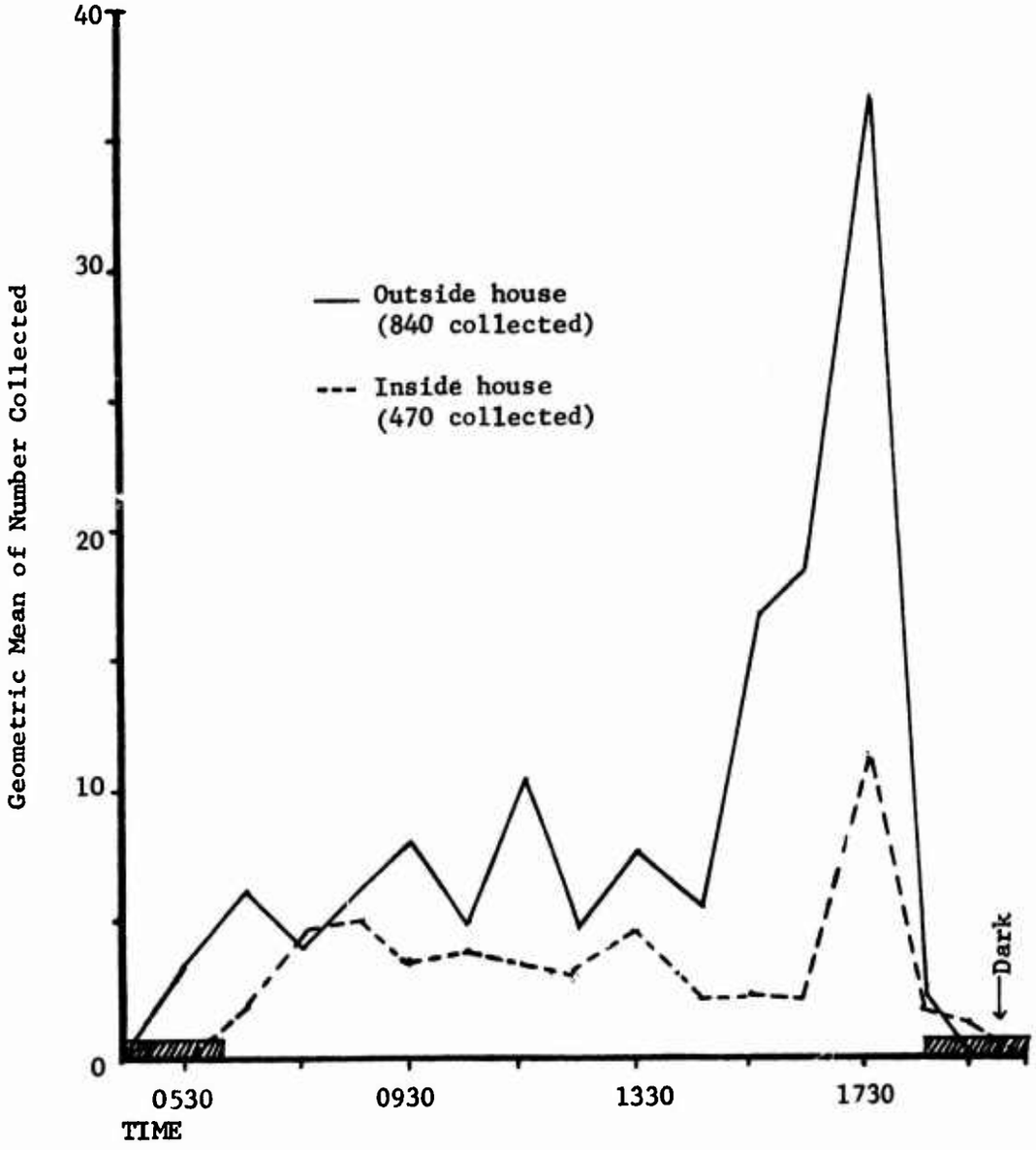


Figure 3. Temporal distribution of host-seeking Culicoides at Mojui dos Campos, Para, Brazil, March 1975. Based on 4 days of continuous collections with 2 collectors inside the houses and 2 outside. Collections were conducted for 50 min. each hour.

3. The inside to outside ratio of Culicoides in biting collections was roughly 50%.

4. The I.E.C. obtained 2 isolates of Oropouche virus from man-biting captures of mixed engorged and unengorged Culicoides. The value of these isolates is questionable, since one collector was incubating Oropouche virus during the time the specimens were collected.

Itupiranga, Para, Brazil

1. The insect fauna in Itupiranga was represented by dense populations of mosquitoes, black flies and biting-midges.

2. Within the city, only 2.57 C. paraensis were collected per man-hr. of collecting; compared to 33.8 black flies per man-hr.

3. Peak biting activity was found to occur between 1700-1800 hr. for C. paraensis (Fig. 4).

4. Dense populations of Culex (including C. p. quinquefasciatus) Mansonia and Anopheles were also encountered within the city and will be processed for virus isolation.

Santarem, Para, Brazil

Results from Santarem indicate a correlation in distribution between the more dense populations of C. paraensis and prevalence of human Oropouche virus seropositivity (Table 1). All collection data for C. paraensis for the 1700-1800 hr. were normalized with a square root transformation and employed in a 3-way ANOVA for areas, days and houses (Sokal and Rohlf, 1969). Main factor analysis revealed significance ($P < .001$) between areas and between houses within areas. Collections between days by area were not significant.

Whether the correlation between population densities and Oropouche virus antibody prevalence is spurious can be better determined after all epidemiological and virological data are analyzed. Correlations between populations of C. p. quinquefasciatus and Oropouche prevalence data will also be of importance.

The presence of large populations of C. paraensis along the highway (Table 2), away from large human populations, refutes the hypothesis that this species is completely restricted in distribution to human population centers. This finding has recently been verified by collecting C. paraensis at one rural site along the Transamazon Highway.

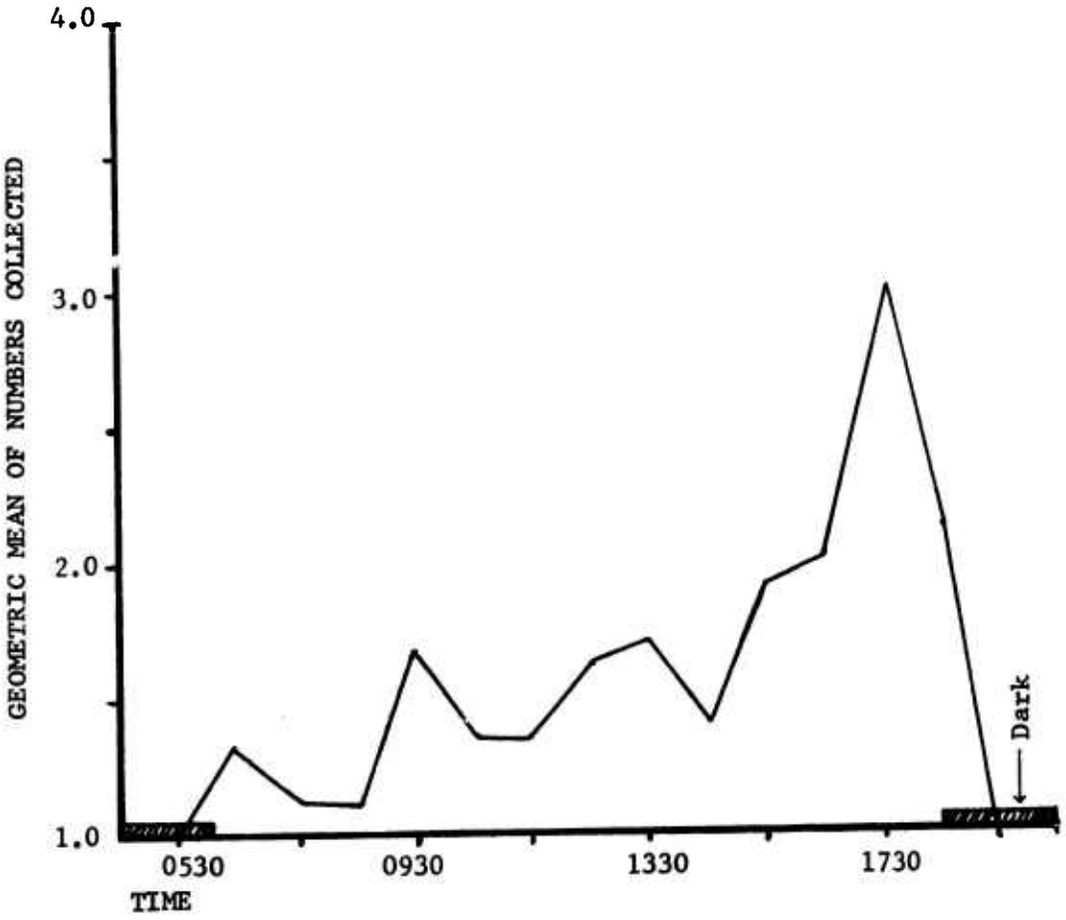


Figure 4. Temporal distribution of host-seeking Culicoides paraensis (Goeldi) in a peridomiciliary environment at Itupiranga, Para, Brazil. Based on 9 days of continuous collections with 2 collectors collecting 50 min. each hr., June 1975.

Table 1. Correlation of population densities of Culicoides paraensis (Goeldi) and Oropouche HI seropositivity within the City of Santarem, Para, Brazil, July 1975.

	TOTALS COLLECTED BY AREAS					
	AREAS					
	4	2	1	5	6	3
	5	5	55[a]	1	38	42
	0	0	46[a]	52[b]	27	17
	0	9	4	20	37	170
	3	1	4	18	11	41
	4	6	3	17	9	36
	2	1	1	1	54	55
TOTAL COLLECTED	14	22	113	109	176	361
OROPOUCHE HI SEROPOSITIVITY	0%	0%	0%	40%	40%	18%

[a] Houses in proximity to areas 5 and 6.

[b] House located adjacent to area 6.

Table 2. Population densities of Culicoides paraensis (Goeldi) in an urban (Santarem, Brazil) vs. a rural area along the Santarem-Curua-una Highway. Each value represents total numbers collected in Man-biting collections at each house for 3 days. Collections were made at 6 houses in each area during June 1975.

COLLECTION AREAS	No. COLLECTED AT EACH OF 6 HOUSES PER AREA 7	8
	23[a]	57[d]
	29[a]	25[c]
	16[a]	19[c]
	3[b]	23[c]
	55[c]	18[c]
	86[d]	2[d]
TOTAL COLLECTED	212	144

[a]houses located in City of Santarem
[b]house located on edge of Santarem
[c]houses located singly at various distances from Santarem
[d]houses located in villages of 5-10 houses at Km 10 and Km 21.

Culex pipiens quinquefasciatus Say

This urban mosquito is anthropophilic, endophilic and abundant in all population centers in the study areas. It not only has potential as a vector of Oropouche virus, but is also the vector of filariasis and alergic reactions are caused by its bite. The allergic reactions seem limited to infants and recent arrivals. No reportable biological information for this species is available from the Oropouche epidemic investigations. The following information was obtained from malaria investigations in Palestina, Para, Brazil.

1. Peak biting activity indoors occurs around 2400 hr. (Fig. 5).

2. Three periods of high frequency biting activity were found in the peridomiciliary environment (Fig. 5).

3. Generally, peak numbers resting on the walls indoors correlated with the indoor peak in biting activity (Figs. 5 and 6). There was no drastic decline in numbers resting on the wall during late morning.

4. Inside to outside biting ratio for paired collections during 4 nights was 1.2.

CONCLUSIONS: The available data is insufficient to determine the vector of Oropouche virus in the urban areas of the Amazon basin. Due to poor success at virus isolation from field programs, a laboratory transmission study with both C. p. quinquefasciatus and C. paraensis is indicated.

D. OBSERVATIONS ON BLACK FLY PROBLEMS IN THE AMAZON BASIN

BACKGROUND: During the rainy season, January-April, black flies (Simuliidae) are an omni-present pest throughout the Amazon basin. In some areas the residents leave their land due to allergic reactions to bites and overwhelming populations densities of these voracious insects. Allergic manifestations seem common to persons new to the areas and vary from annoying local reactions to incapacitating and life threatening episodes, e.g., hemorrhagic syndrome of Altamira. In the course of the routine surveillance program, large numbers of Simuliidae were captured and general observations were made on these pests along the Transamazon Highway. In other parts of the Amazon, black flies have been incriminated as vectors of Mansonella ozzardi and Oncocerca volvulus.

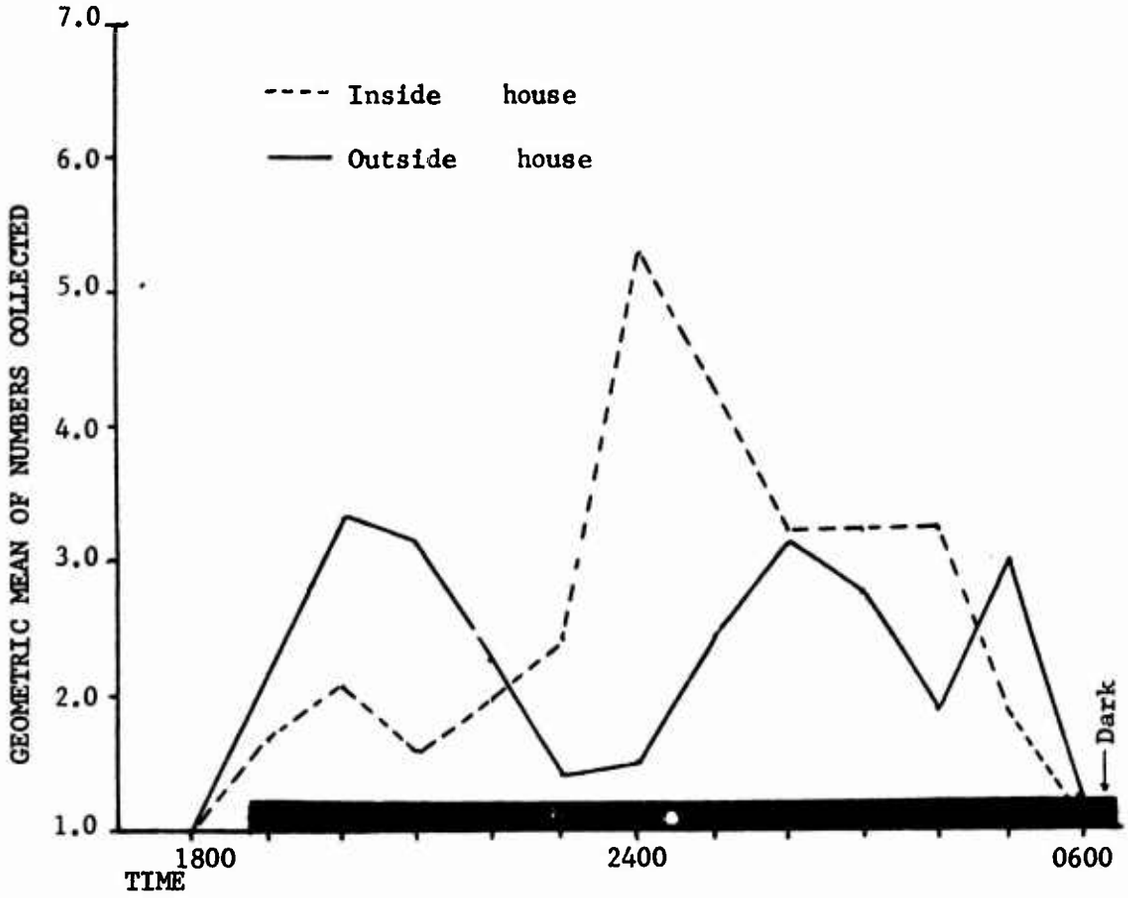


Fig. 5. Temporal distribution of man-biting Culex pipiens quinquefasciatus Say in Palestina, Para, Brazil. Data from four all-night collections for 30 min. per hr., March 1975.

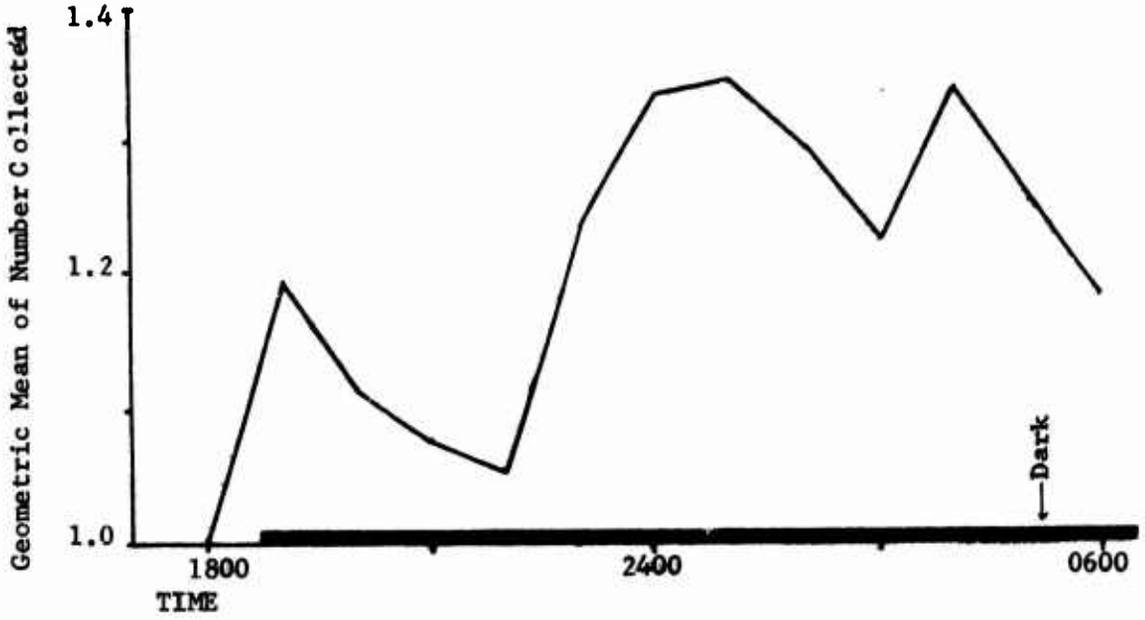


Figure 6. Temporal distribution of resting Culex pipiens quinquefasciatus Say in Palestina, Para, Brazil. Data from 8 nights of collecting mosquitoes on the walls inside a house; collected for 10 minutes each hr. (March and and April 1975).

WORK ACCOMPLISHED: Specimens of Simuliidae have been obtained from every collecting site in the Maraba and Altamira areas. They are very abundant during the rainy season; but abundance varies greatly from site to site with time. Densities of man-bting populations sometimes exceeded 150 in a 15 min. collection. Fifty or more per collection were common occurrences. During an epidemic investigation in Itupiranga it was found that, with about 500 man-collection hrs, density of Simuliidae throughout the city averaged 33.8 per hr. Peak biting activity occurred during early morning and late afternoon (Figure 1).

The black flies readily enter houses and bite most intensely during peak work hrs of the day. Although black flies have been observed to bite under clothing; long sleeves and pants tucked into tops of boots will greatly reduce the number of bites received. This is not routinely practiced by the colonists. Available repellents do not seem effective deterrents.

COMMENTS: During the coming year the capability of doing routine identifications of Simuliidae will be developed. A reference collection of known species will be deposited at the I.E.C. Special efforts in conjunction with the routine surveillance program will be undertaken to quantitatively evaluate the pest value of these insects, determine which species are more likely to cause allergic reactions and to identify the allergens involved. Of great interest is the fact that the same species seem to occur in high densities in both the Maraba and Altamira areas, but the hemorrhagic syndrome has not been observed in Maraba.

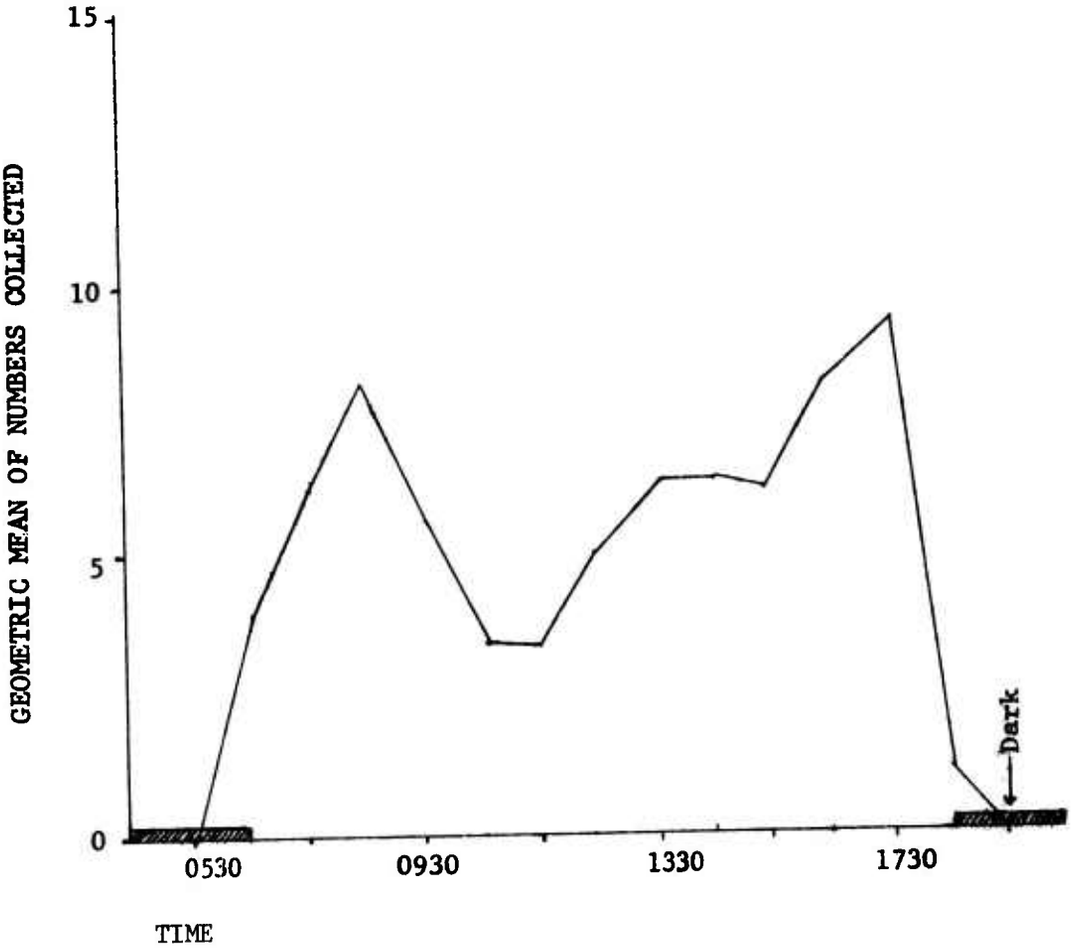


Figure 1. Temporal distribution of host-seeking Simuliidae in peridomiciliary environments of Itupiranga, Para, Brazil. Based on 9 days of continuous collections with 7 collectors collecting 50 min. each hr, June 1975.

III. WILDLIFE ECOLOGY PROGRAM

A. INTRODUCTION

BACKGROUND: Recent ecological data from the Amazon basin is scanty and is generally derived from studies of small areas in the easily accessible major riverine environments. The Transamazon Highway route is 90% non-riverine. No comprehensive data relating to flora, fauna, terrain, habitats, meteorology or changes induced by man are available for this recently penetrated area. Valid data in these categories are essential for understanding present and potential mechanisms of maintenance and transmission of various infectious agents either native to the region or capable of being imported.

The objectives of these studies are:

1. To describe the study areas;

2. To obtain meteorologic data from multiple sites in these areas;

3. To sample the marmalian populations and define their relative abundance with relationship to time and habitat.

4. To obtain specimens from these mammals for laboratory examination.

5. To describe the domestic animals extant and entering the study areas and to obtain specimens for laboratory examination.

Important observations included the variable nature of the forest as to height, density and type within a short distance (swamp forests, lowland forests and upland forests) similarly the terrain changes rapidly over short distance (flat land to rolling hills to steep hills less than 250 in. in height).

B. STUDY AREA RECONNAISSANCE, SITE SELECTION AND DESCRIPTION

Trips were made to the Maraba and Altamira areas to observe the various vegetative formations along the Transamazon Highway. Along most of the Transamazon, the topography alternated between flatland, rolling hills, and fairly steep hills within short distances. Swamp forests, lowland forests, and upland forests also alternated throughout. The entire area was covered by forest, except where cleared by colonists for agricultural purposes. The upland forests were generally taller in the hilly areas than in the flatland areas. Swamps and open water occurred both in the flatland and in the hills.

A trip was also made from Santarem, Para, west to Humaita, Amazonas, a distance of 1397 Km along which the topography varied from flatland to steep and high hills. The highest and steepest hills were located between 150 Km west of Itaituba to Jacareacanga, a distance of 225 Km. The hilltops were not over 250 m in elevation. The terrain near Humaita was usually flat.

The majority of the colonists were concentrated in the Maraba-Altamira area. West of Itaituba to Humaita the colonists were widely scattered, and various long portions of the Transamazon had none whatsoever.

Two sites in the Maraba area and two in the Altamira area were selected for intensive surveillance of mammalian populations. These sites were selected on the basis of obtaining a representative sample of mammals from as many habitat types as possible. Entomological and epidemiological data were being collected from the same areas in order to observe the disease agents present in insects and humans as well as other mammals.

The impressions gained from the reconnaissance concerning seasonal differences and differences in flora between Maraba and Altamira during the same month indicated a need for monthly photographic documentation of each trapping site. A photographic documentation program was begun and has demonstrated the rapid and frequent changes in both natural flora and agricultural plants occurring in the trapping sites.

TRAPPING SITES:

Maraba

a. The trapping site in Gleba 05 Lote 05 (Fig. 1) was located 26 Km north and 30 Km west of Maraba near Itupiranga (5^{o} 06' S, 49^{o} 24' W). Three types of forest were sampled in this site; lowland sidehill, low flatland, and upland forest. The lowland sidehill forest was located on the side of a small valley and had a narrow stream slowly flowing through. The soil was a reddish-brown clay-sand. The A_1 horizon (humus layer) was very thin and contained many fine hair-like roots. The A_0 horizon (litter layer) was also very thin ($\pm$1 cm) and consisted of fallen leaves, palm fronds, and twigs. Many small woody plants less than 1 m tall formed the ground vegetation. The small trees and palms formed an almost closed canopy. Acai palms (Euterpe oleracea) were plentiful along the stream, and spiny palms were scattered throughout. Trees and palms of up to 20 m in height formed a more or less open canopy. The emergents were up to 30 m tall, and widely scattered. Their trunks were not buttressed. Lianas were found in the larger trees, but did not form a network. Very few epiphytes were observed.

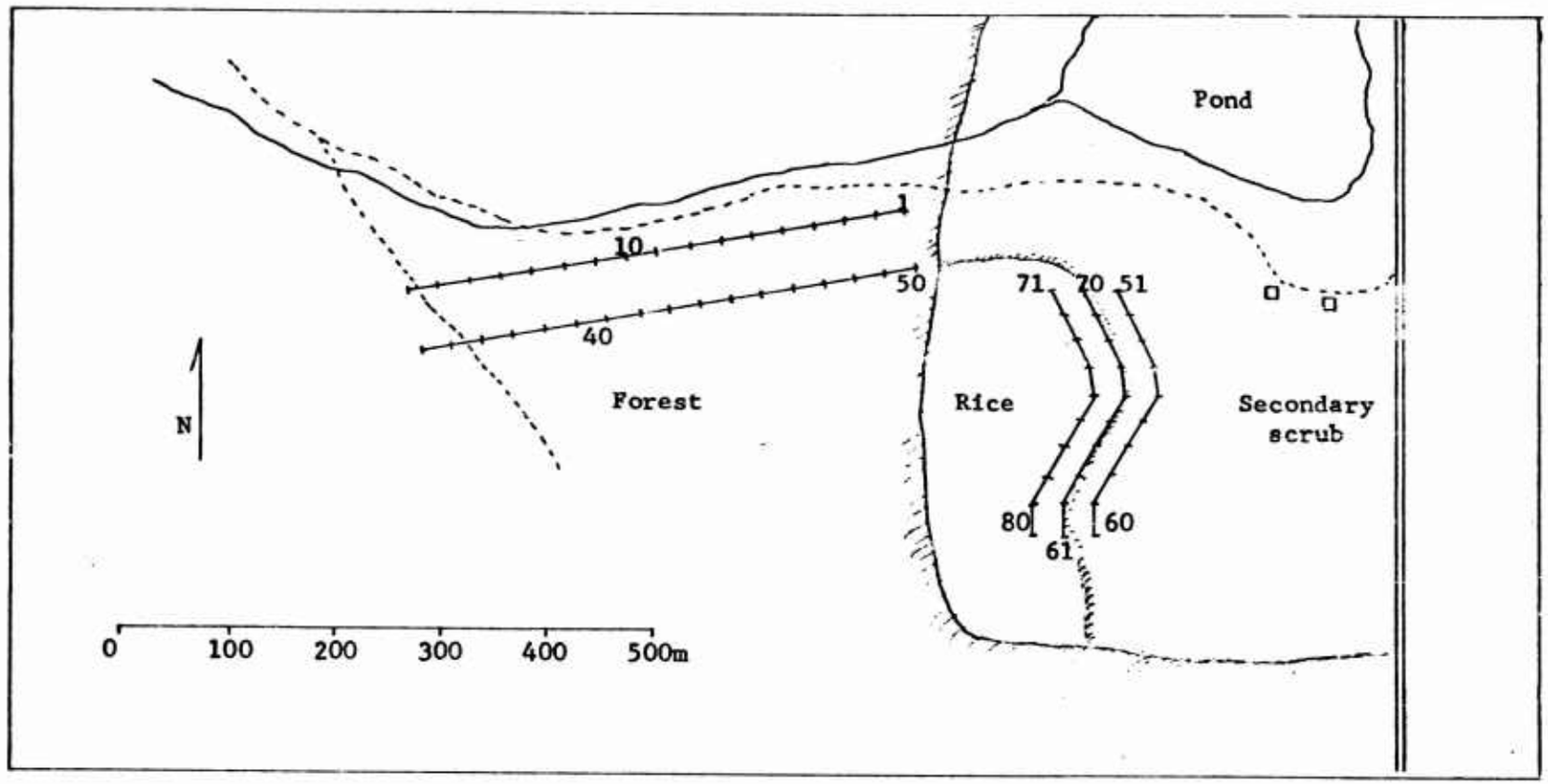


Figure 1. Trapping sites in Gleba 05 Lote 05 west of Maraba. Located Brazil: Para; 26 km north and 30 km west of Maraba (near Itupiranga). Latitude 5° 06' S and longitude 49° 24' W.

Trap line
Trail
Transamazon Highway

The low flatland forest was located in the valley bottom. It was not inundated, although small pools of water were present. The soil and A_1 horizon was similar to the lowland sidehill, but the A_0 horizon was from 2-4 cm thick and composed of leaves, palm fronds and twigs. An almost continuous growth of small woody plants, shrubs, and palms from ground level to about 8 m in height was present. A low canopy of trees and palms was from 15 to 20 m tall. The emergents were very widely scattered. Many small vines were present, but few large lianas were observed. A considerable amount of light reached the ground, and no difficulty was encountered walking without the aid of a machete.

The upland forest was located on the low ridge above the valley on a slightly hilly terrain. The soil was a reddish clay with a very thin A_1 horizon which contained a network of fine hair-like roots. The A_0 horizon was from 1-3 cm thick, and made up of leaves, palm fronds, twigs and fallen bamboo. There was an almost continuous growth of small shrubs, palms and in many areas, bamboo from ground level to a height of about 8m. Two types of bamboo were present, a free-standing type which grew in clumps, and a thin vine-like type which formed impenetrable thickets 20 to 40 m in diameter where trees had fallen making an opening in the canopy. Small trees and palms formed a canopy about 15 m in height. The scattered emergents, some with buttress roots, were 20 to 30 m tall. Many small vines, but few lianas were observed on the trees and shrubs [illegible] few epiphytes were found. As the canopies were not closed, a [illegible]ount of light reached the ground.

Traps were also placed in a thick [illegible]ondary scrub which was impossible to penetrate without cutting a trail. This area had been cleared about three years before, and the spiny shrubs had grown to a height of about 3 m. Traps were placed 30 m from the edge, as well as along the edge of the scrub. Traps were also placed in a corn, rice and cassava field.

b. The trapping site in Gleba 29 Lote 03 (Fig. 2) was located 73 Km north and 45 Km west of Maraba near Jatobal (4^{o} 41' S and 49^{o} 32' W). Three types of forest were sampled in this site; lowland, lowland but not inundated, and upland forest. In the lowland forest, the terrain was flat with many pools of standing water in the wet season. Numerous small waterways flowed through also. The clay soil was a very dark grey, almost black in color. The A_1 horizon contained many hair-like roots, and many large roots were found on top of the ground. The A_0 horizon was composed of leaves, palm fronds, and many fruit husks. The low woody shrubs from ground level to about 3 m in height were moderately thick. Many small palms, to 4 m in height, grew in clumps. The canopy, composed of trees and palms to 20 m tall, was nearly closed. The larger emergents ranged in height to over 30 m, and many had buttress roots. Many lianas of different types were present on the larger trees, and some small vines were supported by the smaller trees. Moss was common on the tree trunks and on some shrubs. Very few epiphytes were in evidence.

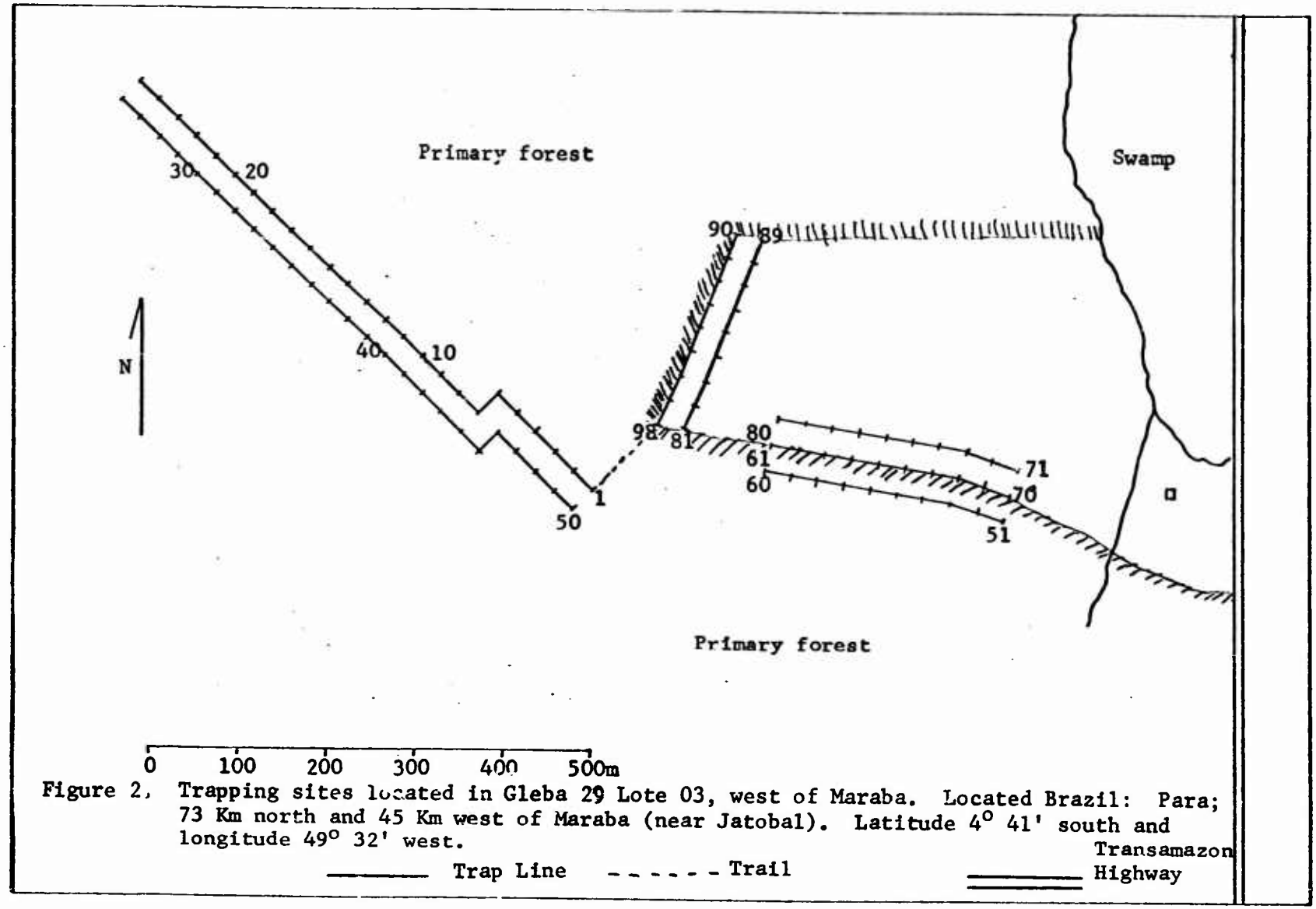


Figure 2. Trapping sites located in Gleba 29 Lote 03, west of Maraba. Located Brazil: Para; 73 Km north and 45 Km west of Maraba (near Jatobal). Latitude 4° 41' south and longitude 49° 32' west.

The lowland but not inundated forest was located on gently sloping terrain, and contained some pools of standing water during the rainy season. It was similar to the lowland forest, but the trees were generally taller, and it contained more trees and less palms; stilt root and buttress root trees were common. More light reached the ground than in the lowland wet forest.

The upland forest was located in a very hilly terrain with some steep slopes. The clay soil ranged from reddish to a light grey in color, and the thin A_1 horizon contained many hair-like roots. The A_0 horizon was about 5 cm thick and consisted of fallen leaves, branches, twigs, and some palm fronds. Small woody plants to 1 m tall were moderately thick. Many small trees and shrubs ranged up to 10 m in height. The larger trees and a few palms were from 20 to 30 m tall. Although some emergents had buttress roots, most did not. The emergents were up to 40 m in height. Moss and lianas were found on the taller trees, but not as much as in the lower forest. This forest was more open than the lowland forest, and more light reached the ground. Traps were also placed along the edge of the forest, and in the agricultural areas.

Altamira

a. The trapping site in Agrovila da União (Fig. 3) was located 19 Km south and 18 Km west of Altamira (3^{o} 22' S, 52^{o} 23' W). Four types of forest were sampled in this site; acai swamp, lowland, sidehill, and upland forest. The acai swamp was a low flat area with streams and pools of standing water in the wet season. The soil was a dark brown clay. Many fine hair-like roots and large roots were in the A_0 horizon, which was usually less than 1 cm thick and consisted of leaves and palm fronds. A continuous growth of low woody plants, shrubs, small trees and palms from ground level to a height of 12 m was not very thick. The acai palm was the most common specie in this canopy which had a height of 20 m, although other palms and some trees were present also. A number of emergents over 40 m tall were present. A considerable amount of moss was found on the trees. The lianas were not numerous, and were generally small. The underbrush was not thick, and it was easy to walk without cutting a trail.

The lowland forest was in a flat area with very few pools of water. The clay soil was reddish-brown in color. Many fine hair-like roots were found in the A_0 horizon, which was composed of leaves, palm fronds, and twigs, and from 3 to 5 cm thick. A low woody vegetation was present on the ground and ranged to about 4 m in height. Small trees and palms were common and formed a canopy 15 to 25 m tall above the ground. The emergents were up to more than 40 m tall and some had large buttresses. The lianas were very numerous and formed a network in the trees. Small vines were common on the shrubs. A liberal amount of moss was on the trees. The forest was thick and a machete was needed to cut a trail in most places.

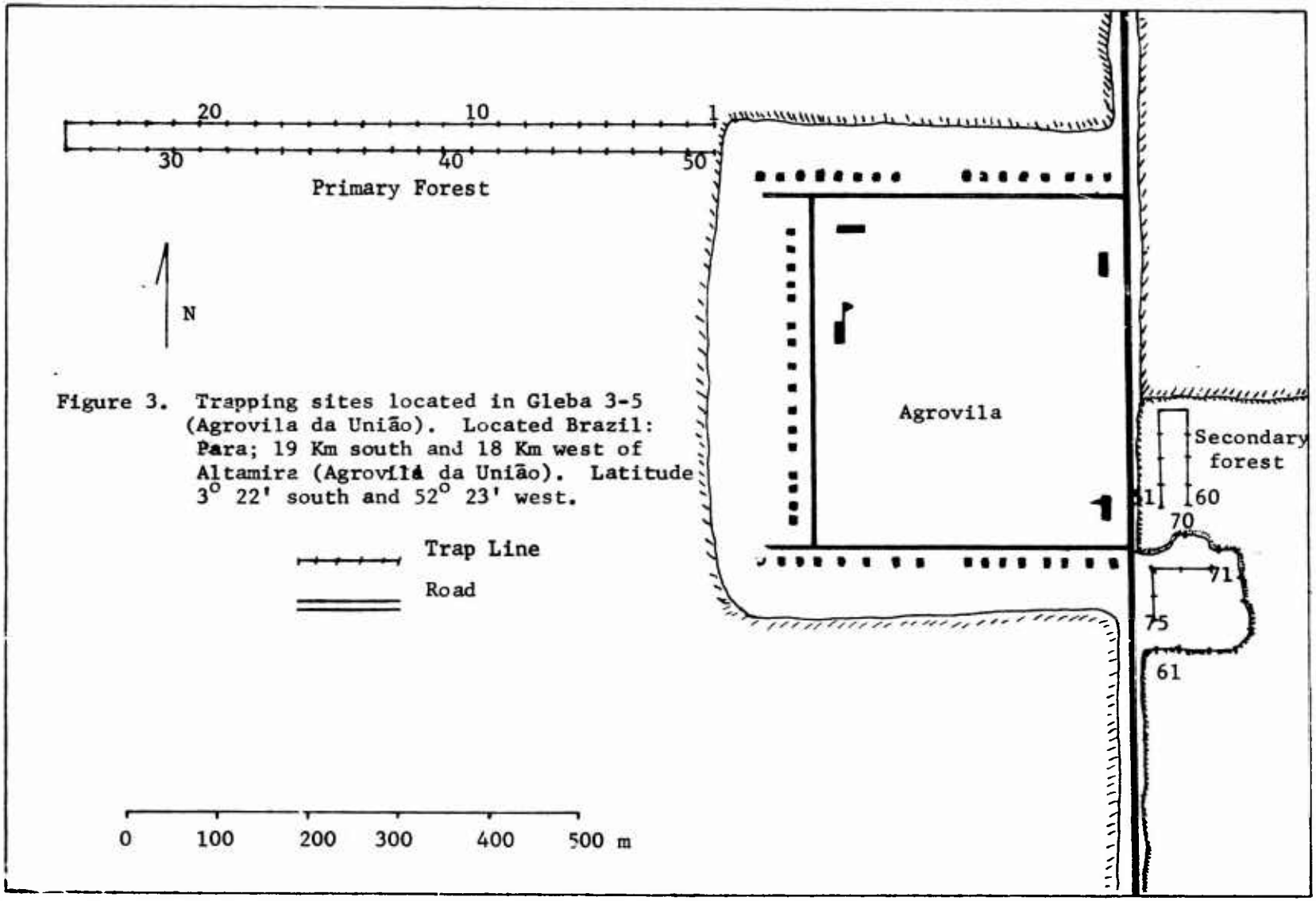


Figure 3. Trapping sites located in Gleba 3-5 (Agrovila da União). Located Brazil: Para; 19 Km south and 18 Km west of Altamira (Agrovila da União). Latitude 3° 22' south and 52° 23' west.

The sidehill forest was very similar to the upland forest, but the terrain was occasionally very steep.

The upland forest was located in an almost flat terrain. The soil was a red clay, and the A_1 horizon was very thin with many fine hair-like roots. The A_o horizon was from 2 to 4 mm thick and was composed of leaves, palm fronds, bamboo, and twigs. A thicket of woody plants and a few palms ranged from ground level to over 2 m in height. The small trees and palms formed a canopy about 15 m above the ground. The larger trees and palms were up to 25 m in height. Some of the emergents were over 40 m tall, and many had buttress roots. Mosses were very common on the trees. The numerous lianas formed a thick network from the ground to the tree tops in many areas. Small vines were common on the small trees and shrubs. Thickets of the viny type bamboo were scattered throughout. It was very difficult to walk in most areas without cutting a trail.

Traps were also placed in a tall secondary scrub forest which had been cleared over three years before, and in the agriculture areas.

b. The trapping site in Gleba 61 Lote 02 (Fig. 4) was located 54 Km south and 150 Km west of Altamira (3^o 41' S, 53^o 45' W). Two types of terrain were sampled, lowland and sidehill, although the forest appeared similar in both types. The lowland forest was in a very flat area, and the sidehill forest was located on a gentle slope. Some areas of standing water in the lowland forest were extensive, over 30 m wide with water to 75 cm deep. The soil was a reddish clay-sand with a thin A_1 horizon which contained many fine hair-like roots. The A_o horizon was from 1 to 3 cm thick and consisted of leaves, palm fronds, some bamboo and twigs. Many fallen trees were present also. The small shrubs, large shrubs, and palms, which were from 25 cm to approximately 10 m in height, formed a thicket of vegetation. Spiny palms were also present in this thicket. Low trees and palms from 15 to 20 m in height made up a fairly closed canopy. Emergents to over 30 m tall, some with buttress roots, were scattered throughout. The many lianas and small vines formed a thick network between the trees and shrubs. Epiphytes were present both on the emergents, and on some of the smaller trees. Open areas, where large trees had fallen and pulled smaller trees which were connected by lianas with them, contained some cecropia and other secondary species. Thick stands of heliconia were growing in some of the open water areas. The heliconia supported lianas and vines which passed between the trees providing runways for arboreal animals. A moderate amount of light reached the ground usually. Traps were also placed in secondary scrub and in agricultural areas.

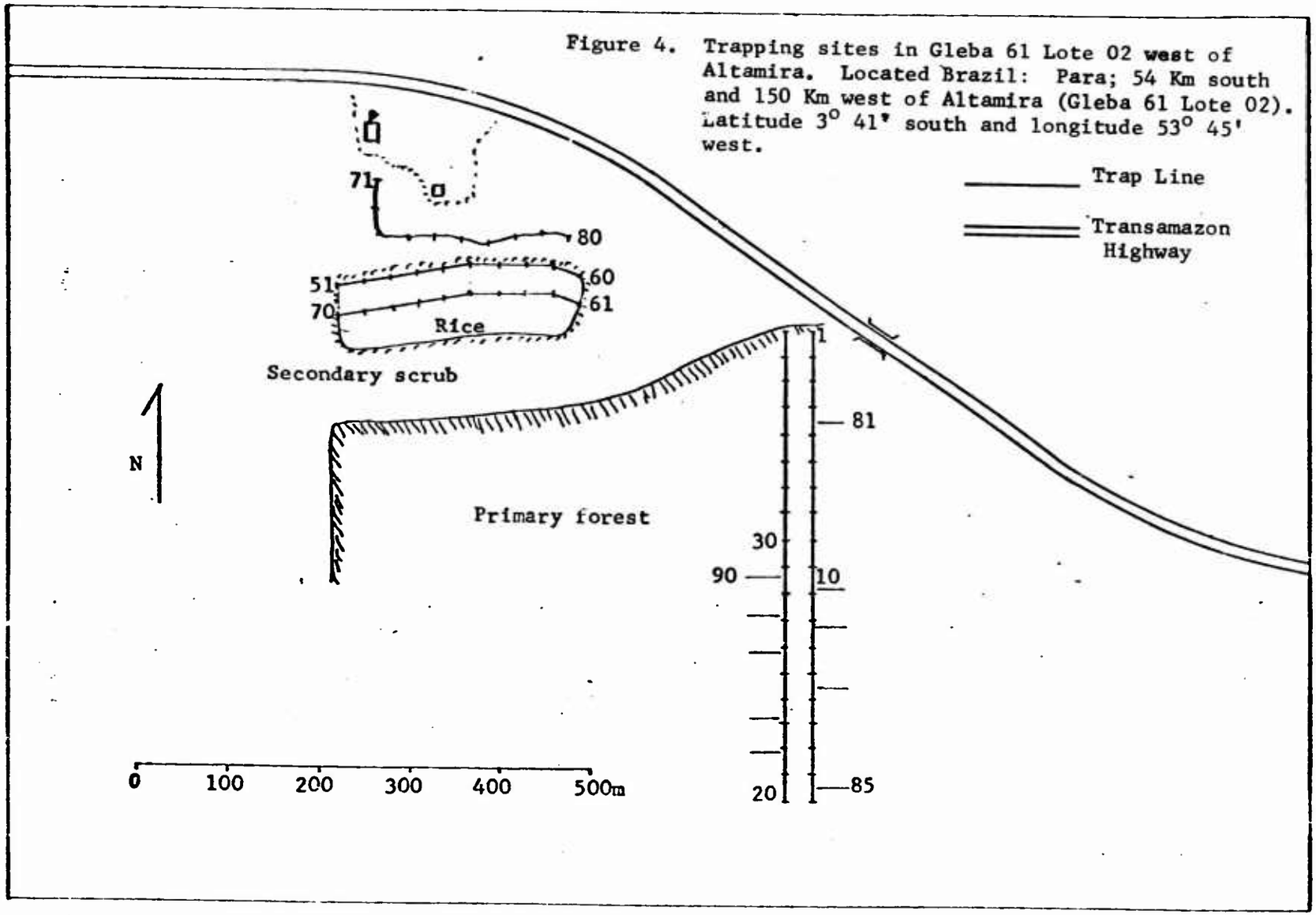


Figure 4. Trapping sites in Gleba 61 Lote 02 west of Altamira. Located Brazil: Para; 54 Km south and 150 Km west of Altamira (Gleba 61 Lote 02). Latitude 3° 41' south and longitude 53° 45' west.

C. MAMMALIAN ECOLOGY AND SURVEILLANCE

BACKGROUND: Reported mammalian studies in the Amazon region have been mainly taxonomic, carried out in small areas, and conducted in riverine ecology. Little work has been reported on the influence of seasonal changes in climate and vegetation on animal populations. The opening of the Transamazon Highway has permitted access to a variety of ecologically different areas. The mammalian ecology program has been developed to monitor the changes in animal populations along the Transamazon as man changes the habitat from forest to cropland and pasture.

Many arboviruses and leishmaniasis have been described in the Transamazon, and plague and rickettsial agents have been described elsewhere in Brazil with both sylvatic and domestic mammals implicated in the transmission cycles. As important as the identification of these agents in various animals, is information concerning the kinds of species present and their relative abundance in various habitats throughout the year. This information when compared with simultaneous epidemiological and entomological data from the same areas will assist in better understanding vector/reservoir interactions and the present and potential disease risk to humans from these zoonoses.

PROGRAM DESCRIPTION: The trapping program compares the relative abundance of various animals in different habitats over time. Mammals in the Transamazon region are highly diversified, and small changes in the habitats may cause changes in the types and relative numbers of species found in each. Seasonal, climatic and vegetational changes play an important role in the reproductive cycles and numbers of mammals present in a population during any one period of the year.

Because of the importance of different habitats, traps were dispersed in cropland, orchards, and in as many forest types as possible. The edge between forest and cleared areas were similarly covered. Trapping was carried out for two weeks of every month in each of four sites in the Maraba and Altamira study areas. Wooden live traps (155 x 175 x 450 mm) and Rinker live traps (80 x 80 x 255 mm) were placed 30 m apart along grids both in the forest and agricultural areas. Because some mammals are essentially arboreal, the Rinker traps were also placed in trees and on lianas. Traps were also placed in colonists' houses to sample the mammals living there. Larger mammals were hunted to obtain necessary specimens for evidence of disease agents. A mixture of corn and bananas was the most common trap bait used.

Each trap location in each trapping area has a permanent number enabling the location of the exact capture site of each mammal. Thus, we may determine exactly where a potential zoonoses may exist, and also

the preferred habitat type of a certain species. This information is invaluable in combating future disease problems in which animals from a similar habitat are involved.

The mammalogy field form number 2 (Figure 1) was completed in duplicate for each night of trapping, thus providing a record of the quantity and types of traps set out in the various areas. The kinds of baits used and cloud cover were also recorded. The individual field form (Figure 2), was completed for every mammal collected and processed. The traps were checked early in the morning to reduce the number of animals dying. The captured mammals were transferred to cloth bags, and taken to the field laboratory for processing. At the field laboratory, located near the trapping site, each mammal was given a collection number which corresponded to the date and area (example: M-010675-01, which is decoded as M-Maraba, 010675 - 1 June 1975, 01 - first specimen processed on this date). All specimens taken from one animal were labelled with the same number. The mammal was exsanguinated, 1cc of whole blood preserved in liquid nitrogen for virus isolation attempts, a thick and a thin smear for blood parasite examination, and the remainder centrifuged and serum preserved in liquid nitrogen for virus, plague, tularemia and rickettsia screening. The animal was then placed in a paper bag with chloroform, and the ectoparasites removed and preserved in liquid nitrogen. The standard measurements (total length, tail length, hind foot length, ear length, and weight) were recorded. Organs were removed and preserved in liquid nitrogen for isolation attempts, and also in 10% formalin for pathological studies. Endoparasites were preserved in 10% formalin also. The preserved specimens were sent to the base laboratory in Belem for processing or reshipment to WRAIR. Each mammal specimen was preserved either as skin and skull, skull only, or in formalin, shipped to Belem for tentative identification, and later to taxonomists specializing in South American mammals for confirmation.

PROGRESS TO DATE: Although more information is needed before any kind of statement may be made concerning the habitat preference and distribution of mammals along the Transamazon Highway, the following information, along with Table 1, indicates a high density of certain species in some areas and a very low density in other areas.

A panel of sera from 300 mammals collected along the Transamazon Highway are presently being tested for rickettsia, plague and tularemia at the Walter Reed Army Institute of Research.

MAMMAL FORM NO. 2

1. Date:_____

2. Location:_____ Gleba:_____ Lote:_____

3. No. of traps set: _____

Numbers	Bait
_____	_____
_____	_____
_____	_____
_____	_____

4. No. of traps set in houses: _____

5. Sky conditions:_____

_____

_____

_____

6. Trap number in which a mammal escaped:_____

7. Trap number sprung without mammal: _____

8. Number of mammals collected: _____

9. Observations:_____

_____

_____

_____

Figure 1. Daily mammal form

(1) Species_____ (2) Sex M F (3) Collection No._____

Measurements
(4) TL _____
(5) T _____
(6) HF _____
(7) E _____
(8) Weight _____
(9) Forearm_____
(10) Testis _____
_____

(11) Age
1 Ad
2 Juv
3 Yng

(12) Reproduction
1 Lactating
2 Pregnant
3 Embryos_____
4 Nothing

(13) Capture method
1 Wooden trap
2 Metal trap
3 Snap trap
4 Mist net
5 Shot
6 Insect net
7 Hand caught
8 In roost
9 Found dead
10 Purchased

Location
(14) Jurisdiction_____
(15) Gleba _____
(16) Lote _____
(17) Trap No. _____
(18) Other_____
_____
_____
(19) Elevation_____

(21) Time of capture

1	Day	9	2400
2	Night	10	0100
3	1800	11	0200
4	1900	12	0300
5	2000	13	0400
6	2100	14	0500
7	2200	15	0600
8	2300	16	0700

(22) Capture location
1 Ground
2 Tree
3 Cave
4 House

(23) Sky
1 Clear
2 Partly cloudy
3 Overcast
4 Showers
5 Steady rain
6 Heavy rain

(24) Topography
1 Hill top
2 Hill side
3 Valley
4 Flatland

(25) Terrain
1 Upland
2 Lowland
3 Swamp

(26) Environment
1 Forest
2 Scrub
3 Savanna
4 Orchard
5 Cropland
6 Clearing
7 Town

(27) Vegetation
1 Trees
2 Trees and palms
3 Palms
4 Acai palms
5 Scrub
6 Grassland
7 Mixed orchard
8 Orchard_____
9 Cropland_____
10 House
11 Other

(28) Bait
_____

Figure 2. Individual field form (front)

Laboratory Specimens

N_2	Formalin	Various
(29) Kidney	Kidney	
(30) Liver	Liver	
(31) Spleen	Spleen	
(32) Heart	Heart	
(33) Brain	--	
(34) --	Lung	
(35) --	--	Blood_____
(36) --	--	Blood slides
(37) --	--	Urine
(38) --	--	Throat swab
(39) --	--	Rectal swab
(40) --	--	Other_____

Ectoparasites

N_2	Alcohol
(41) Ticks	Ticks
(42) Mites	Mites
(43) Fleas	Fleas
(44) Lice	Lice
(45) Diptera	Diptera
(46) Other_____	Other_____

Endoparasites

Formalin

(47) Intestinal
(48) Liver
(49) Heart
(50) Other_____

(52) Mammal specimen

1 Skin and skull
2 Formalin
3 Skull only
4 Skin only
5 Discard

(51) Observations_____

Figure 2. Individual field form (back)

Table 1. Mammals Collected in the Maraba and Altamira Study Areas.

Species	G-05 L-05 1380*	G-29 L-03 1808*	Agrovila 3/5 888*	G-61 L-02 1366*	Total
Marsupialia					
Didelphidae					
Caluromys philander	-	1	2	-	3
Monodelphis brevicaudata	1	3	7	10	21
Marmosa cinerea	-	-	-	2	2
M. murina	1	2	-	-	3
Philander opossum	-	3	-	11	14
Metachirus nudicaudatus	-	-	1	-	1
Didelphis marsupialis	3	9	5	10	27
Primates					
Callithricidae					
Saguinus tamarin	-	1	-	-	1
Rodentia					
Cricetidae					
Oryzomys bicolor	-	3	-	-	3
O. capito	62	109	26	10	207
O. concolor	2	3	-	-	5
O. delicatus	1	8	-	-	9
O. macconnelli	-	29	-	-	29
Neacomys guianae	3	9	3	1	16
N. spinosus	-	-	2	1	3
Nectomys squamipes	1	9	-	-	10
Zygodontomys lasiurus	24	-	-	-	24
Oxymycterus sp.	-	1	9	36	46
Holochilus brasiliensis	-	1	-	-	1
Echimyidae					
Proechimys sp.	25	67	27	46	165
Mesomys sp.	-	1	-	-	1
TOTAL	123	259	82	127	591

* Trap Nights

Mammal Trapping Program in the Maraba Area

1. Gleba 05, Lote 05

Vegetation Type	Number of Traps
Lowland sidehill forest	15 wooden traps
Low flatland forest	7 wooden traps
Upland forest	12 wooden traps
Secondary scrub	10 Rinker traps
Edge of secondary scrub & cropland	10 Rinker traps
Cropland	10 Rinker traps
Total	64

Trapping Success

March 1975
256 Trap Nights plus 20 trap nights in house
Collected: 1 mammal per 7.8 trap nights

Monodelphis brevicaudata	1
Marmosa murina	1
Didelphis marsupialis	2
Oryzomys capito	16
Neacomys guianae	1
Zygodontomys lasiurus	5
Proechimys sp.	7
Total	33

April 1975
336 Trap Nights plus 30 trap nights in house
Collected: 1 mammal per 9.6 trap nights

Oryzomys capito	22
Neacomys guianae	2
Nectomys squamipes	1
Zygodontomys lasiurus	5
Proechimys sp.	5
Total	35

January 1975
196 Trap nights plus 20 trap nights in house
Collected: 1 mammal per 32.7 trap nights

Philander opossum	1
Didelphis marsupialis	1
Saguinus tamarin (shot)	1
Oryzomys capito	1
O. delicatus	1
Rattus rattus (in house)	2
Proechimys sp.	2
Total	9

February 1975
294 Trap nights plus 30 trap nights in house
Collected: 1 mammal per 5.6 trap nights

Caluromys philander	1
Monodelphis brevicaudata	1
Philander opossum	1
Didelphis marsupialis	4
Oryzomys capito	18
O. delicatus	2
O. macconnelli	3
Neacomys guianae	2
Nectomys squamipes	3
Holochilus brasiliensis	1
Proechimys sp.	16
Total	52

March 1975
274 Trap nights plus 20 trap nights in house
Collected: 1 mammal per 3.8 trap nights

Didelphis marsupialis	4
Oryzomys bicolor	3
O. capito	29
O. macconnelli	7
Neacomys guianae	2
Nectomys squamipes	1
Oxymycterus sp.	1
Rattus rattus (in house)	1
Proechimys sp.	23
Mesomys sp.	1
Total	72

May 1975
292 Trap nights
Collected: 1 mammal per 15.4 trap nights

Didelphis marsupialis	1
Oryzomys capito	10
Zygodontomys lasiurus	3
Proechimys sp.	5
Total	19

June 1975
496 Trap nights plus 40 trap nights in house
Collected: 1 mammal per 13.8 trap nights

Oryzomys capito	14
O. concolor	2
O. delicatus	1
Zygodo tomys lasiurus	11
Proechimys sp.	8
Total	36

2. Gleba 29 Lote 03

Vegetation Type	Number of Traps
Lowland forest	11 wooden traps
Lowland, but not inundated, forest	24 wooden traps
Upland forest	15 wooden traps
Edge of forest and cropland	19 Rinker traps
Cropland	29 Rinker traps
Total	98

Trapping Success

November 1974
298 Trap Nights plus 25 trap nights in house
Collected: 1 mammal per 74.5 trap nights

Monodelphis brevicaudata	1
Marmosa murina	2
Philander opossum	1
Rattus rattus (in house)	3
Total	7

May 1975
218 Trap Nights plus 20 trap nights in house
Collected: 1 mammal per 4.4 trap nights

Oryzomys capito	22
O. delicatus	3
O. macconnelli	12
Nectomys squamipes	1
Rattus rattus (in house)	1
Proechimys sp.	11
escaped	1
Total	51

June 1975
528 Trap nights plus 30 trap nights in house
Collecced: 1 mammal per 6.9 trap nights

Monodelphis brevicaudata	1
Oryzomys capito	39
O. concolor	3
O. delicatus	2
O. macconnelli	6
Neacomys guianae	5
Nectomys squamipes	4
Rattus rattus	1
Proechimys sp.	15
escaped	1
Total	77

Mammal Trapping Program in the Altamira Area

1. Agrovila 3/5 (da União)

Vegetation Type	Number of Traps
Acai swamp forest	9 wooden traps
Lowland forest	15 wooden traps
Sidehill Forest	10 wooden traps
Upland Forest	16 wooden traps
Secondary Scrub	10 Rinker traps
Edge of secondary scrub & cropland	10 Rinker traps
Cropland	5 Rinker Traps
Total	75

Trapping Success

June 1975
592 Trap nights
Collected: 1 mammal per 10.4 trap nights

Caluromys philander	2
Monodelphis brevicaudata	5
Metachirus nudicaudatus	1
Didelphis marsupialis	5
Oryzomys capito	16
Neacomys spinosus	2
Oxymycterus sp.	5
Proechimys sp.	21
Total	57

July 1975
296 Trap nights
Collected: 1 mammal per 11.8 trap nights

Monodelphis brevicaudata	2
Oryzomys capito	10
Neacomys guianae	3
Oxymycterus sp.	4
Proechimys sp.	6
Total	25

2. Gleba 61 Lote 02

Vegetation Type	Number of Traps
Lowland forest	18 wooden traps
Sidehill forest	20 wooden traps
Secondary Scrub	10 Rinker traps
Edge of secondary scrub & cropland	10 Rinker traps
Cropland	10 Rinker traps
Total	68

Trapping Success

June 1975
519 Trap nights plus 35 trap nights in house
Collected: 1 mammal per 11.3 trap nights

Monodelphis brevicaudata	6
Marmosa cinerea	2
Philander opossum	7
Didelphis marsupialis	3
Neacomys spinosus	1
Oxymycterus sp.	8
Proechimys sp.	19
Total	46

July 1975
847 Trap nights
Collected: 1 mammal per 10.5 trap nights

Monodelphis brevicaudata	4
Philander opossum	4
Didelphis marsupialis	7
Oryzomys capito	10
Neacomys guianae	1
Oxymycterus sp.	28
Proechimys sp.	27
Total	81

III. WILDLIFE ECOLOGY

D. METEOROLOGICAL PROGRAM

BACKGROUND: The amount and pattern of rainfall plays an important role in determining the type of vegetation and fauna present in an area. Diseases such as malaria and yellow fever are also dependent on rainfall to provide a breeding area for insect vectors. The meteorolgical program is designed to record daily rainfall and temperatures in various locations along the Transamazon Highway.

The wet and dry seasons, as well as the amounts of annual rainfall differ within relatively small distances in the Amazon Basin (Figures 1 and 2). Two areas located 5.3 Km apart in the city of Belem averaged an 8% difference in annual rainfall over a period of five years (Table 1). The average monthly rainfall over a period of five years from three sites in the Belem area also differed (Figure 3).

Aside from the Air Force meteorological stations located near airports in the larger cities such as Maraba and Altamira, very little climatic data from the Transamazon area is available.

PROGRAM DESCRIPTION; Maximum-minimum thermometers and rain gauges are presently being monitored daily in seven locations between Maraba and Itaituba. The rain gauges are set in clearings away from buildings, trees, and other obstructions which may bias the readings. The thermometers are located under roofs, for protection from the sun, in open areas to allow comparisons of the maximum and minimum temperatures between the sites. The rain gauges are read early in the morning and the amount of rain recorded on a form. The maximum and minimum temperatures are read late in the afternoon and recorded on the proper form. A recording hygrothermograph is located near Maraba, and another near Altamira to record the hourly changes in temperature and relative humidity.

OBSERVATIONS: The common assumption by most persons that the Amazon basin is mainly a lowland region subjected to seasonal flooding is false. The classic works of H. W. Bates, Alexander Von Humboldt, and others describing their travels up the navigable waters of the Amazonian rivers has led to this belief. The opening of the Transamazon Highway has demonstrated that the topography and vegetation away from the rivers is very different. The annual rainfall within the area between Maraba and Humaita ranges from 1,400 to 2,700 mm (Figure 1). This, according to Holdridge (1947) would be classified either as a tropical moist or tropical wet forest formation. Richards (1952) considers almost all of the Amazon basin as a tropical rain forest, disagreeing with Beard (1944) whose definition of a rain forest requires that moisture must be available throughout the year

Figure 1. Average Rainfall, in Millimeters, at Various Sites in the Amazon Basin.

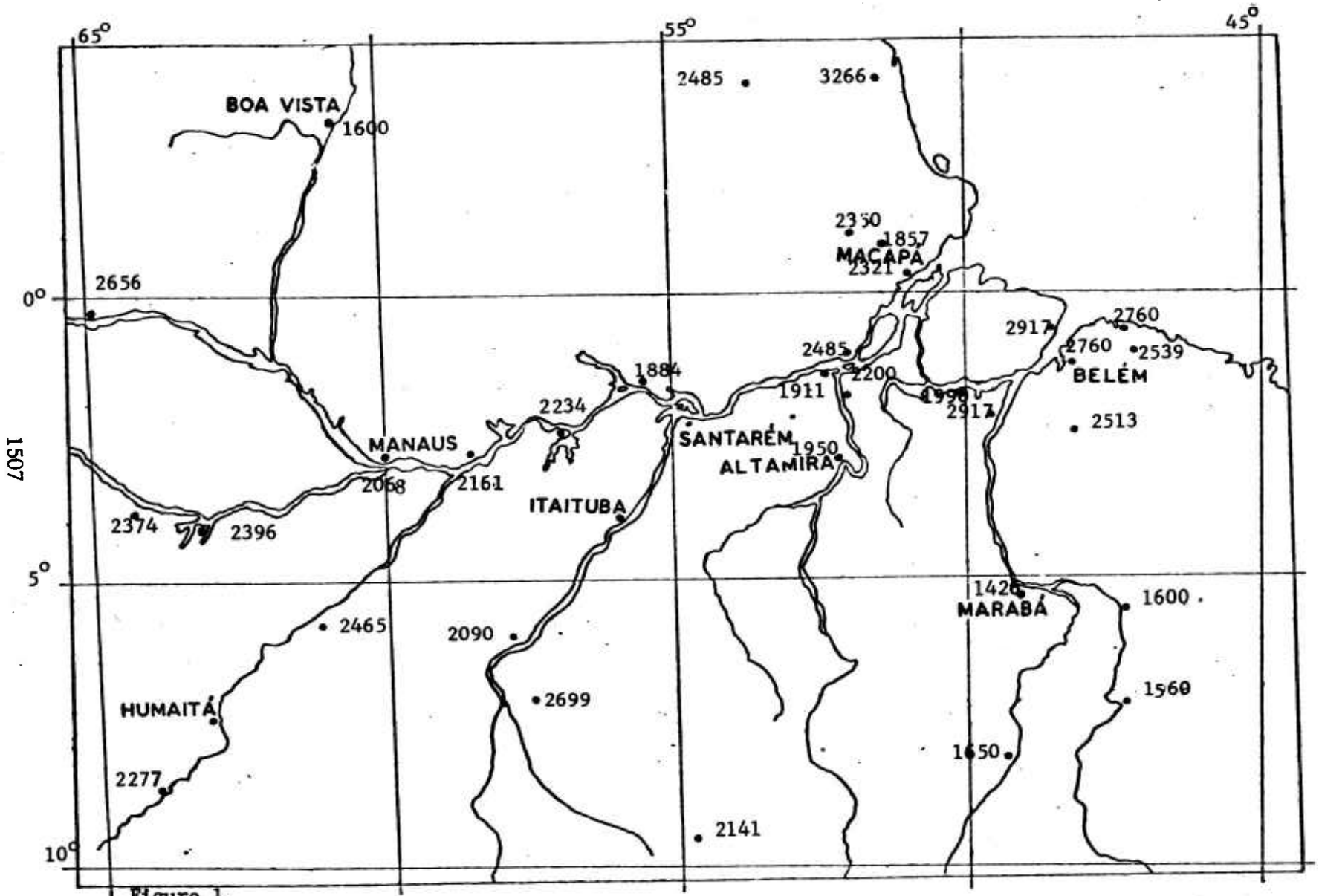

65°
55°
45°
0°
5°
10°
1507
BOA VISTA
1600
2656
2485
3266
2330
1857
MACAPÁ
2321
2917
2760
2760
2539
BELÉM
2513
2485
1884
2200
1911
1998
2917
2234
SANTARÉM
1950
ALTAMIRA
MANAUS
2068
2161
ITAITUBA
2374
2396
1426
MARABÁ
1600
2465
2090
2699
1560
HUMAITÁ
1650
2277
2141

Figure 1.

Figure 2. Months during which the Average Rainfall is more than 100 mm at various sites in the Amazon Basin. (12-8 means the wet season is from December through August).

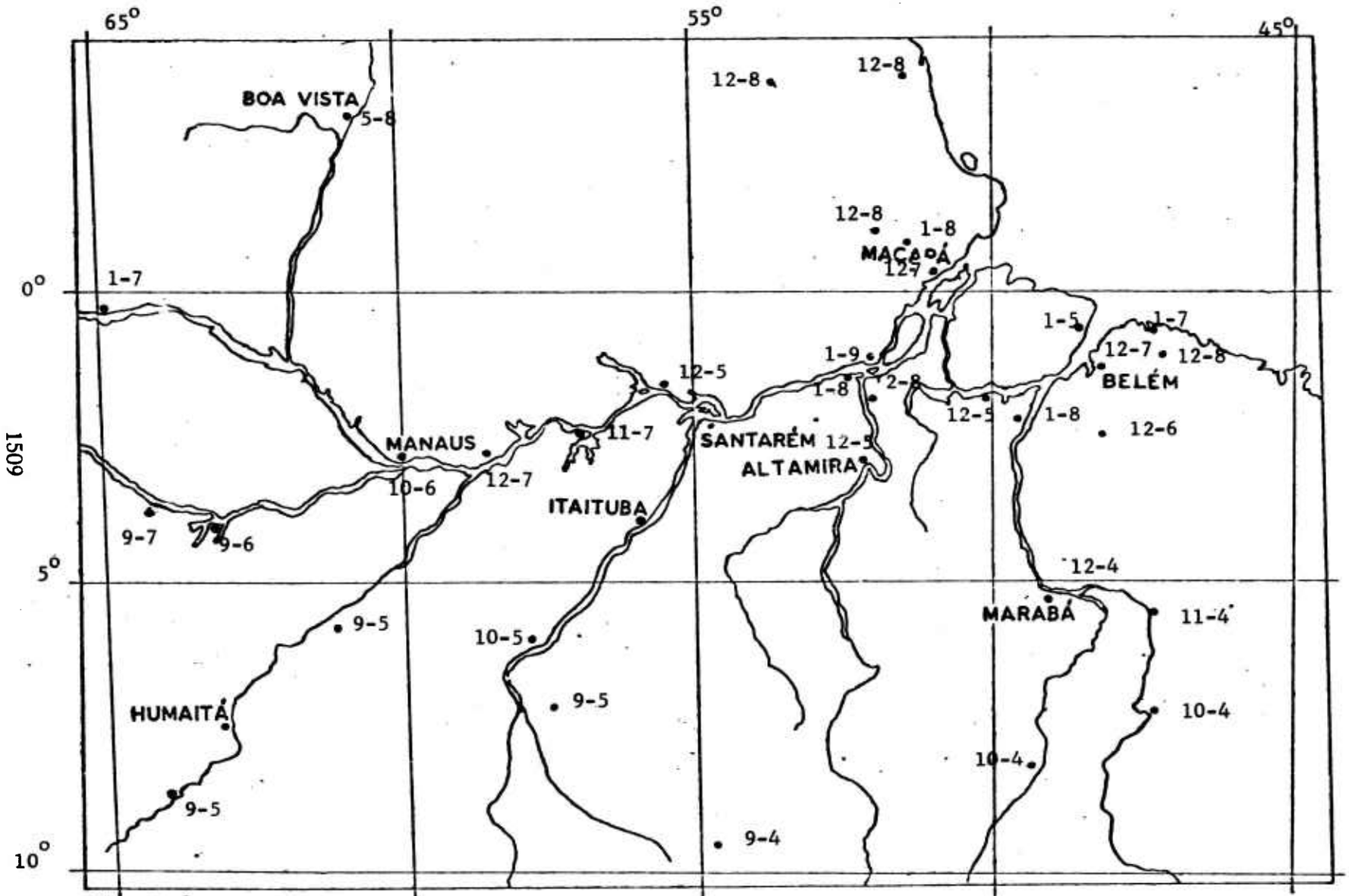

65°
55°
45°
0°
5°
10°
1509
BOA VISTA
5-8
1-7
MANAUS
10-6
12-7
11-7
12-5
ITAITUBA
9-7
9-6
9-5
HUMAITÁ
9-5
12-8
12-8
12-8
1-8
MACAPÁ
12-7
1-9
1-8
2-8
SANTARÉM
12-5
ALTAMIRA
12-5
1-5
1-7
12-7
12-8
BELÉM
1-8
12-6
12-4
MARABÁ
11-4
10-4
10-4
10-5
9-5
9-4

Figure 2.

TABLE 1. Percentage difference in Rainfall per Year between Two Locations in Belem.

Area	Millimeters of Rainfall					
	1969	1970	1971	1972	1973	Average
Utinga	2846	2967	3076	2991	3254	3027
IEC	2466	2538	2827	2999	3088	2784
% Difference	13%	14%	8%	0%	5%	8%

without inundation or seasonal drought. The potential evaporation rate in this area exceeds 100 mm per month (Silva Rosatelle, et al., 1974), and the months during the dry season receive less than 100 mm of rain (Figure 2). Using Beard's (1944) classification, this area would range from evergreen seasonal to semi-evergreen seasonal tropical forests.

Recording daily temperature fluctuations and rainfall from various points along the Transamazon Highway will enable us to compare local climatic conditions between areas. This information will be of assistnace in documenting seasonal changes in flora and in relative abundance of mammals and insects.

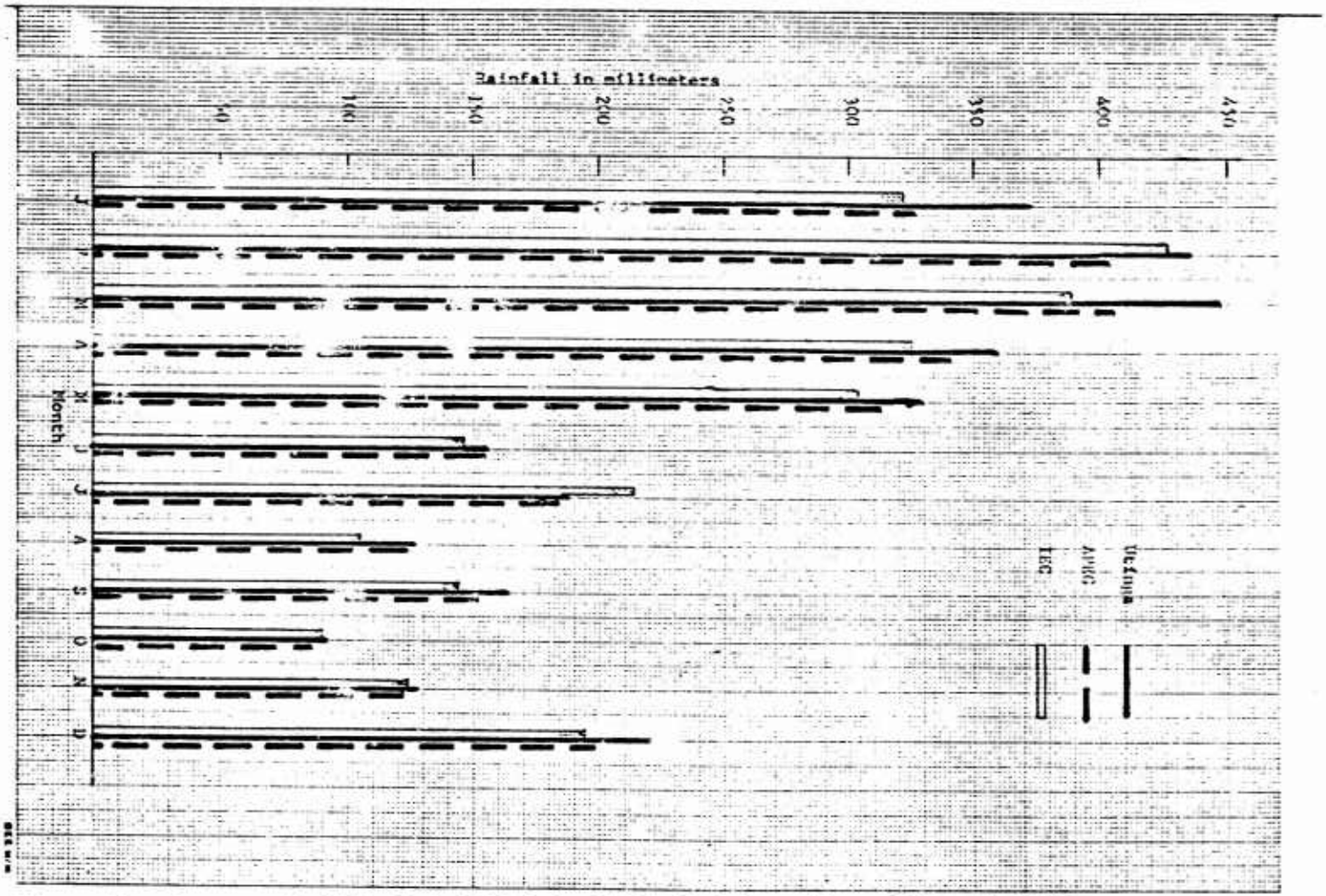


Figure 3. Average Monthly Rainfall Data Collected from Three Sites in Belem from 1969-1973.

IV. PARASITOLOGY/LABORATORY PROGRAM

The objectives of this program are:

1. to provide agent isolation and serologic procedures both in the field and in Belem to support USAMRU studies;

2. to conduct studies of endemic parasitic diseases;

3. to assess the potential for importation of parasitic diseases from other parts of Brazil.

BACKGROUND: No laboratory diagnostic facilities exist in the study areas along the Transamazon Highway with the exception of laboratories reading malaria blood smears and direct stool smears. Prevalence surveys have indicated the presence of various bacterial, viral, parasitic and mycotic agents in this area; but no incidence data are available, nor have any studies been carried out in patients attending outpatient facilities and hospitals in the area.

The arrival of a parasitologist/laboratory officer in December 1974 and opening of a base laboratory (Belem) in April 1975 permitted the initiation of laboratory work.

PROGRESS TO DATE:

Parasitic Serology

During the period 27 April - 15 May 1975, Mrs. Irma de Arjona of USAMRU-Panama visited Belem and demonstrated indirect flourescent antibody procedures for the detection of toxoplasmosis, leishmaniasis, and American trypanosomiasis. The production of antigen for these procedures was also demonstrated. In order to give technical personnel an opportunity to become proficient in immunoflourescent technique, a battery of sera from colonists in the area of Maraba were tested with the indirect flourescent antibody test for toxoplasmosis (IFAT). Sera were screened at a 1:8 dilution. Those sera which were positive at this dilution were subsequently tested at dilutions of 1:32, 1:128, 1:512 and 1:2048. Results of this trial are listed in Table 1. Complete results on the Maraba study group are presented in Figure 1 and Table 2. Collation of these results with epidemiologic data concerning acute disease episodes, seroconversion, and various demographic variables is in progress. The indirect hemagglutination (IHA) test will be used for routine screening of sera for toxoplasmosis, with the IFAT being reserved for confirmatory testing of a percentage of sera positive by IHA and in cases of suspected acute disease.

An attempt is being made at present to choose appropriate screening tests for leishmaniasis and American trypanosomiasis. An evaluation of the direct agglutination test for Chagas' disease (Allain and Kagan, 1972) and the direct agglutination test for leishmaniasis (Allain and Kagan, 1975) in cooperation with the Wellcome Parasitology Unit of the Institute Evandro Chagas is under consideration.

Bacterial Serology

Leptospirosis - Serum specimens from the Maraba study group were examined by a macroagglutination procedure employing six antigen pools (DIFCO - Table 3). Specimens collected at initiation of the epidemiology, July 1974, showed 14.1% positive to either one or multiple pools. Positive results were observed in 19.1% of specimens collected in January 1975 from the same individuals. Results by individual and multiple pools are shown in Table 4. Collation of these results with epidemiologic data is in progress. The Division of Veterinary Medicine, WRAIR, will conduct serotyping of positive specimens.

Brucellosis is shown in Table 5. Brucellosis has been reported in some livestock being introduced into the study area.

Imported Parasitic Disease

Schistosomiasis - The names and addresses of approximately 300 colonists examined and treated with Hycanthone by the Superintendencia de Campanhas de Saude Publica (SUCAM) for Schistosoma mansoni infection were obtained. All are former residents of areas of Brazil where schistosomiasis is endemic, and all now live along the Transamazon Highway or in villages in the vicinity of Altamira. Schistosomiasis is not endemic in this region of Brazil. However, the planorbid species, Biomphalaria straminea, which is capable of being infected with S. mansoni has been found in the Altamira and Itaituba areas (Lacaz et al., 1972). Twenty of the SUCAM patients were located and a single stool specimen from each was examined by direct smear and formalin-ether concentration. All were negative for schistosome ova. An attempt will be made to locate and examine a larger number of these people along with members of their families. The results of these examinations along with observations on snail populations and colonists' activities will determine what efforts will be directed toward a study of the potential for importation and maintenance of schistosomiasis in the area of the Transamazon Highway. Serologic testing of the study population employing slide flocculation and complement fixation tests is in progress, with support from Department of Immunology, WRAIR.

Chagas' Disease - Two cases of Chagas' disease among colonists arriving in the Altamira area (Gleba 5, Km 23) in July 1974, were reported to USAMRU by INCRA. The information concerning these cases available to USAMRU is incomplete, but it is known that both patients were hospitalized in Belem where their sera was tested by complement fixation (reacao de Mechado Guerreira) for antibodies to Trypanosoma cruzi. One patient had a C.F. titer of 1:6000; the other's titer was 1:24,000. The final disposition of these patients is unknown.

One specimen of Panstrongylus sp was captured in Agrovila 3/5 outside Altamira when it attempted to feed on the leg of a sleeping man. It is assumed that the triatomids in this area are exclusively sylvatic. A large number of colonists come from areas where Chagas' disease is endemic. An analysis of the potential significance of this infection along the Transamazon awaits serologic and entomologic study. Serologic testing (Latex agglutination and complement fixation) will be carried out in the study population with support from Department of Immunology, WRAIR).

Onchocerciasis - Prior to 1972, only one autochthonous case of onchocerciasis had been found in Brazil; it was reported from the Federal Territory of Roraima and described by Bearzoti et al., 1967. Moraes and Dias (1972) and Moraes and Chaves (1973) reported a total of three cases in American missionaries living among groups of Yanomama Indians in the extreme north of Brazil near the Venezuelan border. In 1973, Moraes et al., examined 91 Yanomamas from three villages along the Toototabi River in northern Amazonas and found the microfilariae of Onchocerca volvulus in the skin of 57 cases.

In an unpublished presentation to the Brazilian Society of Tropical Medicine in 1975, Dorado reported finding a high percentage of infected Ticuna Indians living in several villages along the Solimoes River in the state of Amazonas. In order to confirm or deny this report, one investigator from USAMRU accompanied Drs. Moraes, Fraiha, and Chaves (I.E.C.) to the villages in question. Skin biopsies from a total of 616 Indians from 7 villages were examined. No Onchocerca microfilariae were found; neither were characteristic cutaneous nodules observed. Blood films and sera were collected during the course of this investigation for the study of other infections, including mansonellosis, toxoplasmosis, and pinta.

Endemic Enteropathogens

As part of a collaborative program begun in May 1975 among USAMRU, IEC, SUCAM and FSESP in Altamira, Para, feces are being collected from patients admitted to the FSESP hospital and from colonists along the Transamazon Highway for subsequent parasitologic and virologic examination. Each stool specimen is divided, part being fixed in formalin for parasitologic study and part being frozen in liquid

nitrogen for virologic study. A sample of each frozen specimen will be provided to WRAIR, and the virology section of the I.E.C.; one sample of each specimen will be stored at USAMRU. Clinical and epidemiologic data are provided by the USAMRU epidemiology program.

Collection is being carried out according to the following plan:

1. Hospital patients:
   Feces for parasitology and virology is being collected from the following classes of patients:
   A. Febrile history
   B. Diarrhea syndrome
   C. Icteric syndrome
      All icteric syndrome patients will be visited 32 days after admission and a follow-up specimen collected.
   Parasitologic examination is performed at USAMRU.

2. Colonists:

   Feces are collected from colonists by USAMRU field epidemiology teams and submitted to SUCAM for parasitologic examination. Results are returned to USAMRU for entry on colonists' health card and review by USAMRU parasitologist.

Bacterial cultures will be included in this study when the field laboratory becomes operational.

Outside Laboratory Support

Division of Hazardous Microorganisms, WRAIR, is examining a panel of sera from mammals captured in USAMRU study areas for sero-positivity to rickettsial, plague and tularemia antigens. Each of these agents is endemic in other parts of Brazil.

Department of Virus Diseases, WRAIR, and the Institute Evandro Chagas, Belem, are performing arbovirus isolation attempts and serologic testing on specimens from the USAMRU surveillance programs. Initial results are in process of analysis.

These same two units are providing hepatitis B antigen and antibody testing.

Table 1. Toxoplasmosis Titers of Trial Battery of Sera Tested by IFA Technique.

			REACTORS AT DILUTION:									
TOTAL SERA	1:8		1:8		1:32		1:128		1:512		1:2048	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
297	112	37.7	73	24.6	36	12.1	42	14.1	26	8.8	8	2.790

Table 2. Toxoplasmosis IFAT Distribution. Original and Sixth Month Specimens from Maraba Colonists.

RECIPROCAL TITER	ORIGINAL		6th MONTH	
	SERA	%	SERA	%
< 8	271	46.9	202	43.0
8	107	18.5	66	14.0
32	80	13.8	72	15.3
512	32	5.5	35	7.4
2048	15	2.2	18	3.8
TOTALS	578	100.0	470	100.0

Fig. 1 TOXOPLASMOSIS IFA TITER DISTRIBUTIONS MARABA/ALTAMIRA

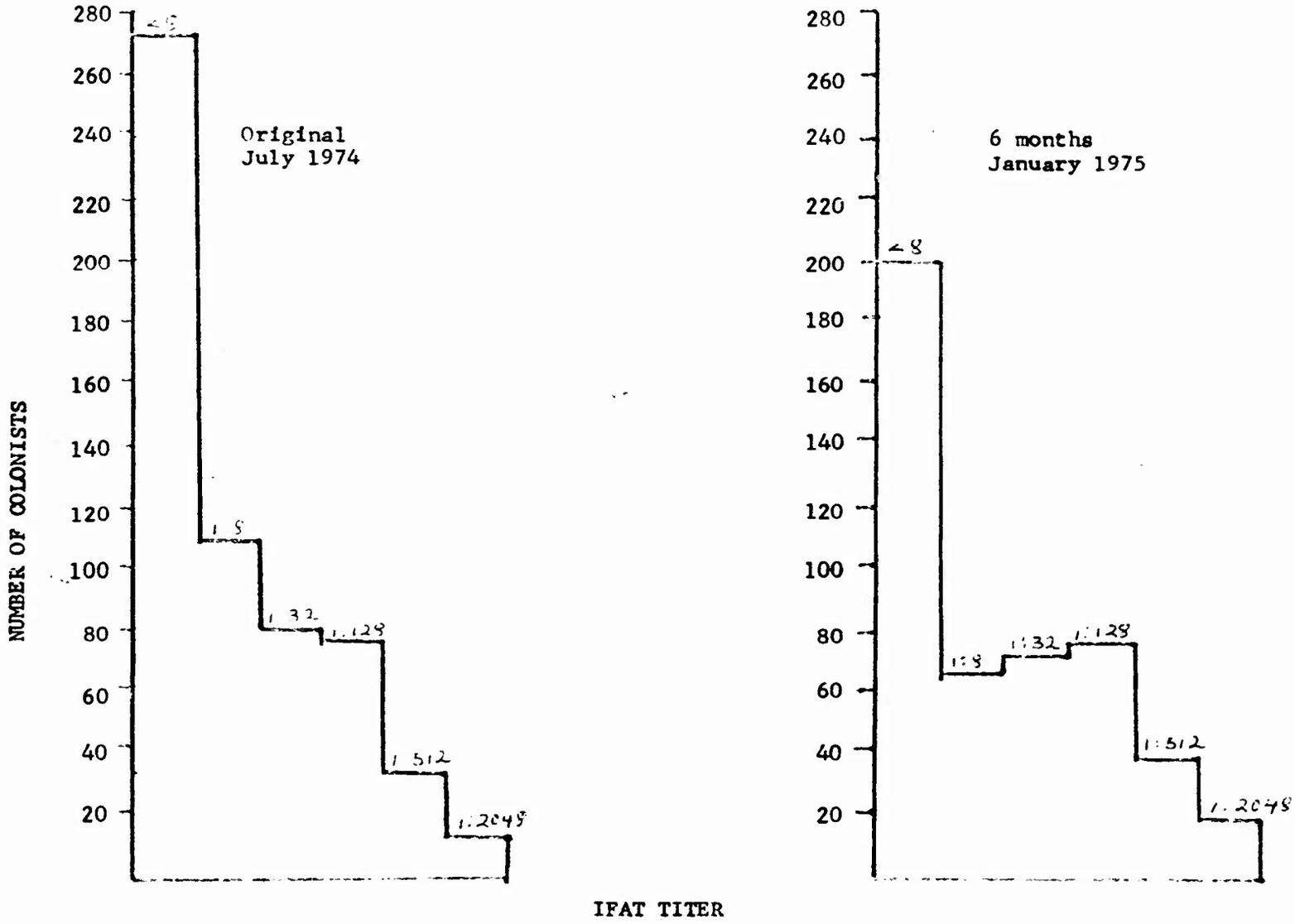


Table 3. Leptospirosis Macroagglutination Test.

DIFCO Leptospiral Antigen Pools Contain the Following Antigens:

Pool 1

L. ballum
L. canicola
L. icterohemorrhagiae

Pool 2

L. bataviae
L. grippotyphosa
L. pyrogenes

Pool 3

L. autumnalis
L. pomona
L. wolffii

Pool 4

L. australis
L. hyos
L. mini, georgia

Pool 5

L. cynopteri
L. celledoni
L. javanica

Pool 6

L. cynopteri
L. panama
L. shermani
L. ictero - kremastos pool
L. kremastos
L. mendanensis
L. seiroe
L. biflexa
L. biflexa patoc

Table 4. Leptospirosis Serology: Maraba Original and Sixth Month Specimens, Including Acute Disease Specimens.

Number of sera	908	
Negative	744	
Positive - one pool only	79	(8.7%)
Pool #1	0	
Pool #2	7	
Pool #3	68	
Pool #4	2	
Pool #5	1	
Pool #6	1	
Positive - Multiple pools	85	(1.36%)
Distribution of reactions		
Pool #1	56	
Pool #2	45	
Pool #3	83	
Pool #4	60	
Pool #5	42	
Pool #6	39	

Table 5. Brucellosis Serology - Maraba Original and Sixth Month Specimens Including Acute Disease Specimens.

No. of SERA	NEGATIVE	1:20	1:40	1:80	1:160	1:320	N.T.*
919	586	10	5	4	2	3	309
PERCENT	63.8	1.1	0.5	0.4	0.2	0.3	33.6

*No Test:
Test Sera agglutinated on the slide before addition of the antigen.

PROJECT 3A762759A831 TROPICAL MEDICINE

Task 00, TROPICAL MEDICINE

Work Unit 073 Disease transmission in tropical populations

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6465	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A821	00	074
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with security classification code)

(U) Tropical and Subtropical Diseases in Military Medicine

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	14.0	1,300
FISCAL YEAR CURRENT 76	10.0	1,100

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security classification code)

(U) Infectious Diseases; (U) Virus Diseases; (U) Bacterial Diseases; (U) Parasitic Diseases; (U) Veterinary Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23. (U) To define the ecology and basic biology of causal agents of tropical diseases, and to study environmental variables that may affect the performance of U.S. servicemen in tropical areas.

24. (U) Routine diagnostic, epidemiological, serological, biochemical, microbiological, entomological and psychological procedures are being utilized. Field studies are emphasized and are supplemented by appropriate laboratory investigations.

25. (U) 74 07 - 75 06 Hepatitis, dengue and tick-borne virus infections in man were studied, with emphasis on improved methods of virus isolation and identification. Dengue virus was isolated from more hemorrhagic fever patients (24%) if the acute plasma was inoculated into mosquitoes, and the mosquitoes inoculated into tissue culture, than by tissue culture alone (10%). The use of sensitive radioimmune assays revealed an incidence of hepatitis B infections in US enlisted personnel of 18/1000/year with 82% asymptomatic infections. Tick-borne Langat virus was identified, but no evidence of human infection was found. An antigenically new strain of Influenza A virus was recovered from an outbreak in remote hill tribe villages. The mean penicillin minimum inhibitory concentration of gonococcal strains in Thailand doubled (.58 to 1.05 U/ml) in 2 years. Epidemiologic studies of filariasis identified six species of naturally infected mosquitoes; studies of larval habitats and human infections are underway. An epizootic of Tropical Canine Pancytopenia affecting 161/316 military dogs was successfully treated with a mortality of only 3.2%. Results of a questionnaire survey for predicting drug abuse are being analyzed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

* Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 074 Tropical and Subtropical disease in military medicine

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Associate: Apinya Asananig, M.S.; COL Majuree Balankura, MC, RTA; MAJ Michael W. Benenson, MC; Prakorb Boonthai, M.D.; Natth Bhamrapavati, M.D.; D.Sc.: Chalam Chatrasri, B.Sc.; Tawee Chottipitayasunodh, M.D.; Vina Churboonchart, M.Sc.; SP5 Ernest Comer; Pairatana Gunakasem, M.D.; Valee Harisdangkul, M.D.; Ph.D.; CPT Bruce A. Harrison, MSC; Siriwan Horosethkul, M.D.; Y.M. Huang, Ph.D.; Suchart Jetanasen, M.D.; Sukachard Kirdpol, M.D.; SP6 Jerome J. Karwacki; Karoon Mansuwan, M.D.; Pethai Mansuwan, M.D.; LTC La-or Nguen, DVM, RTA; Suchitra Nimanitaya, M.D.; Vandee Ningsanonda, M.D.; E.L. Peyton; COL Suchai Premabutra, DVM, RTA; SSG Richard M. Price; MAJ John F. Reinert, MSC; Miodrag Ristic, Ph.D.; Leon Rosen, Ph.D.; MAJ Harry Rosmiarek, VC; COL Philip K. Russell, MC; CPT John W. Sagartz, VC; Stitaya Sirisinha, DMD, Ph.D.; Phinit Simasathien, M.D.; SSG Peter W. Symth; LTC Pramoon Sookwatana, MD, RTA; LTC Edward H. Stevensen, VC; Pramuan Sunnakorn, M.D.; Banchang Tanagkul, M.D.; Panita Tansakul, M.Sc.; Chaufah Thongthai, Ph.D.; Sophon Tirajitara, M.D.; COL Franklin H. Top, Jr.,MC; MAJ Wanitch Vanapruk, MC, RTA; COL Somnuk Vibulsek, MC,RTA; Santisook Vibulbuditket,M.D.; Suwana Vithanomsat,B.Sc.; SFC Robert E. Weaver;CPT Ronald N. Wilkinson, MSC.

I. VIRUS DISEASES OF MAN AND ANIMALS

A. DENGUE HEMORRHAGIC FEVER

1. Dengue Infection at the Children's Hospital of Bangkok

OBJECTIVE: To provide viral diagnostic and laboratory expertise to the Children's Hospital of Bangkok and to collect specimens for specialized dengue virus isolation and serology.

BACKGROUND: Dengue virus infections continue to be an annoying and potentially critical problem for military forces stationed in many tropical areas. Dengue infections are also a major cause of morbidity and mortality among children in Southeast Asia. As in previous years SEATO Medical Research Laboratory has collaborated with the Children's Hospital of Bangkok in the study of dengue hemorrhagic fever. This has been mutually beneficial allowing for improved patient care through diagnostic and laboratory work provided by SEATO Laboratory and allowing for the collection of specialized samples from dengue patients to allow for investigations of the pathogenesis and clinical expressions of this infection.

DESCRIPTION: Patients with a hospital admission diagnosis compatible with dengue infections (dengue hemorrhagic fever, dengue fever or undifferentiated fever) were selected from the infectious disease wards of the Bangkok Children's Hospital. A standardized chart of pertinent signs, symptoms and laboratory findings was instituted on each patient.

An attempt was made to collect blood on at least the day of diagnosis and on the third, fifth, fifteenth and thirtieth days after hospitalization. Blood was allowed to clot or was collected using heparinized tubes (~20 u heparin/ml blood). Studies were done on either serum or plasma. During the month of July and August plasma was removed from heparinized blood and the cellular components were separated using a dextran sedimentation technique. Peripheral blood leukocytes were used by Dr. Nyven J. Marchette, University of Hawaii, in an investigation of the occurrence and specificity of in vitro antibody production, and to study the phenomenon of permissive peripheral blood leukocytes previously identified in monkeys. Platelets and, in a few cases, leukocytes as well as plasma were submitted for virus isolation.

Virus isolation was performed using a direct and delayed plaque technique. Plasma was also inoculated into mosquitoes, both at SEATO Medical Research Laboratory and at the University of Hawaii, to test the mosquito isolation technique of Rosen et al (see elsewhere in this report). Sera or plasma were used for serology;

hemagglutination inhibition (HI) tests were performed using suckling mouse brain antigens prepared from dengue 1 (Hawaii), dengue 2 (New Guinea C), dengue 3 (H-87), dengue 4 (H-241), Japanese Encephalitis (Nakayama) and Chikungunya (Ross). Sera were extracted with acetone and tested against 8 units of antigen. All sera collected from one patient were tested simultaneously.

At the conclusion of hospitalization, a clinical discharge diagnosis was made and the severity of the illness was independently classified by clinicians in charge of the case.

Grading of severity of dengue hemorrhagic fever used criteria established by one of us.

Grade I: Fever accompanied by non-specific constitutional symptoms. The only hemorrhagic manifestation is a positive tourniquet test.

Grade II: Fever and skin hemorrhage or other bleeding such as epistaxis or gingival hemorrhage.

Grade III: Circulatory failure manifested by weak, rapid pulse with narrowing of pulse pressure (less than 20 mm Hg) or hypotension (systolic pressure 90 mm Hg or less).

Grade IV: Moribund patients with undetectable blood pressure or pulse.

Following grading, isolation and serological data were used to identify those patients infected with dengue and to determine the type of antibody response. Patients were considered to have had a dengue infection if a four-fold rise in HI antibody titer to at least two of the group B antigens was found between the acute and convalescent sera or if convalescent antibody titers to at least two antigens equaled or exceeded 1:640. Criteria for the identification of primary or secondary dengue have been previously reported. Patients with convalescent HI titers of 1:640 or more to at least two dengue antigens were considered to have secondary infections while those with convalescent HI titers of less than 1:640 were considered to have primary infections. Where necessary to clarify the occurrence of a primary or secondary infection, plaque reduction neutralization by selected sera of appropriate Group A and Group B seed viruses were performed.

In a few cases immunoglobulin separation were performed using sucrose gradient ultracentrifugation. The original sera and the fractions obtained from the sucrose gradients were tested for

IgM, IgG and B1C/B1A concentrations using radialdiffusion plates (Hyland Laboratories). Antibody contained in these fractions was assayed by hemagglutination inhibition with and without treatment with 2 mercaptoethanol (2 ME). The ? ME treatment was used to reduce IgM antibody activity.

One hundred and thirty four patients with admission diagnoses compatible with dengue infection were seen on the ward. One hundred and twenty seven of these were adequately followed and 114 (90%) were diagnosed as dengue infection by viral isolation, by serological criteria or both. Sixteen strains of dengue virus were isolated by direct or delayed plaque technique from either the plasma or the cellular components from the blood of the 114 patients with evidence of dengue infection (Table 1). In some cases isolations were made from the cellular components only. This represents an isolation rate of 14%. Five strains were dengue 1, five were dengue 2, three were dengue 3 and three are as yet unclassified. No dengue 4 was identified in Bangkok in 1974. Further details of isolation by the direct and delayed plaque technique and the mosquito isolation procedure will be found elsewhere in this report.

Of the 114 patients diagnosed as dengue, 13 (11.4%) patients had low level antibody responses detected by HI and were considered primary dengue infections, 94 (84%) had high HI titers and were considered secondary infections. Of the latter, 10 patients exhibited high fixed titers and 84 showed rising titers. Seven (6%) patients died, usually before the fifth day of disease. As no convalescent sera could be collected in these cases, the patients could not be classified on serological grounds as having primary or secondary infections. Laboratory findings on the 107 patients on whom classification could be completed may be found in Table 2. Dengue hemorrhagic fever occurred in patients with either primary or secondary infections and the distribution of clinical grades was essentially similar to that found in previous studies of hospitalized patients with one major exception.

This year, close clinical observation allowed for the detection of shock in three patients (D74-77, 91 and 103) exhibiting an antibody pattern characteristic of primary dengue infection (Table 3). These three patients were investigated further. Figure 1 is a flow diagram of the clinical course of one of them (D74-77). In this patient shock occurred in the morning of the sixth day at a time when the fever was subsiding. The rising hematocrit and the falling platelet counts occurred over a short period of time just prior to the onset of shock. HI antibody titers at this time were between <1:20 and 1:80 against dengue 1

Table 1. Dengue Isolations by Direct and Delayed Plaque Technique - 1974

Patient	Source[a]	Plaque Technique[b]			Identification
		Direct	Delayed	Secondary Delayed	
D74-16	Plasma	11[c]	-	-	D2
D74-33	Plasma	28	-	-	D3
	Platelets	106	-	-	
D74-38	Plasma	16	-	-	D2
	Platelets	15	-	-	
D74-44	Plasma	7	-	-	D1
D74-61	Platelets	0	6	-	D1
D74-63	Plasma	0	22	-	D1
	Platelets	2	-	-	
D74-66	Platelets	0	TNTC[d]	-	D2
D74-74	Leukocytes	2	-	-	D2
D74-90	Plasma	0	72	-	?
D74-95	Plasma	63	-	-	D1
D74-103	Plasma	121	-	-	D3
D74-104	Plasma	0	0	66	?
D74-112	Plasma	118	-	-	D1
D74-137	Plasma	0	17	-	?
D74-150	Plasma	TNTC	-	-	D2
D74-151	Plasma	TNTC	-	-	D3

a - Isolation from plasma was attempted in every case.
b - Direct, Delayed and Second delayed plaque techniques were used; using 0.3 ml of plasma
c - Number of plaques counted.
d - TNTC = too numerous to count.

Table 2. Hemagglutination Inhibition Antibody Levels in Convalescent Sera from 107 Patients with Dengue Infection

Grade of Disease	Primary Infection Titer <1:640	Secondary Infection Titer ≥1:640
UC[a]	0	1 (0.9%)
UF[b]	5 (4.7%)	8 (7.4%)
I & II	5 (4.7%)	50 (46.7%)
III	3 (2.8%)	29 (27.1%)
IV	0	6 (5.6%)
TOTAL	13 (12.1%)	94 (87.8%)

a. UC indicates that the patient was unclassified.

b. UF indicates undifferentiated fever.

Table 3. Hemagglutination Inhibition and Plaque Reduction Neutralization Tests of Selected Dengue Hemorrhagic Fever Patients Seen in 1974

Study Number	Day of Disease	Reciprocal Hemagglutination Inhibition Titer				Reciprocal Plaque Reduction Neutralization Titer				
		D1	D2	D3	D4	D1	D2	D3	D4	Chik
D74-77	5	<20	<20	<20	<20	<20	<20	<20	~40	<10
	17	80	40	160	160	>1280	~40	~120	<40	~160
	36	40	20	40	40	>1280	~40	~20	≤40	~120
D74-91	5	40	<20	20	<20	<20	<20	<20	<20	ND
	11	160	40	80	160	<20	<20	<20	<20	ND
	67	160	80	80	160	500	<40	<40	<40	<10
D74-103	3	<20	<20	<20	<20	NC	NC	NC	NC	NC
	7	<20	20	40	<20	NC	NC	NC	NC	NC
	58	20	20	160	80	NC	NC	NC	NC	NC

ND = Not done

NC = Not complete

virus and were lower against other dengue types. Complement factor three, as estimated from the B1C/B1A concentrations, was 74 mg% on day 5; it fell to 54 mg% on day 7 and rose slightly to 64 mg% by day 9. It was measured again on days 19 and 34 when levels had returned to 136 mg%; a value within the normal range of 143 ± 22 mg% reported by Hyland Laboratories. The clinical course of D74-77 was essentially similar to those of the other two cases investigated.

From one of these patients (D74-103) a dengue 3 virus was isolated. No virus was identified in the other two (D74-77 and 91). Plaque reduction neutralization tests of all four dengue types and Chikungunya were performed on acute and convalescent sera from each patient. The results are currently available from two of them (D74-77, 91) (Table 3). There was no appreciable antibody to any of the viruses in either of the acute sera obtained on the fifth day of disease. In the convalescent sera from both cases high titered antibody to dengue 1 was found. In one (D74-77), low level antibody was also found to dengue type 3 and to Chikungunya. This patient received several units of plasma which may have contained antibody to group A and B arboviruses and might have caused the serological findings.

In order to determine whether dengue specific IgM was produced in these patients as would be expected in primary infection, sucrose gradient ultracentrifugation was performed on convalescent sera (Table 4). IgM antibody was found in the second through the fourth sucrose fraction (35-31% sucrose) of each serum studied. Specific IgM (reduced by 2 ME) was found against all four dengue types when tested by HI. In D74-77 and 91 the highest titers were found against dengue 1. In a convalescent serum from a case of secondary dengue (identified by HI antibody titers $\geq$1:20480), which was centrifuged simultaneously, IgM antibody was again found in the second, third and fourth fraction. A low level of antibody activity was also found in these fractions against all four dengue types but it could not be reduced by 2 ME indicating that the antibody activity was possibly due to IgG contamination. In none of the sera tested in this manner was any 2 ME reducible antibody to Chikungunya detected in the IgM portion of the gradient. In the convalescent serum from D74-77, low level antibody to Chikungunya was found in the IgG portion of the gradient; this activity was not reduced by 2 ME.

Because of the association of shock in dengue with low complement levels, B1C/B1A concentrations were determined on the acute and convalescent sera of all three patients. In all cases the B1C/B1A concentrations were reduced to less than 50% of normal in the

Table 4. IgM Antibody Titrations of Sera Taken from Selected Dengue Hemorrhagic Fever Patients Seen in 1974

Patient No.	Day of Disease	Sucrose Fraction	IgM* mg %	IgG* mg %	Reciprocal HI Antibody Titer							
					D1		D2		D3		D4	
					B**	2ME***	B	2ME	B	2ME	B	2ME
D74-77	9 (1725)	2	15	0	16	0	0	0	4	0	8	0
		3	24	0	32	0	4	0	16	0	8	0
		4	>50	0	64	0	32	0	8	0	64	0
		5	20	0	16	0	8	0	8	0	16	0
D74-91	6 (1993)	2	20	0	32	0	0	0	8	0	4	0
		3	44	0	64	0	4	0	8	0	8	0
		4	7	0	32	0	0	0	4	0	8	0
		5	0	0	32	0	0	0	4	0	8	0
D74-103	7 (2112)	2	11	0	0	0	0	0	0	0	0	0
		3	>50	0	0	0	0	0	32	4	0	0
		4	14	0	0	0	0	0	8	0	0	0
		5	0	4.5	0	0	0	0	0	0	0	0
D74-6	11 (1087)	2	18	0	8	8	8	4	16	16	8	4
		3	35	0	16	16	16	8	32	32	16	16
		4	12.5	0	64	32	16	16	64	64	32	32
		5	0	8.5	256	512	128	256	256	512	256	256

* Concentrations of IgM and IgG as detected by radialimmunodiffusion

** Sucrose gradient fractions pretreated with buffer

*** Sucrose gradient fractions pretreated with 2 mercaptoethanol

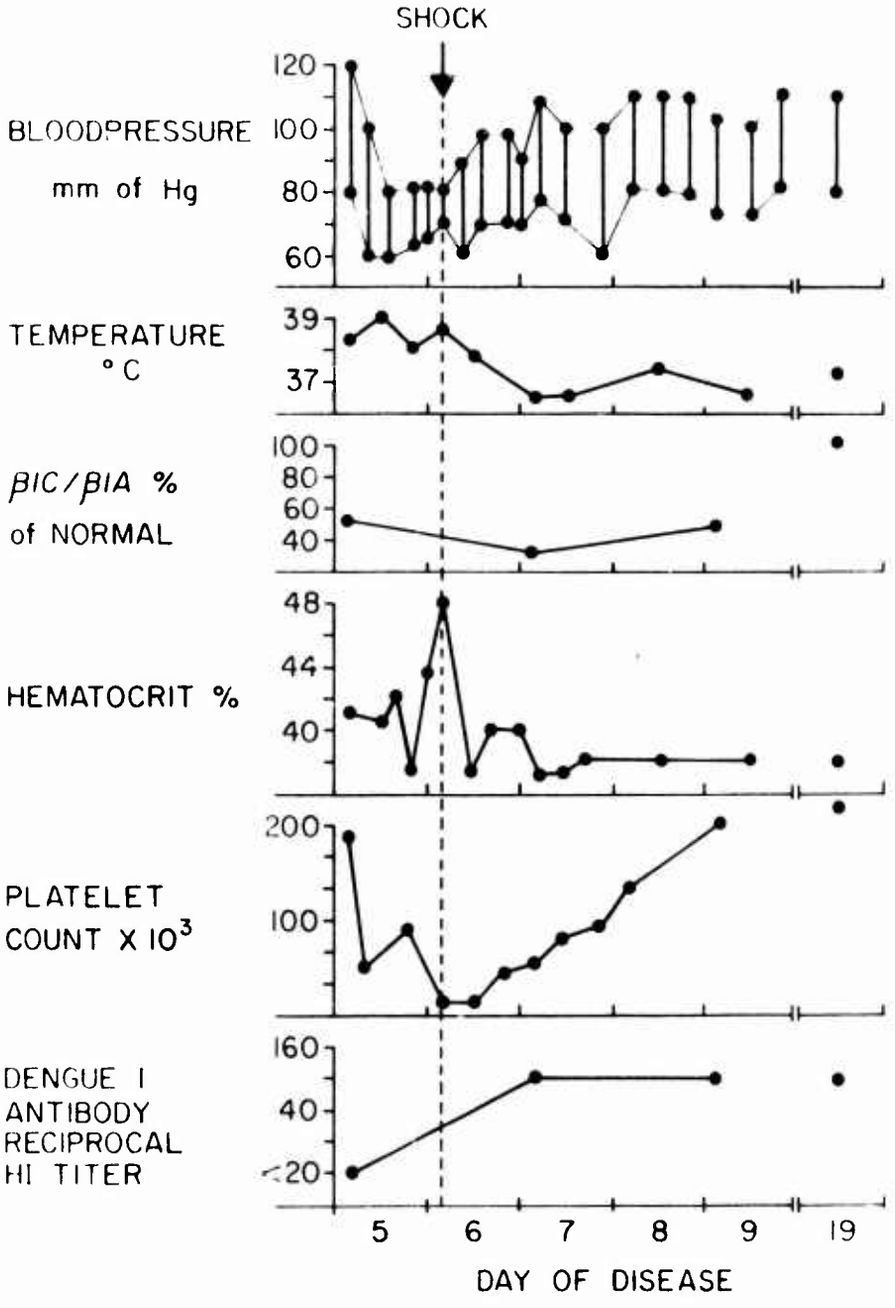

Figure 1 Diagram of the clinical course of patient D74-77 showing the relationship of several clinical and laboratory variables to the onset of shock

acute sera and in sera taken at the time of shock. The concentration increased to the normal range during convalescence. B1C/B1A levels in five patients with primary disease without shock were either in the normal range or only slightly depressed throughout the course of the disease.

DISCUSSION: As in previous years SEATO Medical Research Laboratory has provided laboratory and diagnostic support to the Children's Hospital of Bangkok in an attempt to delineate dengue infection in Bangkok. This year, through close clinical observation, shock was detected in three patients whose HI antibody titers indicated a primary dengue infection. Efforts to firmly establish the nature of these infections are presently underway.

2. Surveillance of Dengue Hemorrhagic Fever Cases in Thailand, 1973 and 1974

OBJECTIVE: The purpose of this study is to confirm the clinically diagnosed dengue hemorrhagic fever (DHF) cases reported to the Ministry of Public Health by using a hemagglutination inhibition (HI) serum screening technique.

BACKGROUND: Dengue hemorrhagic fever remains a major infectious disease in every province and town of Thailand, manifested by high mortality and morbidity in children under 14 years old. The social and demographic features of Thailand make effective control of DHF a complex problem. This program is a long-range study to help in planning public health DHF control measures. An earlier report contains the results obtained from a study of acute and convalescent blood of clinical DHF cases (1). This report compares the results from the first two years of this surveillance program.

DESCRIPTION: In 1974, 70 provincial hospitals submitted acute and convalescent dried blood for testing compared to only 60 provinces in 1973. The methods for blood collection on filter paper discs and HI tests have been described (2).

PROGRESS: The localities of provinces and towns contributing to the study are shown in Figure 1. The total number of cases tested in 1974 was 2850, a 130% increase over the 1236 cases of 1973. At the same time, the total number of cases reported to the Ministry of Health was nearly the same for both years. It appeared that in 1974 more communities participated in the program and many provinces submitted more specimens for testing.

Dengue infections were confirmed for 491 patients in 1973 and 1042 in 1974, but the frequency of confirmation fell from 40% to 37%

Table 1. Results of HI Tests for Dengue Antibody - 1973, 1974

Region	No. Patients		Dengue Positive				Undetermined			
			1973		1974		1973		1974	
	1973	1974	No.	(%)	No.	(%)	No.	(%)	No.	(%)
North	249	419	77	(31)	419	(44)	40	(16)	2	(0.5)
Northeast	125	945	62	(50)	945	(26)	9	(7)	3	(0.3)
Central	689	1132	288	(42)	1132	(41)	120	(17)	9	(0.1)
South	173	354	64	(37)	354	(41)	9	(5)	3	(0.8)
Total	1236	2850	491	(40)	2850	(37)	178	(14)	17	(0.6)

Table 2. Results of HI Tests for JEV Antibody - 1973, 1974

Region	No. Patients		JEV Positive				Undetermined			
			1973		1974		1973		1974	
	1973	1974	No.	(%)	No.	(%)	No.	(%)	No.	(%)
North	84	81	22	(26)	21	(26)	10	(12)	0	(0)
Northeast	11	79	3	(27)	13	(16)	1	(9)	0	(0)
Central	17	113	5	(29)	32	(28)	0	(0)	2	(1.8)
South	14	26	1	(7)	6	(23)	0	(0)	0	(0)
Total	126	299	31	(25)	72	(24)	11	(9)	2	(0.7)

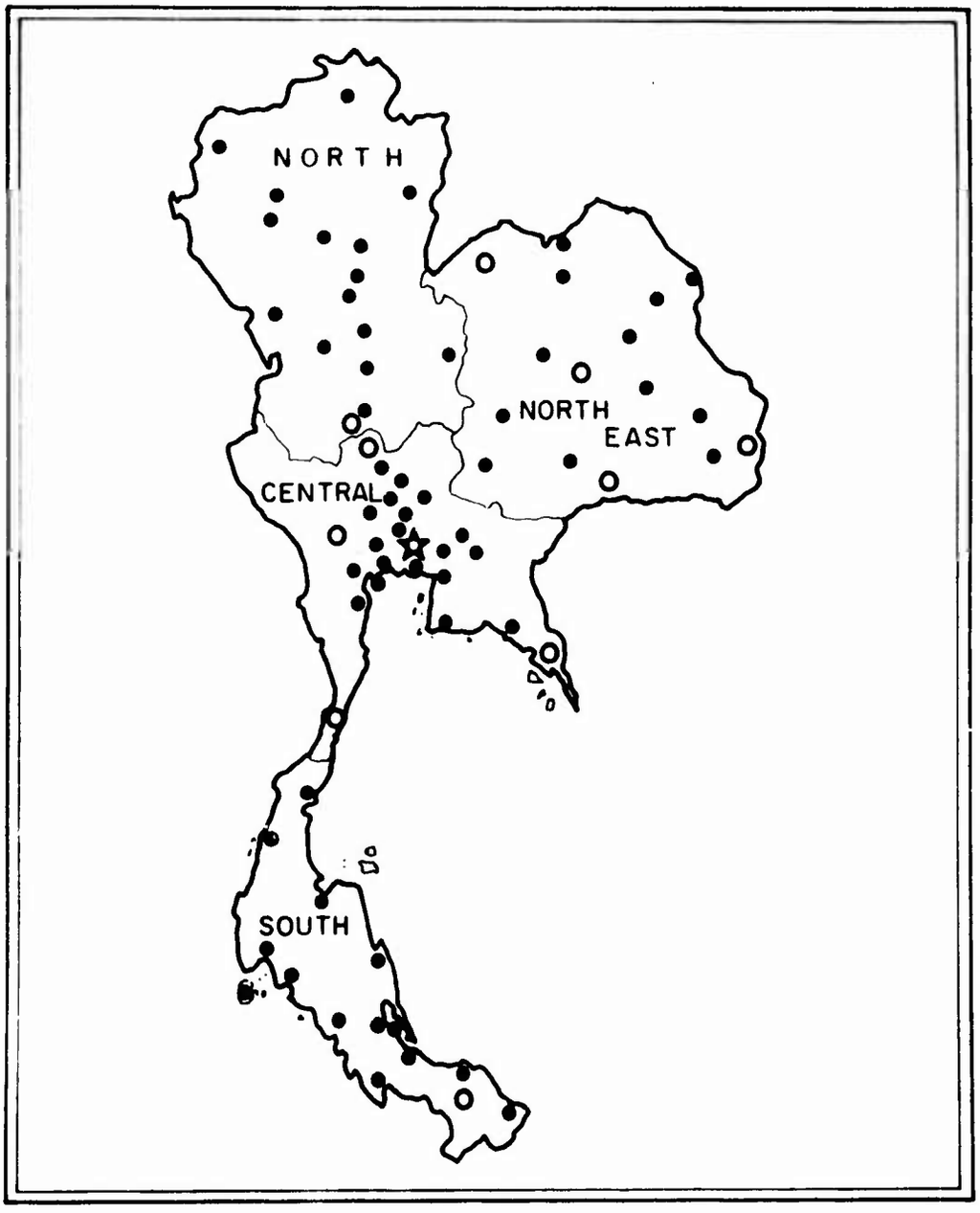

FIGURE I. MAP DEMONSTRATING PROVINCES OR TOWNS OF STUDY (●),(○)

● STUDY AREAS 1973 (60)
●○ STUDY AREAS 1974 (60 + 10)

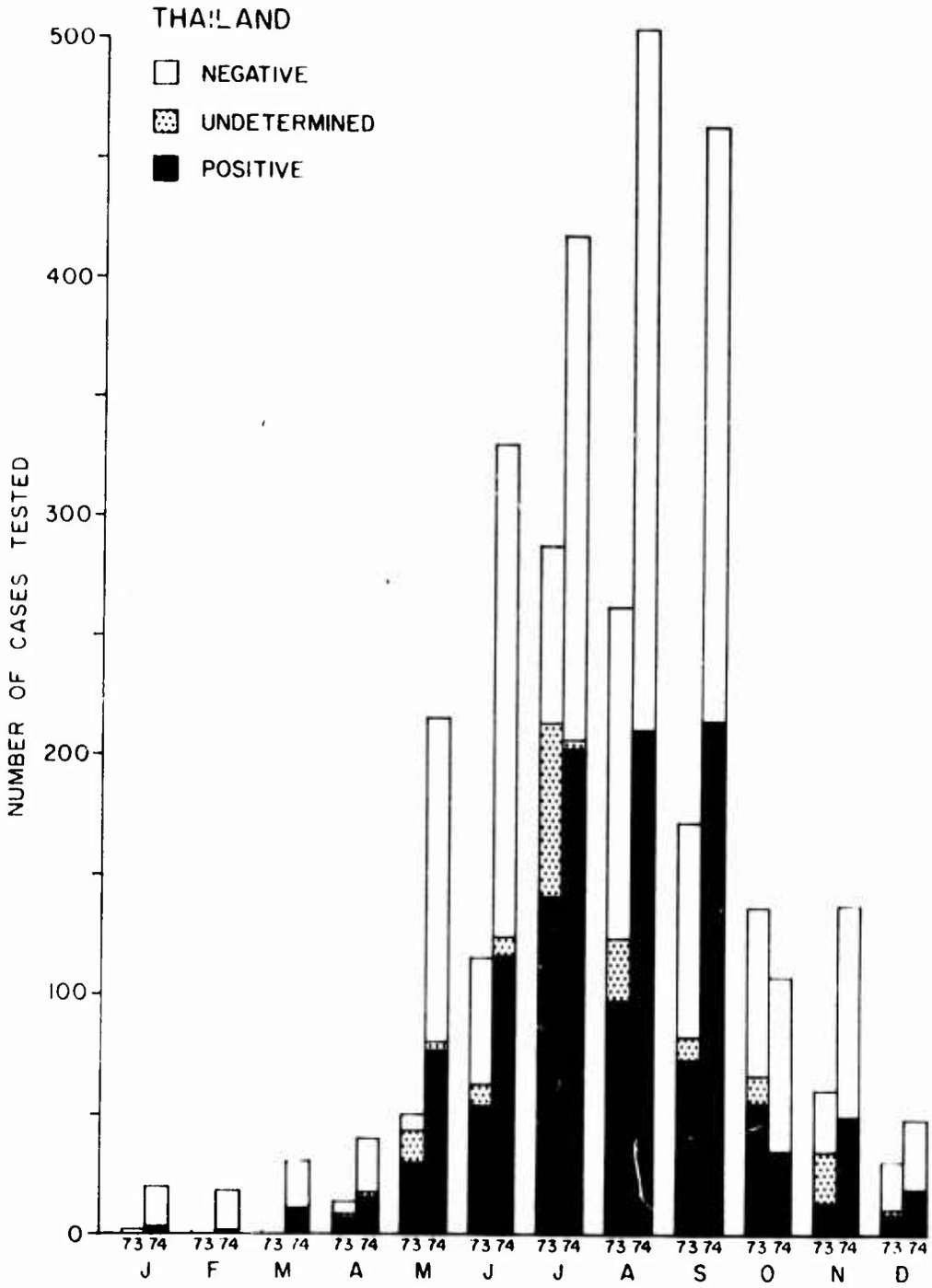

FIGURE 2. CONFIRMATION OF DHF CASES BY HI

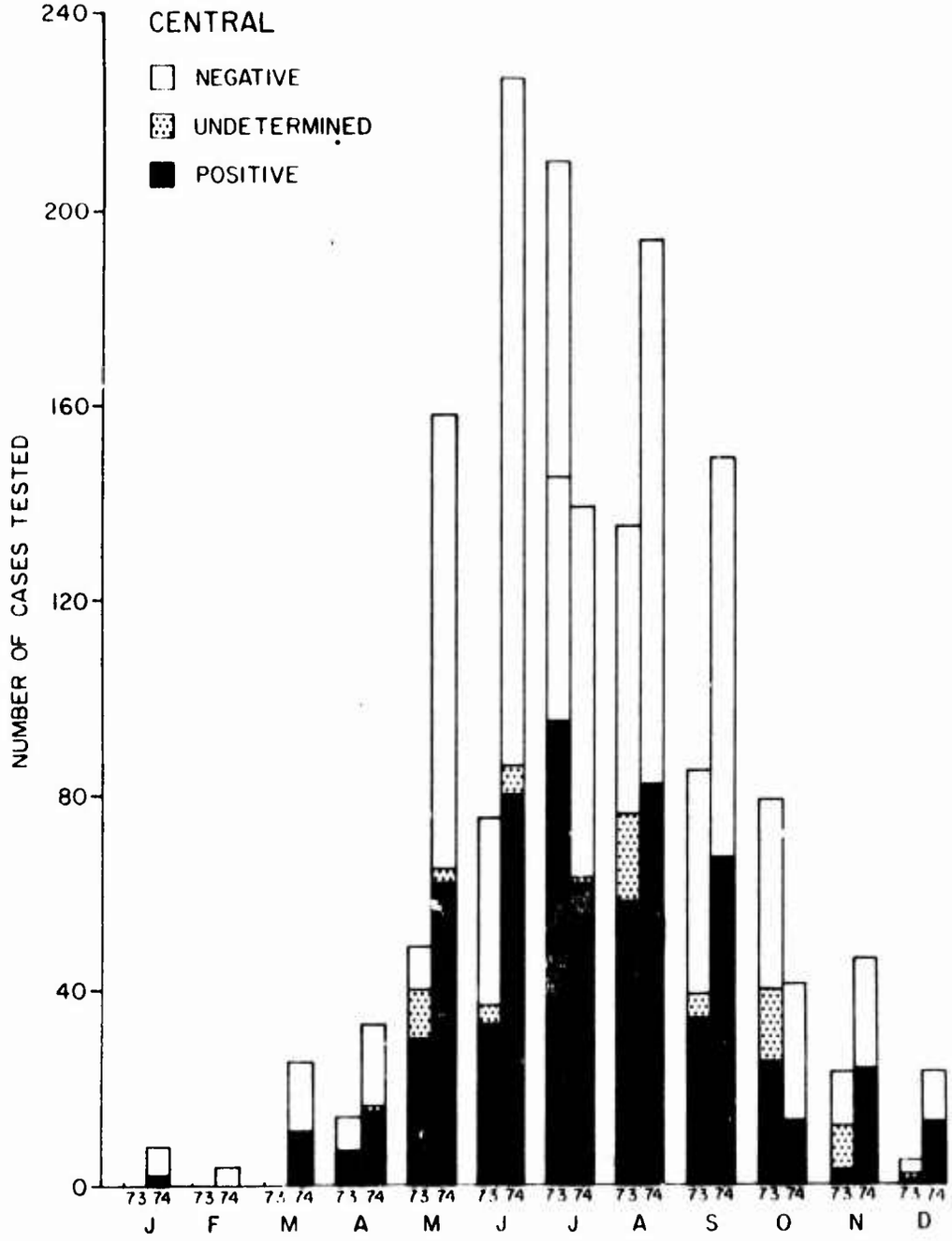

FIGURE 3. CONFIRMATION OF DHF CASES BY HI

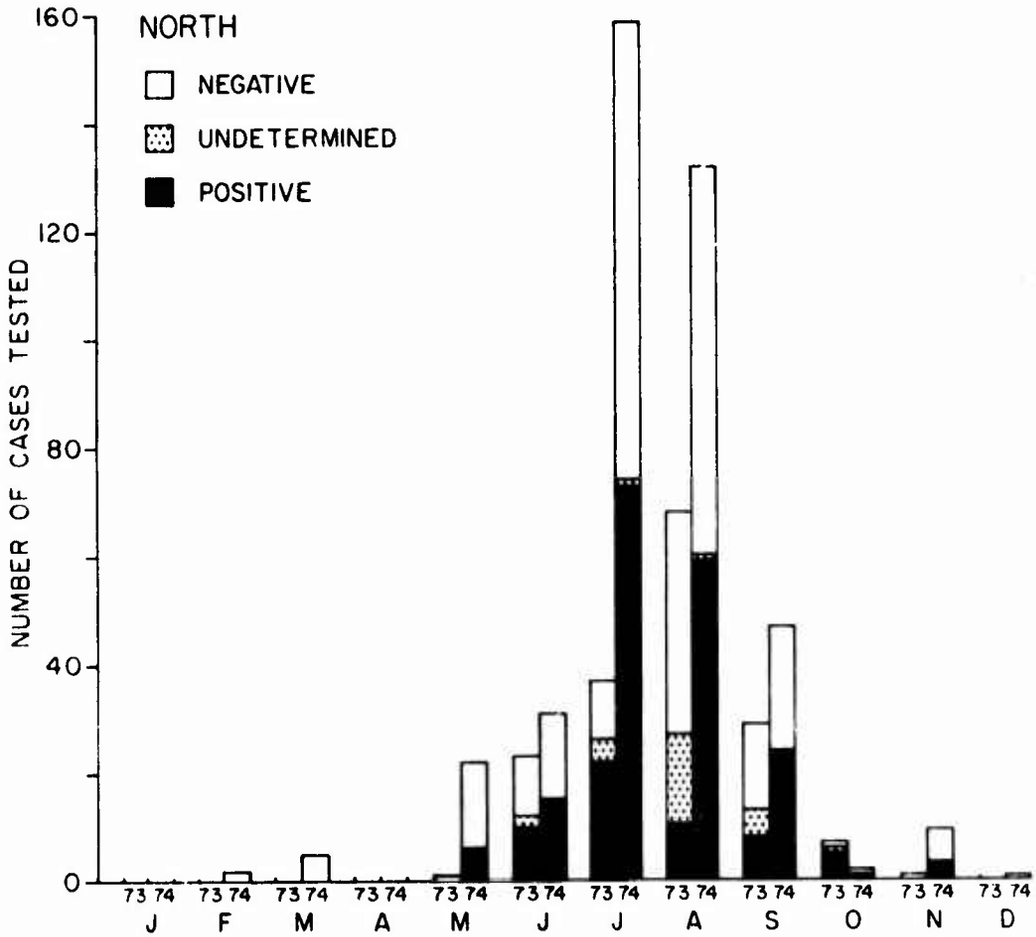

FIGURE 4. CONFIRMATION OF DHF CASES BY HI

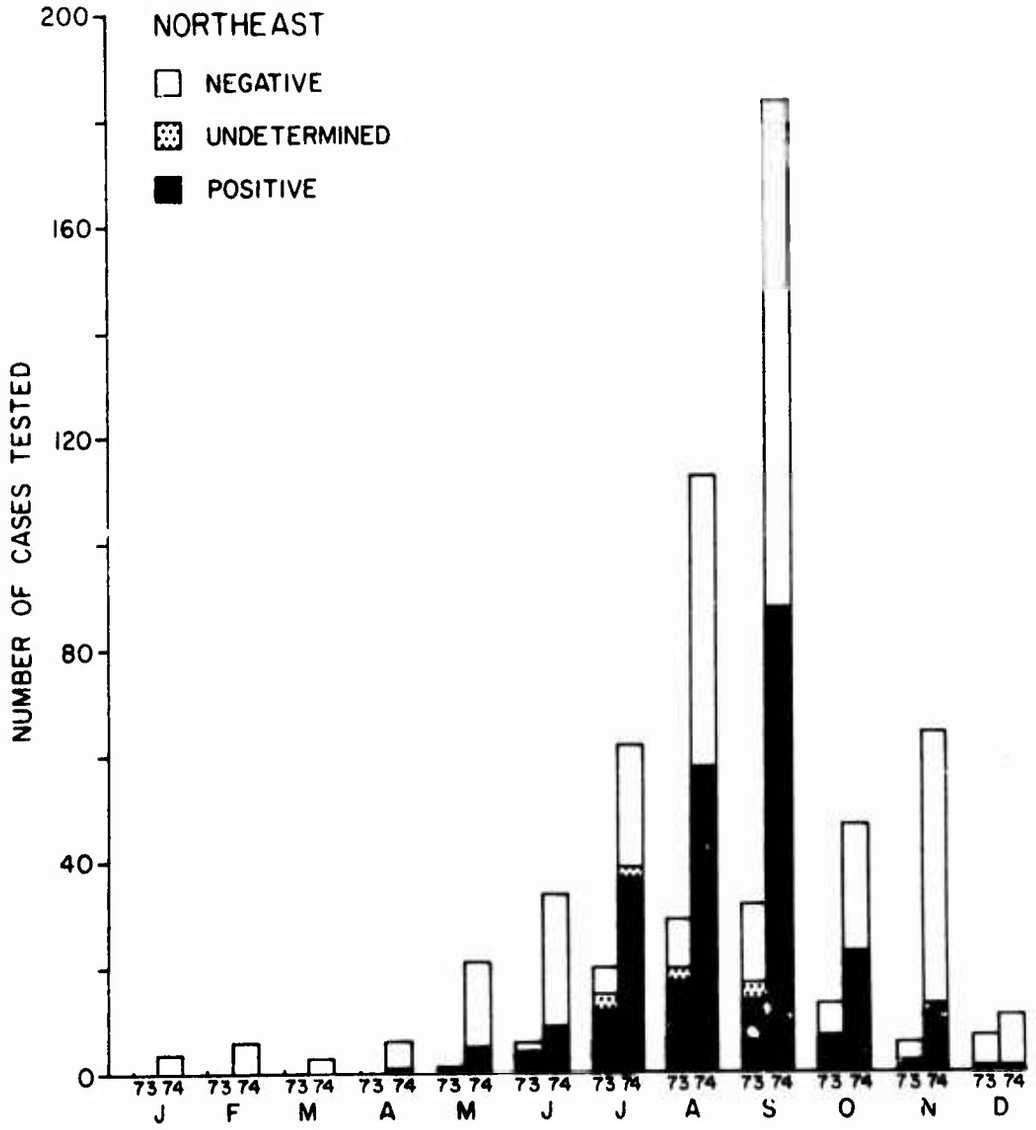

FIGURE 5. CONFIRMATION OF DHF CASES BY HI

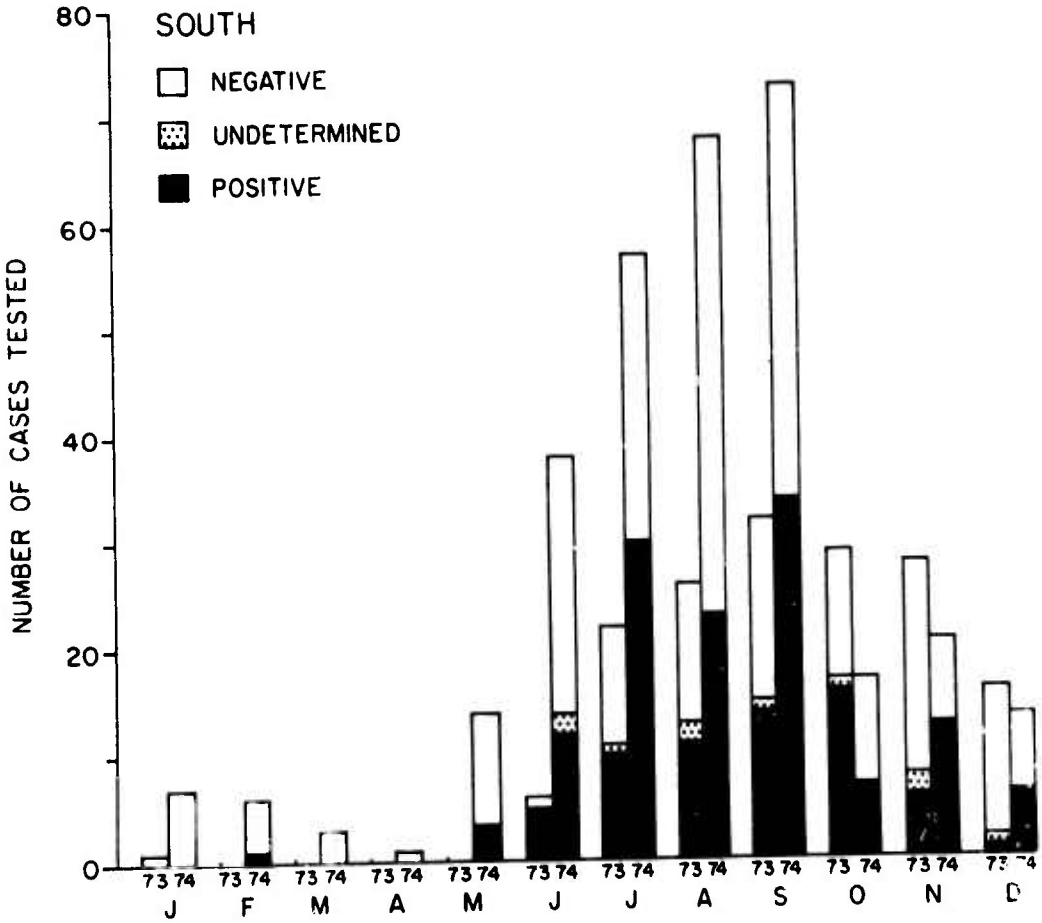

FIGURE 6. CONFIRMATION OF DHF CASES BY HI

since many more negative specimens were also obtained during the second year (Table 1). Specimens from 178 patients (13%) were unsatisfactory for testing in 1973, because of insufficient blood, incomplete clinical information, unpaired blood specimens or laboratory technical problems; these specimens were considered undetermined. In 1974, only 17 patients (0.6%) fell into the undetermined category. The striking decrease in the number of patients with undetermined results also reflects improved operation of the surveillance system.

Figures 2 to 6 illustrate the frequency of positive dengue cases by region and month. Although cases of dengue were confirmed for each month of 1974, the highest frequency of positive results was found during the usual epidemic season of May to November. Even then, positive cases rarely represented half of all of the cases tested. As seen in other reports, the epidemic period for the Central region was more prolonged than for the other regions (1).

The experience with testing encephalitis patients for JEV infection was similar to that with testing DHF patients. In 1974, more patients were submitted from all regions except the North (Table 2). The overall frequency of JEV infections was the same for both years, but the number of undetermined specimens fell substantially.

DISCUSSION: The results of 1974 support the value of the surveillance system. During the 1974 dengue season, the physicians in the provincial hospitals submitted more and better specimens from a greater number of provinces than in 1973. This suggests an increasing awareness of the system on the part of referring physicians and a favorable reaction on their part.

3. Dengue Virus Isolation from Human Plasma Inoculated Into Mosquitoes

OBJECTIVE: To compare the results of three techniques of dengue virus isolation from human plasma.

BACKGROUND: An earlier report (1) showed preliminary infection of *Aedes aegypti* mosquitoes with dengue seed virus followed by incubation for 10 days yielded 1 to 3 logs more virus per pool of mosquitoes than was inoculated. Pools of mosquitoes were ground and inoculated into LLC-MK_2 tissue culture for virus isolation and identification by a standard plaque assay. The preliminary results suggested this combined mosquito inoculation/tissue culture (MI/TC) assay was more sensitive than tissue culture alone, but information was needed on the usefullness of this technique for the isolation of dengue virus from human blood.

Table 1. Dengue HI Antibody Titers of Plasma Specimens Yielding Virus Isolates

Dengue Antibody Titer [a]	No. Tested	No. of Virus Isolates	
		SMRL [b] LLC-MK_2	PRS [c] MI/LLC-MK_2
<20	7	6	6
20	2	2	2
40	2		1
80	3		2
160	3	2	3
320	7		6
640	11		2
1280	7		1
2560	15		3
5120	11	1	2
10240	5		
≥20480	3		
TOTAL	76	11	28

a Reciprocal plasma dilution

b Virus isolates made at SEATO Medical Research Laboratory by direct inoculation of LLC-MK_2 tissue culture

c Virus isolates made at the Pacific Research Section by inoculation of mosquitoes before attempting virus isolation in LLC-MK_2 tissue culture

Table 2. Diagnosis of Patients Yielding Virus Isolates

Diagnosis	No. Patients	No. of Virus Isolates	
		SMRL [a] LLC-MK_2	PRS [b] Mosquito/LLC-MK_2
Dengue Fever	5	2	3
DHF grade 1	6	2	3
grade 2	31	4	11
grade 3	23	1	7
grade 4	10	2	4
unspecified	1	0	0
Total dengue	76	11	28
Non-dengue	25	0	0

a Virus isolates made at SEATO Medical Research Laboratory by direct inoculation of LLC-MK_2 tissue culture

b Virus isolates made at the Pacific Research Section by inoculation of mosquitoes before attempting virus isolation in LLC-MK_2 tissue culture

Table 3. Relative Number of Dengue Virus Isolates Obtained from 58 Human Plasmas by Three Techniques

PRS MI/TC[a]	SMRL MI/TC	SMRL TC[b]	No. Dengue Isolates
+	0	+	1
+	+	+	5
+	+	0	4
+	0	0	8
0	+	0	0[c]
18	9	6	18

+ means test positive; 0 means test negative

[a] Mosquito inoculation followed by tissue culture isolation technique

[b] Tissue culture only

[c] Five isolates obtained only by SMRL MI/TC have not yet been identified

DESCRIPTION: A collaborative prospective study was done to compare virus isolation results at SEATO Medical Research Laboratory (SMRL) to those at the Pacific Research Section (PRS) of the National Institute of Allergy and Infectious Diseases (NIAID) in Hawaii. Acute plasma samples collected as part of the overall studies of dengue hemorrhagic fever (DHF) at Children's Hospital, Bangkok in 1974 were divided into three identical aliquots and numbered sequentially. Aliquots were frozen promptly at -70°C and not thawed until virus isolation was attempted by: 1) standard plaque assay in LLC-MK_2 tissue culture (TC) cells at SMRL, 2) inoculation of pools of A. aegypti, incubation for 10 days, then standard plaque assay in LLC-MK_2 cells at SMRL (MI/TC), and 3) sent to PRS for isolation attempts by inoculation into A. albopictus followed by a plaque assay of individual mosquitoes in LLC-MK_2 cells. Virus isolates were identified at SMRL by plaque reduction neutralization tests (PRNT) using type specific hyperimmune mouse ascitic fluid (HMAF). Only the isolates from the standard plaque assay have been completed so far. Identification of isolates at PRS was accomplished by PRNT and complement fixation (CF) using inoculated mosquitoes as the CF antigen.

PROGRESS: There was a striking difference in the number of isolates by the two laboratories. The SMRL TC yielded 11 (14%) isolates from 76 patients compared to 28 (37%) at PRS by MI/TC. Only three SMRL isolates were recovered from plasmas with dengue HI antibody titers of 1:40 or greater (Table 1). On the other hand, fully half of the PRS isolates come from plasmas with HI antibody titers of 1:320 to 1:5120. The results suggest that one reason the MI/TC technique used at PRS recovered more virus isolates was that mosquitoes may be able to disassociate neutralizing antibody from infectious virus particles in the plasma. SMRL did not obtain any isolates by TC that were missed by PRS. There was some difference between the clinical diagnosis of the patients yielding virus isolates to either laboratory (Table 2). Many more isolates were obtained by PRS from patients with DHF grade 2 and 3 than SMRL; patients in these categories generally had higher levels of antibody in the acute plasma samples.

A comparison of the results from MI/TC at SMRL can only be made for 58 plasmas which were carried to completion. Of the 58 plasmas, 6 (10%) were positive by TC, 14 (24%) by MI/TC at SMRL and 18 (31%) by MI/TC at PRS (Table 3). Five plasmas yielded isolates by the SMRL MI/TC only; their identity is still in doubt. One plasma was positive by SMRL TC and at PRS but negative by MI/TC at SMRL. On the other hand, 4 plasmas yielded isolates by both mosquito inoculation techniques. Some of the differences in results are probably due to technical problems at SMRL that still need to be solved.

DISCUSSION AND SUMMARY: A collaborative study for the comparison of three techniques for dengue virus isolation was carried out with PRS/NIAID. Techniques using mosquitoes as amplifying hosts for dengue replication prior to isolation of virus in LLC-MK_2 cells yielded a greater number of isolates than a standard tissue culture plaque assay method. The greatest improvement in isolation results was found in plasmas containing dengue HI antibody at titers of 1:40 or higher. It is presumed that inoculation of plasma into mosquitoes permits dissociation of neutralizing antibody from some infectious virus particles. The benefit of mosquito inoculation is two-fold: 1) the mosquito may strip off interfering antibody, 2) the mosquito allows small amounts of virus to multiply to levels that can be more easily detected in a LLC-MK_2 tissue culture system. The mosquito inoculation step will be included in future attempts at dengue virus isolation.

4. Rapid Detection of Dengue Virus Antigen and Antibody by Counterimmunoelectrophoresis (CEP)

OBJECTIVE: To determine the ability of a CEP test to detect dengue antigen and antibody in human serum.

BACKGROUND: Because the manifestations of dengue hemorrhagic fever (DHF) develop very rapidly, a quick screening test for the detection of dengue antigen and antibody in patient serum would be beneficial to physicians. The use of CEP for making a diagnosis of other viral infections led to an attempt to apply the technique to dengue infections.

DESCRIPTION: A collaborative study was conducted in which sera from selected DHF patients in Children's Hospital collected and tested by virus isolation and hemagglutination inhibition tests in the Dept of Virology, SEATO Medical Research Laboratory (SMRL) were tested under code by CEP in the Dept of Pathobiology, Mahidol University. The CEP technique has been described (3). Antibody was detected using 20% suspensions of suckling mouse brain prepared at SMRL representing dengue types 1 to 4 and Japanese encephalitis virus. Dengue antigen was detected by screening with hyperimmune mouse ascitic fluid (dengue 1 and 4) and rabbit antisera (dengue 2 and 3). Few sera were tested with the anti-dengue 3 sera.

PROGRESS: Two to four sera were tested from 10 different patients with dengue infections documented by virus isolation (five cases), a four-fold rise in antibody (four cases) or a high fixed antibody titer (one case). In every patient, the first serum specimen gave a positive reaction for antibody with at least one dengue antigen, whether or not virus was isolated or antibody was detected by

hemagglutination inhibition. On the other hand, antigen was detected by a reaction with dengue antibody in only three acute specimens; dengue virus was isolated from all three (Table 1). The results suggest CEP may be able to detect dengue antigen in some viremic patients but may not be specific enough for the detection of dengue antibody.

Table 1. Detection of Dengue Antigen in Viremic Human Serum

Patient No.	Virus Isolate	CEP Reaction with Type of Antibody			
		Dengue-1	Dengue-2	Dengue-3	Dengue-4
73-9	D1	0	0	ND	0
73-17	D3	0	0	+	0
73-19	D3	0	0	ND	0
73-68	D2	0	0	ND	+
73-41	D2	+	+	ND	0

D = Dengue ND = Not done

5. The Pathogenesis of Dengue Hemorrhagic Fever. The Role of Biological Mediators: Histamine and Serotonin

OBJECTIVE: A study of biological mediators of shock in dengue hemorrhagic fever.

BACKGROUND: Dengue hemorrhagic fever (DHF) differs from dengue fever in the development of hemorrhagic phenomenon (a positive tourniquet test), "hypotension," (a low blood pressure and/or narrowed pulse pressure), a decrease in plasma volume, (a rising hematocrit) and thrombocytopenia (a rapid drop in platelet count). The differences appear to be related to the formation of antigen antibody complexes (4), and it has been suggested that this is related to an individual experiencing a second dengue infection (5). These changes have a rapid onset suggesting the involvement of short-lived biochemical mediators.

At least two phenomena have been observed during the development of this illness which may be the source of these mediators. Observations suggest that there is activation of the complement system by antigen-antibody complexes with liberation of pharmacologically active components C3A and C5A (6). These low molecular weight polypeptides are potently vasoactive and their

release leads to a marked increase in vascular permeability (7). They may act directly on the vasculature or by liberating histamine, slow reacting substance (SRS-A) and/or heparin from mast cells and white blood cells.

The other phenomenon is the decrease in platelets (8). Platelet counts often fall in a matter of hours from a normal level of approximately 250,000/mm^3 to as low as 10,000/mm^3. Platelets also return rapidly to supra-normal levels during the recovery phase (1). As the half life of the platelet is only two to three days this suggests that there is acute lysis of many platelets with supra-normal replacement from a hyperactive bone marrow. A similar but less marked phenomenon has also been noted with white blood cells (8).

The reason for the acute decrease in platelets is not clearly understood and requires further study. The lysis of platelets may lead to the sudden release of several potent vasoactive agents. Histamine, serotonin and heparin are all found in platelets and could be released into the circulation.

C3A and C5A are labile polypeptides and cannot yet be accurately measured (9). SRS-A which is released from mast cells, has not been characterized and can only be measured inaccurately by a biological assay (10). Heparin is stored in both platelets and mast cells; it also is difficult to measure in serum.

Serotonin is manufactured in fairly large amounts in the chromaffin cells of the gastrointestinal tract. This vasoactive amine has a rapid turnover time. Most of the serotonin manufactured appears as metabolites in the urine within 24 hours; however, a small amount of it is taken up and stored in platelets (11). In man no serotonin is found in mast cells (12). The major metabolite of serotonin, 5-Hydroxyindolacetic acid (5 HIAA), can be measured in the urine by colorometric analysis.

Histamine is a major storage product of mast cells with a small amount being stored in platelets (11). In the past it could be measured only by biological assay but recently a sensitive radio-enzymic assay has been developed (13). The purpose of this study was to determine whether the excretion of histamine or 5 HIAA increased in the urine during the development or the course of DHF as compared to other febrile diseases and normal controls.

DESCRIPTION: This study was an integral part of other dengue studies performed in collaboration with the Children's Hospital of Bangkok during 1974. During the dengue epidemic season from 24 June to 26 August 1974 all patients admitted to the ward

service with an admission diagnosis of DHF and appropriate febrile and afebrile controls were accepted for study. Blood samples were taken daily in heparinized syringes for the first five days in hospital and then approximately 15 and 30 days following hospitalization. Plasma was submitted for virus isolation using a direct and delayed plaque technique previously described and a mosquito isolation technique (see elsewhere this report). Plasma was also used for the detection of antibodies to dengue types 1-4 and Japanese Encephalitis virus using a hemagglutination inhibition (HI) technique. Urines, collected over either 8 or 12 hour periods, were obtained from each patient in plastic bottles and stored on wet ice. At the conclusion of the collection period these were divided into aliquots in plastic containers using toluene as a preservative and frozen at -70°C. A white blood count, differential count, platelet count and hematocrit were performed on each blood obtained. The clinical status of each patient was assessed by a physician at least once every 12 hours and usually more often. All clinical and laboratory details were recorded on a flow sheet, which was kept on each case.

Patients to be further studied for the presence of biological mediators were selected retrospectively after all clinical and laboratory information was evaluated.

PROGRESS: During this two month period at the height of the dengue season only 64 patients with an admission diagnosis of DHF were collected. This however represented almost 50% of the dengue patients collected through the entire year, as there was a low incidence of disease during 1974. Of these 64 patients, 11 or 18% did not have laboratory evidence of dengue virus infection, one patient was not adequately followed and three patients died. From the three patients who died no convalescent sera was obtained and therefore no judgement could be made on the patient's prior experience with dengue. Table 1 shows a breakdown on the clinical grading and type of convalescent antibody response seen in the 49 patients who were studied.

Of these patients five were selected who exhibited DHF grades 1 or 2 and five were selected who exhibited DHF grades 3 or 4. Three patients with bacterial infections and five non-infected children were selected as controls. All urine samples collected on these children from the time of admission until two days after the period of shock were submitted for biochemical analysis of creatinine, 5 HIAA and histamine. The 5 HIAA and creatinine assays were done at SEATO Medical Research Laboratory. Urine for histamine analysis was forwarded through Walter Reed Army Institute of Research to Dr. Michael Beaven at the NIH in Bethesda, Maryland to be tested for histamine by radioenzymic assay. When all of the

assays are completed the data will be examined to determine whether the excretion of histamine or serotonin breakdown products was related to the development or severity of DHF.

Table 1. Clinical Grading and Type of Convalescent HI Antibody Responses Seen in 49 Dengue Patients Collected Between June 24 and August 26, 1974.

Grade of Disease	Primary Infection $\leq$ 1:640	Secondary Infection > 1:640
UF*	1	3
1 & 2	5	22
3	2	12
4		4
TOTAL	8	41

* Undifferentiated fever

B. HEPATITIS B VIRUS

1. Hepatitis B Virus Infections in Americans in Southeast Asia

OBJECTIVE: To determine the epidemiology of hepatitis in American military personnel exposed to populations with endemic hepatitis and a high prevalence of HB_sAg carriers.

BACKGROUND: Until recently only historical evidence was available to document infection with agents causing viral hepatitis. In the past 10 years, however, investigations of hepatitis B, initially stimulated by the discovery of hepatitis B surface antigen, have provided serological evidence of infections with hepatitis B virus in a number of populations. In tropical Southeast Asia studies at the SEATO Medical Research Laboratory have documented serological evidence of prior HBV infection in up to 75% of Bangkok residents and a carrier prevalence of HB_sAg of approximately 9% (14). In recent years a large number of Americans, largely military personnel, have been stationed in Southeast Asia. These

Americans came from an area in which HB_sAg is found in only 0.1 to 1.0% of the population and evidence of prior HBV infection in only 5 to 20% (15, 16).

A study of Americans entering and leaving the Republic of Vietnam in 1970 showed that troops arriving for an initial tour and those leaving after approximately one year had had equal experience with hepatitis B virus (Table 1). Troops arriving in Vietnam for a subsequent tour, however, had significantly greater experience with hepatitis B virus suggesting that exposure to hepatitis B virus was considerably increased in Southeast Asia. This study was designed to determine the environmental and host factors which lead to the development of clinical and subclinical hepatitis among American troops in Southeast Asia.

DESCRIPTION: A description of the design of this study appeared in the SEATO Medical Research Laboratory Annual Report 1973-1974. Briefly the population studied was drawn from servicemen aged 18-25 years in grades E1-E5 entering either the United States Army Support Group, Thailand or the United States Air Force 635th Combat Support Group. Shortly after arrival in Thailand a questionnaire was administered to these men to determine, among other things, their previous duty station, previous tropical experience and their prior experience with hepatitis. During the ensuing year these men were interviewed three times at approximately four month intervals. The interviews contained questions

Table 1. Evidence of Hepatitis B Virus Infection in American Military Personnel in the Republic of Vietnam

Military Personnel	HB_sAg	Anti-HB_s
Inprocessing		
Initial tour	0.39% (4/1004)	3.50% (7/200)
Subsequent tour	2.42% (7/289)	11.28% (22/195)
Outprocessing	0.46% (5/1072)	3.1% (6/189)
TOTAL	0.68% (16/2865)	6.0% (35/584)

of social behavior and medical experience. Serum samples were collected at the time of each interview and were submitted for detection of hepatitis B surface antigen (HB_sAg) by complement fixation (CF), immunoelectrophoresis (IEOP) and radioimmune assay (RIA). Antibodies against hepatitis B surface antigen (anti-HB_s) were detected by a radioimmune assay inhibition (RIAI) and confirmed and titered by a passive hemagglutination test (PHA). Methods for these assays have appeared elsewhere (SEATO Medical Research Laboratory Annual Reports 1971-1972, 1972-1973 and 1973-1974).

PROGRESS: Subjects were enrolled in this study between April and December 1972. Initial questionnaires were completed in December 1971. The first of three follow-up interviews was completed in April 1973, the second in August 1973 and the third in December 1973. The three follow-up interviews and bleeds were divided according to timing into groups. The time of the first interview fell between 12 and 24 weeks (3 and 6 months), the second between 24 and 38 weeks (6-9.5 months) and the last between 39 and 65 weeks (9.5-15 months). Individuals whose interviews fell outside of these time periods were excluded from the study. With these stipulations, there were 418 individuals who completed the initial questionnaire and from whom blood was obtained. Of these, 385 (92%) were seen at the first follow-up, 317 (76%) at the second and 326 (78%) at the third. Two hundred and seventy-one people (61%) were completely followed with all three interviews and bleeds. Table 2 shows the prevalence of past experience with hepatitis B virus at each bleed and the incidence of infection for the period of time from the first bleed. Table 3 shows similar data for 271 people who were completely followed with all three interviews and four bleeds.

Table 2. Experience* with Hepatitis B Virus in American Enlisted Men at Three Month Intervals Throughout One Year's Tour in Thailand

Bleed	1st	2nd	3rd	4th
Weeks in country	0	13-25	26-39	40-65
Total number studied	418	385	316	326
Persistant evidence	-	16 (4.2%)	15 (4.8%)	14 (4.3%)
Incidence	-	8 (2.0%)	12 (3.7%)	17 (5.2%)
Prevalence	18 (4.3%)	24 (6.2%)	27 (8.5%)	31 (9.5%)

* Experience is determined by presence of HB_sAg or anti-HB_s.

Table 3. Experience with Hepatitis B Virus in 271 American Enlisted Men Completely Followed Throughout One Year's Tour in Thailand

Bleed	1st	2nd	3rd	4th
Weeks in country	0	13-25	26-39	40-65
Persistant evidence	-	13 (4.7%)	13 (4.7%)	13(4.7%)
Incidence	-	7 (2.5%)	12 (4.4%)	16(5.9%)
Prevalence	13 (4.5%)	20 (7.3%)	25 (9.2%)	29 (10.7%)

Three hundred and twenty-six individuals were followed with at least a first and a fourth bleed. At the risk of falsely inflating the prevalence and incidence of hepatitis B infection, six additional individuals were added. These six men were followed with at least two bleeds; three had evidence of prior HBV infection on the first bleed and three developed antibody during the study. Table 4 illustrates the number of HBV infections recorded in these 332 men over the one year period.

Table 4. Hepatitis B Infections Recorded in 332 American Enlisted Men Followed Through One Year's Tour of Duty in Thailand

HBV Serology	Follow-up Blood Sample				
	1st	2nd	3rd	4th	Total
HB_sAg positive	2 (0.6%)	1 (0.3%)	3 (0 9%)	4 (1.2%)	10 (3.0%)
Anti-HB_s positive	16 (4.8%)	7 (2.1%)	5 (1.5%)	5 (1.5%)	33 (9.9%)
Total HBV Experience (HB_sAg + Anti-HB_s)	18 (5.4%)	8 (2.4%)	7*(2.1%)	8*(2.4%)	41 (12.3%)

*Two persons who developed antigen followed by antibody are counted only once in the total HBV experience.

Six clinical cases of hepatitis were diagnosed in these 332 men during the periods between bleeds (Table 5). There were 4 individuals in whom clinical hepatitis was associated with HBV. No HB_sAg was identified in one of these but anti-HB_s developed in the convalescent period by the time of the second bleed. In the second case, HB_sAg was detected in the second blood specimen at the time of clinical disease and anti-HB_s was found in the third. The two remaining cases were diagnosed in the third period; in both of them HB_sAg was detected in the fourth blood sample. No further follow-up samples were obtained from either of them. There were two cases of hepatitis diagnosed with no detectable evidence of HBV infection. These are listed as HBV non-associated hepatitis; however, these individuals may have had HBV infections which might have been detected by more sensitive tests.

In this group of 332 men, HBV associated hepatitis was diagnosed in four of them. Serological evidence of inapparent infection was documented in an additional 19 men. The incidence of HBV infection over the one year period was 23/332 or 6.9% and the apparent to inapparent infection ratio was 4:19.

In analysing these figures, those men who were entering a tropical area for the first time were compared to those who had prior experience in the tropics. Tables 6 and 7 document the differences seen in these two groups.

Table 5. Clinical Hepatitis Infections Recorded in 332 American Enlisted Men Followed Through One Year's Tour of Duty in Thailand

Type of Hepatitis	Four Month Interval			
	1	2	3	Total
HBV Associated	1	1	2	4
HBV Non-associated	1	1		2
Total Clinical Hepatitis	2	2	2	6

These data have been coded for computer analysis. Cross tabulations of hepatitis experience with variables, such as drug use, mixing with the indigenous population and sexual experience will

Table 6. Prevalence of Demonstrable Experience with Hepatitis B Virus on Entering Thailand: A Comparison of Those With and Without Prior Experience in a High Prevalence Area

Prior Experience	Number of Men	HB_SAg	Anti-HB_S	Total HBV Experience
Yes	119	2[a] (1.6%)	11 (10.1%)	13 (10.9%)
No	213	0 (0.0%)	5 (2.3%)	5 (2.3%)
Total Population	332	2 (0.6%)	16 (4.8%)	18 (5.4%)

a. One man had HB_SAg with a complement fixing titer of 1:64 and carried it throughout his stay in Thailand. The carrier rate = 0.3%

Table 7. Incidence of HBV Infection During a One Year Tour in Thailand: A Comparison of Those With and Without Prior Experience in a High Prevalence Area

Prior Experience	Number of Men Susceptible	HB_SAg	Anti-HB_S	Total HBV Experience
Yes	105	1 (1.0%)	7 (6.6%)	8 (7.6%)
No	208	5 (2.4%)	10 (4.8%)	15 (7.2%)
Total Population	313	6 (2.0%)	17 (5.4%)	23 (7.3%)

be computed to determine if hepatitis in these troops is associated with any identifiable behavioral pattern.

DISCUSSION: In a group of 332 young American military personnel followed in Thailand, clinically recognizable hepatitis occurred in six (18/1000), over a one year period. Of these six, four (12/1000) had detectable serological evidence of association with hepatitis B surface antigen, the remaining two did not. Screening of these men for the development of HB_sAg or anti-HB_s revealed an additional 19 who had inapparent hepatitis B infection. Thus for HBV infections the apparent: inapparent ratio was as high as 4:19 or nearly 1:5. In other words, 82% of all HBV infections were asymptomatic.

The men in this study demonstrated a direct relationship between previous tropical experience and prior HBV infection as was found in the earlier study in Vietnam. In men without serological evidence of prior HBV infection, however, the incidence of new infection during the current study period was the same, whether or not they had previous tropical experience.

Information on the association of hepatitis B virus infection with social and physical behavior has not yet been analyzed. It is possible that routes of transmission of hepatitis B virus between indigenous populations and United States military personnel may be demonstrated and ways of preventing this infection may be suggested.

2. Anti-Hepatitis B Serum Production in Laboratory Animals

OBJECTIVE: To produce antisera to Hepatitis B surface antigen (HB_sAg) for use in the determination of antigen subtypes.

BACKGROUND: This is a continuation of work which was previously reported (1). The detection of subtypes of Hepatitis B surface antigen requires antisera containing specific antibodies to each subtype determinant. Previously these antisera were prepared using rabbits, who were exanguinated six weeks after the initiation of the immunization procedure (17). This method of preparation produced antisera with a high titer against homologous antigen; however, because of impurities in the original immunogen, the antisera were often contaminated with anti-human serum protein activity. This anti-human serum activity interfered with immunodiffusion (ID) tests (18) and required absorption with normal human sera. The present study was designed to determine if subtype specific antisera free of anti-human serum activity could be produced in rabbits by selecting the time of bleeding.

DESCRIPTION: Rabbits of 2.5 to 4.0 Kg bodyweight were used. Five milliliters of blood were taken from the peripheral ear vein of each rabbit before incubation and nearly every week post inoculation. Anti-HB_s activity as well as anti-human serum protein activity were tested by immunoelectroosmophoresis (IEOP) and titered by complement fixation test (CF). A cesium chloride purified fraction of HB_sAg/adr (EH-17) prepared by Electronucleonic, Inc., Bethesda, Maryland was emulsified with an equal volume of Freund's complete adjuvant (17). Four rabbits free from anti-HB_s activity were inoculated with 0.25 ml of antigen-adjuvant emulsion, intradermally, into each of four sites on the thighs and back. An identical dose of the same antigen was given four weeks later. Anti-HB_s and anti-human serum protein activities were studied once a week from two to ten weeks.

All blood was tested for anti-HB_s and anti-human serum protein activities by IEOP and CF. The titer of these antibodies was determined by CF and the specificity of the antisera was identified by ID.

PROGRESS: Four rabbits were immunized with HB_sAg/adr (EH-17). Of these four, one died of unknown cause four weeks after immunization. In the remaining three, antibodies to HB_sAg/adr with CF titers of 1:2 to 1:16 had appeared by the first bleed, two weeks after immunization (Table 1 and Figure 1). CF titers of antibodies increased slowly reaching 1:8 to 1:32 by the fourth week just prior to receiving the booster dose of immunizing antigen. Antisera reached its maximum titer of 1:64 to 1:128 by five weeks, one week after the booster dose. After the fifth week the titer remained stable until the rabbits were exanguinated at 8-9 weeks.

The specificity of individual rabbit sera were determined by ID test. Two rabbit sera (R26 and R28) formed definite precipitin lines with reference antigens, but only specific d spurs were observed (Table 1 and Figure 2) suggesting they contained only ad subtype specific determinant antibodies. Another rabbit serum (R29) gave specific reactions with the reference antigens, with both d and r spurs, suggesting it contained subtype specific a, d and r antibodies.

Antibody to normal human serum protein was observed only transiently and at low titer (Table 1 and Figure 1). The interference of anti-human serum protein disappeared in ID test at 7 to 8 weeks post-inoculation. Anticomplementary activity of all three rabbit antisera was minimal and did not interfere with the interpretation of the tests.

Table 1. Production of Anti-HB_sAg/adr in Rabbits

Rabbit No.	Time After Immunization	Anti-HB_s				Anti normal human protein		
		Immunodiffusion		IEOP	CF Titer	Immunodiffusion	IEOP	CF Titer
		d Spur	r Spur					
R 26	Pre-immunization	ND*	ND	-	ND	ND	-	ND
	2 weeks	-	-	+	1:2	-		<1:2
	3 weeks	ND	ND	+	1:4	ND		<1:2
	4 weeks	ND	ND	+	1:8	ND		<1:2
	5 weeks	+	-	+	1:64	+		1:2
	6 weeks	+	-	+	1:32	+		<1:2
	7 weeks	+	-	+	1:32	+		<1:2
R 28	Pre-immunization	ND	ND	-	ND	ND	-	
	2 weeks	-	-	+	1:8	-		<1:2
	3 weeks	ND	ND	+	1:16	ND		<1:2
	4 weeks	ND	ND	+	1:16	ND		<1:2
	5 weeks	+	-	+	1:128	+		1:2
	6 weeks	+	-	+	1:64	+		1:4
	7 weeks	+	-	+	1:64	+		1:4
	8 weeks	+	-	+	1:64	-		1:2
	9 weeks	+	-	+	1:64	-		1:2
R 29	Pre-immunization			-	ND	ND	-	ND
	2 weeks	-	-	+	1:16	-		<1:2
	3 weeks	ND	ND	+	1:16	ND		<1:2
	4 weeks	ND	ND	+	1:32	ND		<1:2
	5 weeks	+	+	+	1:128	+		1:2
	6 weeks	+	+	+	1:128	+		1:4
	7 weeks	+	+	+	1:128	-		<1:2
	8 weeks	+	+	+	1:128	-		<1:2
	9 weeks	+	+	+	1:128	-		<1:2

Note: ND* = Not done

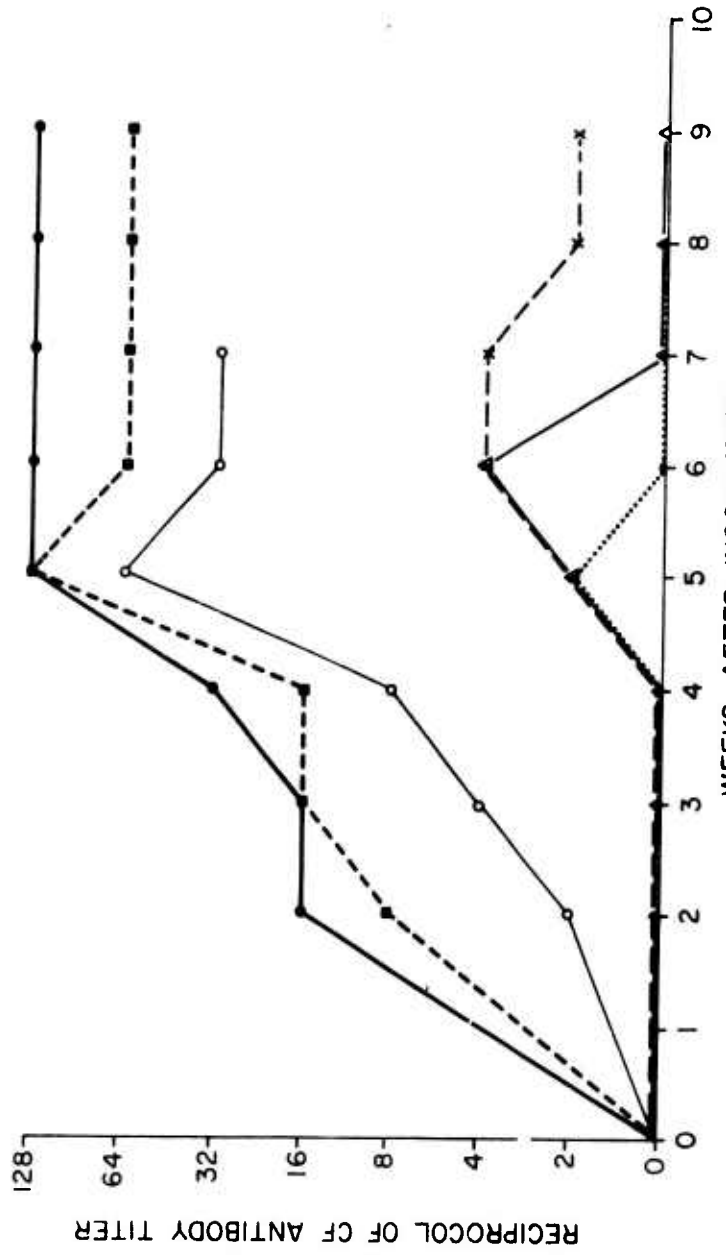

FIGURE 1. Anti-HB_s and anti-human protein responses in rabbits immunized with purified HB_sAg/adr (EH-017).

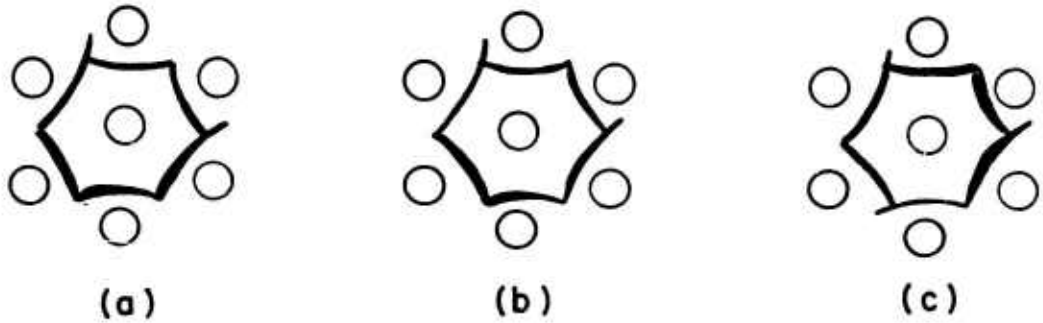

Figure 2. Patterns of immunodiffusion reactions observed with reference HB_sAg. In each pattern, reference HB_sAg/ayw were placed in the top and right upper wells, HB_sAg/adr in right lower and bottom wells and HB_sAg/adw in left upper and left lower wells. The central wells contain: (a) R. 26 anti-adr, (b) R. 28 anti-adr, and (c) R. 29 anti-adr rabbit sera.

DISCUSSION: The method of immunization with HB_sAg/adr in rabbits proved to be satisfactory for the production of subtype specific antisera in one (R29) out of three rabbits. In the other two, (R27 and R28) a strong d spur was produced but no easily discernible reaction with the r antigen was observed. In R29, anti-human serum activity in ID tests disappeared by the seventh week. This allowed the use of antiserum from this rabbit in ID test without preabsorption with normal human serum.

3. Radioimmune Assay Inhibition Test for the Detection of Antibody to Hepatitis B Surface Antigen

OBJECTIVE: To compare the radioimmune assay inhibition (RIAI) test for antibody to hepatitis B surface antigen (anti-HB_s) using two radioimmune assay (RIA) techniques.

BACKGROUND: The RIAI for anti-HB_s was developed in this laboratory using the Ausria I RIA kit manufactured and sold by Abbott Laboratories (SEATO Medical Research Laboratory Annual Report 1973-1974). To the procedure for the Ausria I, an initial absorption step was added. The sera, whose antibody content was to be determined, was used to absorb a standard amount of antigen. A change in the technical aspect of this test was brought about by the discontinuation of production of the Ausria I kit and its replacement by the manufacturer with a newly developed Ausria II kit. This change in material necessitated a series of comparative tests to insure that the results of the RIAI based upon the Ausria II ($RIAI_{II}$) were comparable to those based upon the Ausria I ($RIAI_I$).

DESCRIPTION: In February 1975 the Ausria I kit on which the $RIAI_I$ was based was withdrawn from the market. A final order of Ausria I kits and an equal number of the replacement Ausria II kits were provided by the manufacturer for cross testing and standardization.These kits were used to compare both the RIA and the RIAI tests in our laboratory.

The major technical difference between the Ausria I and the Ausria II was a change in the antibody carrier from a polystyrene tube to a polystyrene bead. The technique for the $RIAI_I$ was presented in last year's annual report. The initial absorption technique for the Ausria II is briefly reported: the RIAI was performed using a standard dilution of serum containing a known amount of antigen. Exactly 0.1 ml of this antigen dilution was incubated with 0.1 ml of each serum to be tested. After 1 1/2 hours incubation a polystyrene bead was introduced into the well and submerged in the mixture. From this point the test was performed using the directions for the Ausria II kit provided by the manufacturer and is essentially the same as with the Ausria I.

Included in each test run were seven negative controls, testing a pool of human serum shown to have neigher HB_sAg or anti-HB_s activity. The standard antigen dilution which was used as maximum for the RIAI, was also tested in seven replicates. The number of counts per minute (CPM) in each test was determined using a gamma ray spectrometer.

The percent radioimmune assay inhibition (% RIAI) was calculated using the following formula:

$$\frac{D - X}{D} \times 100 = \% \text{ RIAI}$$

D is the mean of the CPM of the standard antigen dilution from which the mean of the negative controls had been substracted and X is the CPM of the serum-antigen mixture following a similar manipulation.

Data from the $RIAI_I$ and $RIAI_{II}$ were compared on the basis of the percent RIA inhibition. Also, a positive or negative score was assigned to each test using 50% inhibition as a cut-off point for differentiating positives from negatives.

PROGRESS: In order to establish the appropriate dilution of antigen to use in the RIAI tests, antigen extinction curves using a sera containing HB_sAg/adw were run using both RIA tests. The results were found to be almost identical (Figure 1). Dilutions of 1:400 and 1:800 were selected as candidates for the standardized antigen for use in the RIAI tests. These dilutions were selected because they showed 50% or less of the CPM of the highest counting antigen dilution and were located on the steepest part of the antigen dilution curve. In this part of the curve, small changes in the concentration of antigen in the substance tested should lead to large changes in the amount of I^{125}-labelled antibody complexed to the antigen.

Dilutions of serum known to contain a high titer of anti-HB_s (Serum PT) were tested by both tests using dilutions of HB_sAg/adw of 1:400 and 1:800. Figure 2 illustrates the percent RIAI of the dilutions of antibody using a 1:800 dilution of the HB_sAg/adw antigen. Use of the 1:400 dilution of the HB_sAg/adw antigen produced similar curves with both tests but the RIA was inhibited by lower antibody dilutions and therefore was less sensitive. For this reason a 1:800 dilution of HB_sAg/adw was chosen as the standard antigen dilution for the RIAI with both the Ausria I and the Ausria II kits.

A panel of 100 sera from a Thai population known to have a high prevalence of anti-HB_s was tested by RIAI using both techniques

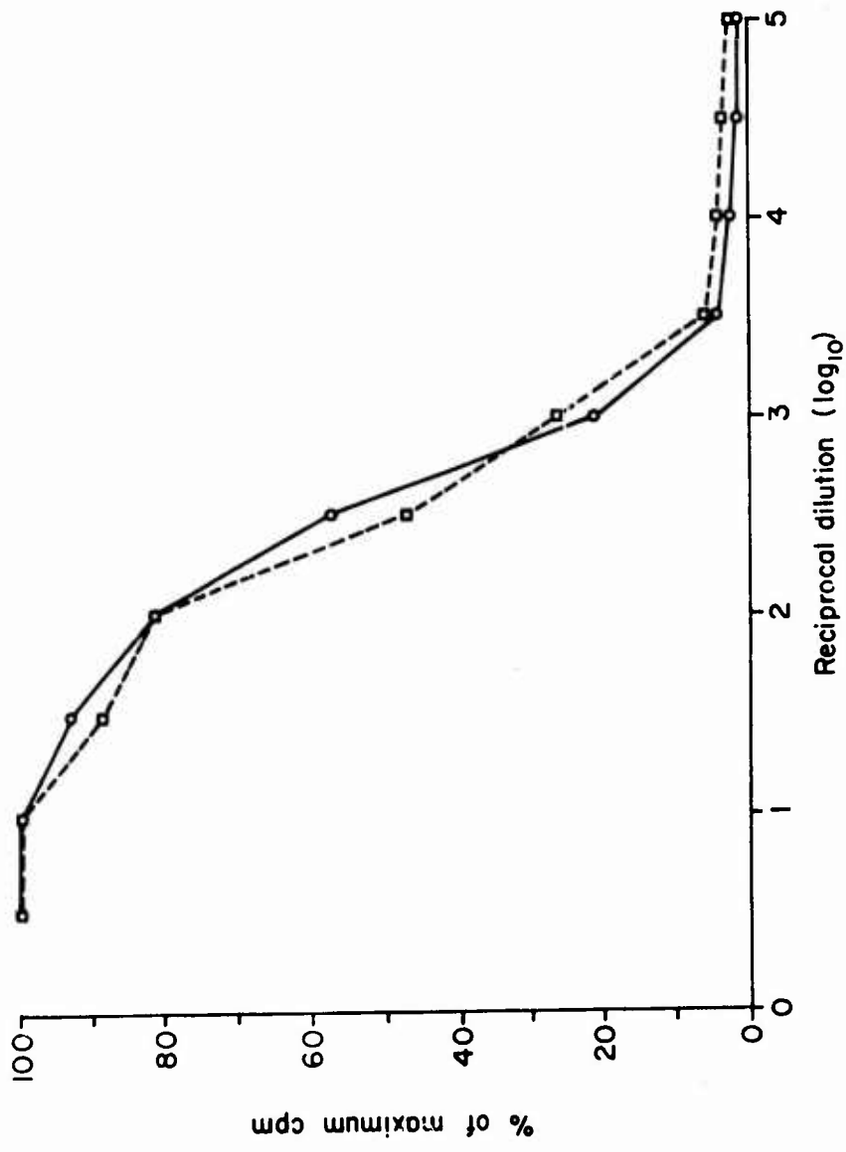

Figure 1. Antigen dilution extinction curves of sera containing HB_sAg/adw (CF titer 1:256) tested by radioimmune assays using both the Ausria I and the Ausria II tests provided by Abbott Laboratories.

▫----▫ Ausria I, o——o Ausria II

and the standard 1:800 dilution of HB_sAg/adw. Figure 3 illustrates the relationship between the percent RIAI on these 100 sera using the $RIAI_I$ versus the $RIAI_{II}$. The correlation coefficient (r) of results obtained from these two techniques was 0.96. This very high correlation indicates that the two tests are measuring the same variable. The proportion of common variance (r^2) was 0.93, indicating that 93% of the variation in one test is accounted for by the variation of the other test.

When the scores assigned to each test on the basis of the 50% cut-off point were examined, 45 of the 100 sera were positive by the $RIAI_{II}$ and 38 of the 100 were positive by the $RIAI_I$ (Table 1). The correlation coefficient (r_{phi}) of the tests scored in this way, was only slightly lower than that derived from the numerical data, again indicating the similarity of the two tests (Table 2).

The 100 sera were also tested by the passive hemagglutination test (PHA, Electronucleonics Inc.) and the immunoelectrosomophoresis test (IEOP) (Table 1). The correlation coefficients (r_{phi}) were also calculated between these tests and both of the RIAI tests (Table 2). The low correlation coefficients between the IEOP and other tests is indicative of the lack of sensitivity of the former as has been shown previously. The PHA identified anti-HB_s in 34 of 100 sera tested. All of these 34 sera were also positive by $RIAI_{II}$.

Table 1. A Comparison of Four Tests for Anti-HB_s Results of 100 Sera

Test	Pattern of Positive Results					Sera positive for each test
RIAI						
AUSRIA II	X	X	X	X	X	45
AUSRIA I	X	X	X			38
PHA	X	X		X		34
IEOP	X					11
Total sera positive by tests indicated	11	20	7	3	4	

Table 2. Correlation Coefficient r_{phi}

AUSRIA II	vs	AUSRIA I	0.87
AUSRIA II	vs	PHA	0.79
AUSRIA I	vs	PHA	0.78
PHA	vs	IEOP	0.49
AUSRIA I	vs	IEOP	0.46
AUSRIA II	vs	IEOP	0.41

Where $r_{phi} = \frac{BC - AD}{\sqrt{(A + B)(C + D)(A + C)(B + D)}}$

In order to increase the confidence in the $RIAI_{II}$ an additional 100 sera, taken from the same population, were added to the original panel (Table 3). In this experiment the $RIAI_{II}$ identified anti-HB_s in 83 (41%) of the 200 sera as compared to 69 (35%) identified by the PHA. The differences seen here were due to 15 sera that were positive only by $RIAI_{II}$ and one serum which was positive only by PHA. The correlation coefficient (r_{phi}) was larger than that found with the original panel of 100 sera indicating an increased between-test reliability.

Table 3. A Comparison of Two Tests for Anti-HB_s Results of 200 Sera

Test	Pattern of Positive Results			Sera positive for each test
RIAI				
AUSRIA II	X	X		83
PHA	X		X	69
Total sera positive by tests indicated	68	15	1	

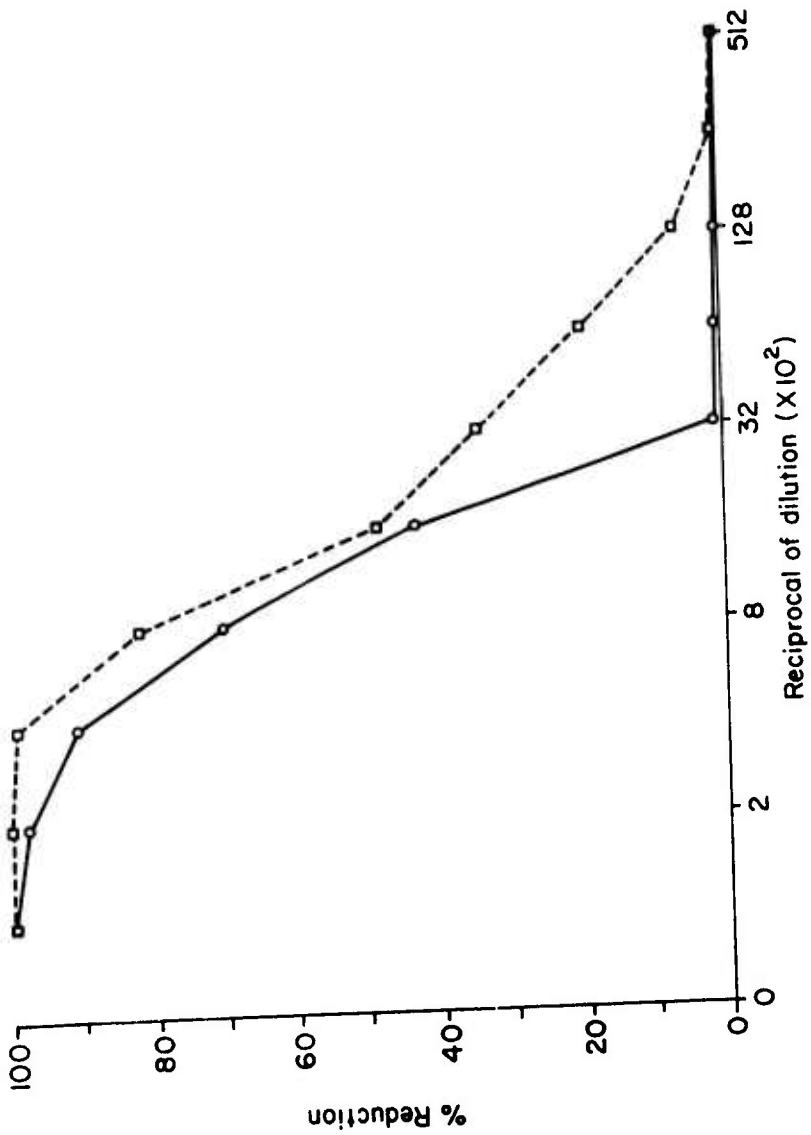

Figure 2. Radioimmune assay inhibition curves on dilutions of a serum containing a high titer of anti-HB_s (PT, CF titer = 1:64). The Ausria I and Ausria II kits were used with a standard antigen dilution of HB_sAg/adw of 1:800.

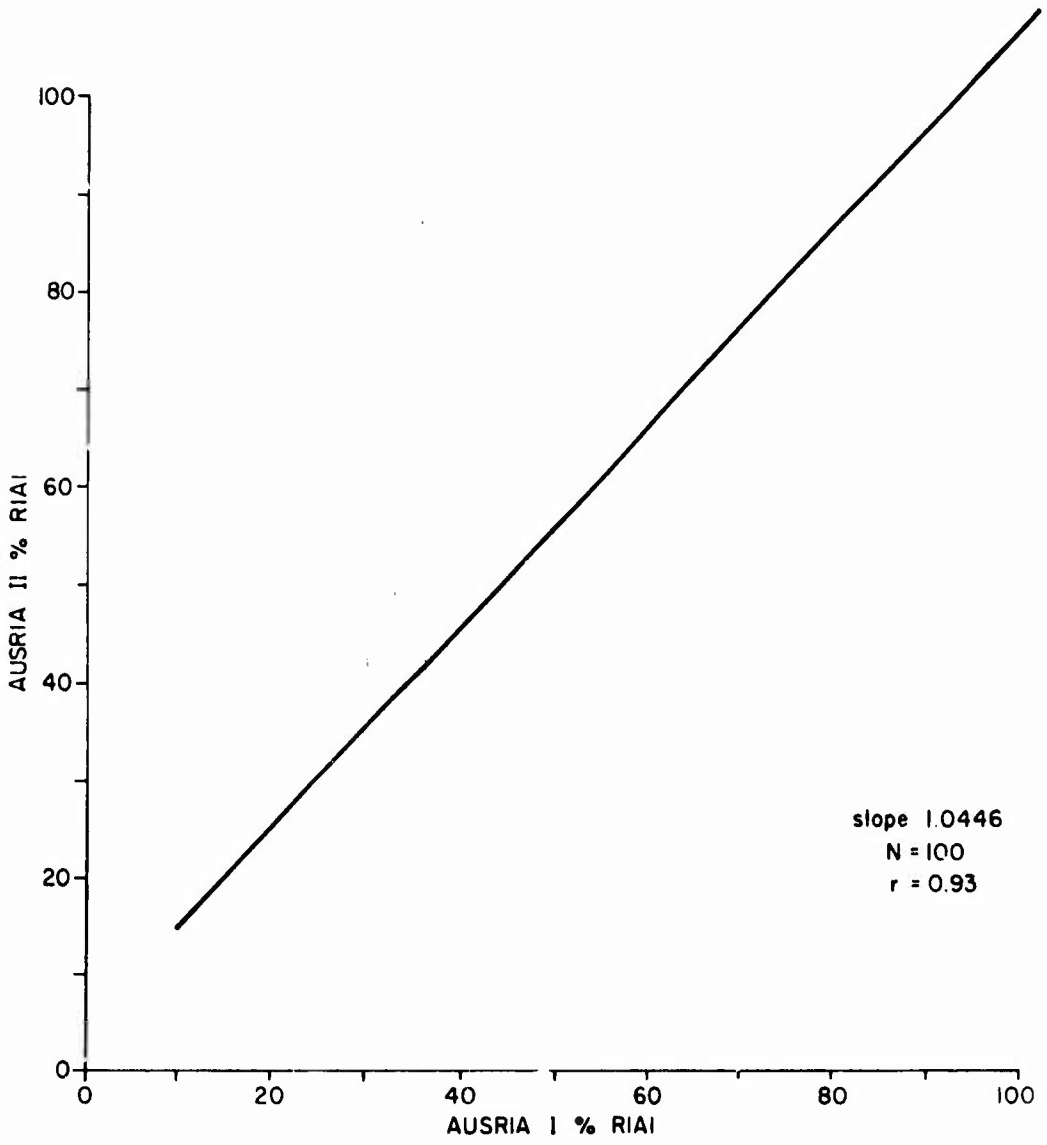

Figure 3. Relationship between the percent radioimmune assay inhibition as shown by the $RIAI_I$ and the $RIAI_{II}$

DISCUSSION AND SUMMARY: The $RIAI_I$ has been used by this laboratory for the past year to identify anti-HB_s in various populations. This test was shown to be slightly less sensitive than the PHA. The introduction of Ausria II test with the withdrawal of the Ausria I test from the market required the series of comparative tests reported here. The $RIAI_{II}$ proved to have an increased sensitivity over the $RIAI_I$. Furthermore, using this test instead of the $RIAI_I$, there appeared to be fewer sera in which anti-HB_s was identified by PHA and not by RIAI. Proving increased sensitivity of the $RIAI_{II}$ remains a problem, as many of these positives cannot be confirmed by PHA. Nonetheless, tne $RIAI_{II}$ has now replaced the $RIAI_I$ in this laboratory for the initial identification of sera containing anti-HB_s.

4. Continuing Studies of Hepatitis B Antigen Carriers in Thailand

OBJECTIVE: To compare the age specific point prevalences of hepatitis B surface antigen (HB_sAg) carriers and the HB_sAg subtype distributions in carriers in different parts of Thailand.

BACKGROUND: The presence of HB_sAg or antibody to HB_sAg (anti-HB_s) serves as evidence of prior exposure of hepatitis B virus (HBV). Difrerences in geographical and environmental status may play an important role in the frequency of HB_sAg carriers and in the distribution of antigen subtypes. This study reports the frequency of HB_sAg carriers found in various parts of Thailand.

DESCRIPTION: HB_sAg carriers were identified using the IEOP test previously described. The method for determining subtypes by immunodiffusion technique using standard reference antigens and hyperimmune rabbit antisera has also been previously described (SMRL Annual Report 1970-1971). The following well defined populations were sampled and tested:

(a) Thai residents of Khao Yai National Park (KYNP) in northeastern Thailand (1),

(b) Randomly selected Thai residents of an urban housing development in Bangkok (14), and

(c) Thai residents of the village of San Kampang, in a semirural area of Chiengmai province in northern Thailand (19).

PROGRESS: Residents of KYNP included the families of employees of three different agencies working in the park: The Forestry Department, the Highway Department and the Tourist Organization

of Thailand (TOT). More than 80% of the estimated total population of the park were sampled and the prevalence of HB_sAg carriers was 9.3%. HB_sAg was found in 10.6% of people associated with the Forestry Department, 9.1% in those associated with the TOT and in only 5.3% of the people associated with the Highway Department.

The prevalence of HB_sAg in males was higher than that in females for every age group. The difference in carrier frequencies between males and females was statistically significant for the total population ($P>.001$). The prevalence of HB_sAg was greatest in children between ages of 10 and 15 years; it fell in older age groups. Of 223 people with multiple blood samples, three acquired HB_sAg between September 1973 and September 1974; the incidence of antigen acquisition was 13.5/1000/year. (Table 1).

Comparison of the above results with the prevalence of HB_sAg carriers in the urban Bangkok housing development (Table 2) revealed that the frequencies of HB_sAg carriers were not significantly different in the two populations. However, in this urban group, there was no significant sex differences in the carrier frequencies of HB_sAg ($0.157>P>0.317$). Children of five to nine years had a slightly higher frequency of antigenemia than other age groups with the exception of the small group of people over 60 years of age.

In a village population drawn from northern Thailand, the prevalence of HB_sAg was 8.6% (Table 3). This was not significantly different from those found in the populations described above. None of the 51 sera collected from females was found to contain HB_sAg, but it was found in 15% of those collected from males. The significantly higher prevalence of HB_sAg carriers among males than females was similar to that seen in KYNP ($0.008>P>0.014$). San Kampang was one of four villages studied in northern Thailand. In the other three, there was no appreciable prevalence of HB_sAg. If all four villages were taken together. the prevalence of HG_sAg fell from 8.6 to 2.9%.

Subtyping of HB_sAg in carriers was studied in 33 residents of KYNP, 48 of Bangkok and 10 of San Kampang (Table 4). Antigens of adr subtype were present in 86% of the Bangkok carriers, 90% of KYNP carriers and 100% of these in the northern Thai village. The findings suggest that within Thailand, there may be differences in the relative frequency of HB_sAg subtypes from place to place.

Several recent studies have shown that subtypes are consistent within families; however, this information was obtained in the temporate zone where antigen carriers are rare and contact by

Table 1. Age Specific Prevalence of HB_sAg in Residents of Khao Yai National Park

(September 1973 - September 1974)

Age (Years)	Male			Female			Total		
	No. Tested	HB_sAg+ No.	(%)	No. Tested	HB_sAg+ No.	(%)	No. Tested	HB_sAg+ No.	(%)
0-4	27	1	(3.7)	35	0	(0.0)	62	1	(1.6)
5-9	27	3	(11.1)	26	0	(0.0)	53	3	(5.7)
10-14	14	5	(35.7)	13	0	(0.0)	27	5	(18.5)
15-19	36	3	(8.3)	18	0	(0.0)	54	3	(5.6)
20-29	130	20	(15.4)	57	3	(5.3)	187	24	(12.8)
30-39	60	7	(11.7)	18	1	(5.6)	78	8	(10.3)
40-59	27	3	(11.1)	8	0	(0.0)	35	3	(8.6)
60+	1	0	(0.0)	0	0	(0.0)	1	0	(0.0)
Total	322	42	(13.0)	175	4	(2.3)	497	46	(9.3)

HB_sAg Acquisition Rate = 3/223/year = 13.5/1000/year

Table 2. Age Specific Prevalence of HB_SAg* in Residents of Huay Khwang, Bangkok (July 1971)

Age (years)	Male			Female			Total		
	No. tested	Prevalence		No. tested	Prevalence		No. tested	Prevalence	
		No.	%		No.	%		No.	%
1-4	35	1	2.8	31	2	6.4	66	3	4.5
5-9	54	5	9.2	56	7	12.5	110	12	10.9
10-14	61	8	13.1	66	2	3.0	127	10	7.9
15-19	39	6	15.4	54	3	5.6	93	9	9.7
20-29	38	5	13.2	71	4	5.6	109	9	8.2
30-39	27	0	0.0	53	4	7.5	80	4	5.0
40-59	37	2	5.4	56	3	5.4	93	5	5.4
60-	6	2	33.3	13	3	23.1	19	5	26.3
TOTAL	297	29	9.8	400	28	7.0	697	57	8.2

*Combined results from IEOP and radioimmunoassay (RIA) tests.

family members with antigens other than those carried in the family would be unlikely. In KYNP, different subtypes of antigen were present in close proximity. Five conjugal families were studied in which the antigen carried by at least two positive individuals could be subtyped, in four families only the adr subtype was identified and only the adw subtype was found in the other family. Only one subtype was found in any one family. Within the urban Bangkok population, the distribution of subtype again fit into family patterns. In four conjugal families with two to seven HB_SAg positive members, only the adr subtype could be detected; in two other families with two and four HBsAg positive members, respectively, only the adw subtype was found. Again in no family was more than one subtype identified (20).

DISCUSSION: Comparison of the three populations revealed no significant differences in the frequency of HB_SAg carriers, suggesting a similar rate of exposure to HBV occurred in these three groups. It was interesting to note, however, the marked local differences in the antigen frequency among the villages of northern Thailand. On the one hand, the number of persons studied were small and therefore the phenomena may represent a sampling error. On the

Table 3. Age Specific Prevalence of HB_SAg in San Kampang (November 1969)

Age (Years)	Male			Female			Total		
	No. Tested	HB_SAg+ No.	(%)	No. Tested	HB_SAg+ No.	(%)	No. Tested	HB_SAg+ No.	(%)
0-4	3	1	(33.3)	8	0	(0.0)	11	1	(9.1)
5-9	15	4	(20.6)	9	0	(0.0)	24	4	(16.7)
10-14, 15-19	21	2	(9.5)	15	0	(0.0)	36	2	(5.9)
20-29, 30-39	15	3	(20.0)	11	0	(0.0)	26	3	(11.6)
40-59, 60+	11	0	(0.0)	8	0	(0.0)	19	0	(0.0)
Total	65	10	(15.4)	51	0	(0.0)	116	10	(8.6)

other hand, these differences may be due to local environmental and social factors. The high prevalence of HB_sAg carriers among all three groups support the finding of high carrier prevalences in the tropics. The striking difference noted in the HB_sAg carrier prevalence among the rural males and females might be explained by two hypotheses. First, the male, through his socially defined role, may have an increased exposure to sources of infection. This has been suggested by the data of Grossman et al (14) who showed increased anti-HB_s prevalences among young urban males. The distribution of anti-HB_s in rural populations remains to be seen. Second, males may be more susceptable than females to the development of the carrier state. This interesting hypothesis could not be evaluated in this study.

The subtype distribution in carriers suggests that local differences in virus subtypes exist within areas of Thailand. Further, the localization of antigen subtypes within family units implies an even smaller unit of HBV transmission.

Table 4. HB_sAg Subtypes in Thais

Population	HB_sAg Subtypes adr %	adw %
Urban Bangkok	86	14
Khao Yai National Park	90	10
Northern Thailand	100	0

5. The Effect on the Offspring of Maternal Hepatitis B Surface Antigenemia

OBJECTIVE: To study the effect on the offspring of chronic hepatitis B antigenemia in the mother.

BACKGROUND: Clinical hepatitis B developing during the latter part of pregnancy has been associated with an increased perinatal mortality, a high incidence of premature delivery and a high frequency of virus transmission from mother to infant (21). Information on the effect of asymptomatic maternal hepatitis B antigenemia is conflicting. It has been suggested that the incidence of prematurity and perinatal mortality is also increased in infants born of these mothers (22). Transmission of virus from antigenemic mothers to their infants appears to be an uncommon event in mother infant pairs studied in the west (23, 24). However,

a strong association between antigenemia in mothers and their children has been shown in cross sectional population studies in the orient (25, 14). This study was designed to investigate the effect of maternal antigenemia on pregnancy and the offspring in a population with a high prevalence of antigen carriers.

DESCRIPTION: This study was divided into two phases. A description of the initial phase may be found in the SEATO Medical Research Laboratory Annual Progress Report, March 1974.

Population: Antigen positive mothers and their families were sought for follow-up 1 1/2 - 2 1/2 years after initial collection in the delivery room. Temporal controls were matched to each family that could be located. Temporal controls were members of families of women who were collected in the delivery room and whose delivery dates were as close as possible to those of the antigen positive mothers. Each control mother was delivered within two days of an antigen positive mother. Interim family histories were obtained on all families studied and these included the duration of breast feeding, the medical and dental history of the child and the person who cares for the child most of the time. Family relationships were determined and other members of the family living in the household were sought. Each infant was examined by a physician and height and weight measurements were recorded.

Sera were collected on antigen positive and control mothers, their infants, and as many family members as could be found. Saliva was also collected from all mothers.

Laboratory Studies: Sera were submitted for determinations of transaminase and bilirubin concentrations. They were tested for hepatitis B antigen by radioimmune assay (Abbott Laboratories, Ausria I), counterelectrophoresis and complement fixation. A radioimmune assay inhibition technique was used to screen for antibody against HB_sAg and positives were titrated and confirmed by a passive hemagglutination test (PHA, Electronucleonics).

PROGRESS: As reported in the SEATO Medical Research Laboratory Annual Report 1973-1974, of 1,625 mothers screened in the delivery room at Women's Hospital, 93 or 5.7% were found to be positive. Of the 93, 47 were located, 30 were not located and 16 lived outside of Bangkok.

Ninety-four families were followed, 47 with antigen positive mothers and 47 temporal controls. There were no significant differences between the antigen positive family and the temporal

control found in family variables such as household size and income. Five families of antigen positive mothers who resided outside of Bangkok, returned to the city for follow-up. If these five were discounted, then the distribution within the city of the families of antigen positive mothers and controls was similar. Maternal factors such as age, parity, history of past abortion, infant mortality and transaminase levels also showed no significant differences. Further, there were no significant differences seen in the weight, length or transaminase levels of the infant at birth, nor in the number of infants born prematurely or the infant mortality rate over the first year of life. In the 94 families followed, five infants had died. Two deaths were recorded within the control families and the other three in families of antigen positive mothers. Three of these deaths occurred at or shortly after delivery. Two were related to complications of delivery and one to prematurity. Two children died during the first year of life. One child of an antigen positive mother died at four months of age during an episode of diarrhea for which no medical aid was sought. A child of a control mother died of pneumonia at six months of age.

All mothers who were antigen positive at delivery were still positive at the time of follow-up 1 1/2 to 2 1/2 years later. In general the complement fixation titers of these mothers were within four-fold of the titer found at delivery. None of the temporal control mothers or their infants had developed antigen; however, 13 of 44 (29.5%) of surviving infants of antigen positive mothers were found to be positive (Table 1).

Table 1. Experience with Hepatitis B Virus 18-30 Months After Birth

Mother	Offspring			
	No. Tested	Evidence of Infection		
		HB_sAg	Anti-HB_s	Total
HB_sAg Positive	44	13 (30%)	3 (7%)	16 (37%)
HB_sAg Negative	45	0 (0%)	1 (2%)	1 (2%)

Table 2. Maternal Antigen Titer and the Percent of Positive Offspring

Mothers		Offspring	
		HB_sAG Positive	
CF Titer	No. Tested	No.	%
≤1:16	61*	0/63*	0
1:32	7	1/7	14
1:64	12	5/12	42
>1:128	10	7/10	70
Total	90	13/92	14

*Differences due to multiple births

Despite what would appear to be abundant hepatitis B virus exposure in infants of HB_sAg positive mothers, the incidence of antibody conversion in this group was low (Table 1). There were no significant differences in the incidence of antibody conversion in infants with positive mothers and in those of control mothers; however, there were three times as many converts in the antigen positive group and the lack of significance might reflect only the small number of infants examined.

There appeared to be a direct relationship between the maternal complement fixation titer at delivery or follow-up and the prevalence of antigen in the serum of offspring for follow-up (Table 2). The maternal titer on all 13 infants found to be positive was greater than or equal to 1:32. None of the offspring of mothers with titers less than 1:32 were positive.

At the time of follow-up, the physical status of children of antigen positive mothers and antigen negative controls was documented. None of these children were chronically ill, nor with one exception did any exhibit any biochemical or physical evidence of hepatitis at the time of examination. When compared to normal values established for Southern Chinese children (26),

average deviations of height and weight for these two groups of children were not significantly different. The one exception was the antigen positive son of an antigen positive mother whose only sign of illness was a moderate elevation in the transaminase concentrations (SGOT to 136 Sigma Frankel units, SGPT to 90 Sigma Frankel units).

DISCUSSION: As reported in the SEATO Medical Research Laboratory Annual Report 1973-1974, there were no gross differences seen in the prematurity and perinatal mortality rates of infants of antigen positive mothers and antigen negative mothers. Further, there were no apparent differences in the maternal history of pregnancy and child birth between these two groups. However, subtle differences could not be excluded.

Transmission of hepatitis B virus occurred from mother to offspring. In this study only infants of positive mothers were found to be positive at 1 1/2 to 2 1/2 years of age. This does not exclude the possibility of virus transmission and development of the carrier state in children of antigen negative mothers; indeed this must happen in order to maintain the high prevalence of antigen carriers seen in this population. These data do suggest that the infection of infants of negative mothers is a relatively rare event when compared to that of children of antigen positive mothers. The phenomenon of hepatitis B virus transmission appears to be directly related to the antigen titer of the positive mothers. The prevalence of antigen positive offspring increased as the HB_sAg titer in the mother increased.

Maternal antigenemia did not grossly effect the growth or the development of the child. Children who developed HB_sAg were not significantly different in height and weight from children without HB_sAg, whether or not they had HB_sAg positive mothers.

6. Hepatitis B Virus in Bangkok Families

OBJECTIVE: To determine when young urban Thai children are first exposed to hepatitis B virus (HBV) and to search for the most common routes of transmission to infants in the first year of life.

BACKGROUND: A recent study showed that 19.9% of the residents of Huay Khwang had Hepatitis B surface antigen (HB_sAg) or antibody (anti-HB_s) between the ages of one to five years (14). HBV infection in children was closely related to the presence of HB_sAg in their mothers. Another study of women who delivered at Women's Hospital, Bangkok, showed 12% of 93 mothers with HB_sAg had antigen in their cord blood by radioimmune assay (RIA). Furthermore, mothers with anti-HB_s always had antibody in their cord bloods (1).

The current study was designed to follow infants in the first year of life and to compare the incidence and affects of HBV infection in infants whose mothers had HB_sAg or anti-HB_s to those whose mothers were negative.

DESCRIPTION: An attempt was made to interview and sample as many women as possible who delivered at Phra Mongkutklao Hospital (PMKH) between 1 February 1974 and 31 January 1975. Blood was collected from the mother and the carefully wiped umbilical cord at the time of delivery for testing for HB_sAg and anti-HB_s. A questionnaire interview of the mother was conducted in the early postpartum period. Study subjects were selected by 1) the presence of HB_sAg or anti-HB_s in the mother's blood; 2) residence within the metropolitan Bangkok area; and 3) willingness to allow home visits and to bring the baby to the PMKH Well Baby Clinic for follow-up. Control mothers were selected if they had no HB_sAg or anti-HB_s in their blood but delivered on the same day as a positive mother. Control mothers also had to meet criteria 2 and 3 listed above.

HBV serology used a solid phase RIA (Ausria I) and immunoelectroosmophoresis (IEOP) as the primary screening tests for HBsAg and a radioimmune assay inhibition (RIAI) test to detect anti-HB_s. Passive hemagglutination (PHA) was used when available to confirm the RIAI results and to test small volume samples. All blood samples were tested for serum transaminase (SGOT and SGPT) levels as well.

Serial serum samples were drawn by venipuncture at approximately two, three, six, nine and 12 months of age in the Well Baby Clinic after examination by a pediatrician. Blood samples were drawn from the mothers at the same time intervals. An attempt is being made to collect blood samples from all other people living in the home during home visits at three, six and 12 months after delivery.

During home visits, information was gathered on the home environment by questionnaire and inspection and samples of breast milk, saliva and mosquitoes were collected from some families. The priority of sample testing is to test sera first, then saliva, breast milk and mosquitoes.

PROGRESS: A comparison was made of 300 women delivering at PMKH to 300 women at Women's Hospital to see if the two hospital populations were similar. The prevalence of HB_sAg detected by IEOP was 4.3% at PMKH and 3.7% at Women's Hospital. The groups were very similar in terms of parent ages, number of people in the home and home location within Bangkok. A notable difference was that the mean family income was 25% greater at PMKH than Women's Hospital. For the purposes of this study, the two hospitals seemed similar.

Table I. Frequency of HB_sAg and Anti-HB_s in 32 Pairs of Mothers.

Mother		Maternal Category								
Blood Spec	Weeks After Delivery	HB_sAg			Anti-HB_s			Negative		
		No.	Ag+	Ab+	No.	Ag+	Ab+	No.	Ag+	Ab+
1	Delivery	12	12	0	20	0	20	32	0	0
2	4-9	8	8	0	16	0	16	24	0	0
3	10-19	9	9	0	18	0	18	27	0	3
4	20-29	7	7	0	14	0	14	21	0	2
5	30-39	2	2	0	1	0	1	3	0	0

Table 2. Frequency of HB_S Ag and Anti-HB_S in 32 Pairs of Infants.

Infant		Maternal Category								
		HB_SAg			Anti-HB_S			Negative		
Blood Spec	Weeks After Delivery	No.	Ag+	Ab+	No.	Ag+	Ab+	No.	Ag+	Ab+
1	Delivery	12	1	0	20	0	20	32	0	0
2	4-9	8	0	0	16	0	15	24	0	0
3	10-19	9	3	1	18	0	10	27	0	0
4	20-29	7	4	1	14	0	2	21	0	0
5	30-39	2	1	0	1	0	0	3	0	0

Interviews and blood samples were obtained from 1042 (43%) of the women who delivered over a 12 month period. From this group, 42 women with HB_sAg, 44 with anti-HB_s and 77 negative controls are being followed. For most mothers with antigen or antibody, a satisfactory negative control was identified who delivered two days before to two days later. In seven instances, mothers who were initially thought to be negative were later shown to actually have antibody at the time of delivery after they had been matched to HB_sAg positive mothers. These seven pairs of antigen positive mothers mismatched to antibody positive mothers are being followed that way.

A preliminary review was made of the serological results for 64 mothers, including 12 with HB_sAg, 20 with anti-HB_s and 32 time-matched controls. Only families that had been followed at least six months or seroconverted before being lost to follow-up were reviewed. All mothers with antigen or antibody remained positive (Table 1). Three negative mothers developed low level antibody activity by 10-19 weeks suggesting they may have been exposed to HBV in the recent past.

The infants showed dramatic serological changes (Table 2). One infant of an HB_sAg positive mother was found to have antigen in the cord blood and in every follow-up serum throughout the next 12 months. This infant had the only antigen positive cord blood detected by IEOP. Other infants of antigen positive mothers frequently developed antigen or antibody by the age of 20-29 weeks, indicating that these infants are at risk of infection very early in life.

All of the infants with antibody positive mothers had anti-HB_s in their cord bloods. The frequency of anti-HB_s declined steadily during the first six months as was expected for passively acquired maternal antibody. Several of these infants came from families with an HB_sAg positive father, sibling or other member; some may show evidence of infection with HBV after the maternal antibody is gone. None of the infants of negative mothers have developed antigen or antibody yet. The prevalence of HB_sAg carriers seems to be the lowest in this group of families.

SUMMARY: A prospective study was started of HBV infection of infants selected on the basis of their mother's serological findings at the time of delivery. A preliminary review indicates children of HB_sAg positive mothers have a high likelihood of becoming infected in the first six months of life. Children of antibody positive mothers have maternal antibody at birth which may afford protection during the first six months. After losing their maternal antibody, these children as a group may be at a

higher risk of infection with HBV than children of negative mothers since the families of the former often include an antigen carrier.

7. Hepatitis B Surface Antigen in Laboratory Reared Mosquitoes

OBJECTIVE: To determine the duration of carriage of hepatitis B surface antigen (HB_sAg) by laboratory reared mosquitoes fed on a HB_sAg carrier.

BACKGROUND: This is the completion of a study reported in the SEATO Medical Research Laboratory Progress Report 1973-1974. This report concerns the completion of radioimmune assay testing of mosquitoes following feeding on an antigen positive donor.

DESCRIPTION: All mosquitoes used in this study were reared from eggs in the laboratory. After the adults emerged they were held for 48 hours and were deprived of fluids for 12 hours prior to use. Mosquitoes were fed on a known carrier of HB_sAg/adr with a constant complement fixation titer of 1:512. Engorged mosquitoes were then removed and unfed mosquitoes discarded. A sample of 10 fed mosquitoes were quick-frozen and stored at -70°C; the remainder were placed in cages and allowed to feed on sugar water. Samples of 10 mosquitoes were withdrawn from the cages at 1, 3, 5, 7, 10, 15 and 21 days after feeding, quick-forzen and stored at -70°C.

All mosquitoes were tested by radioimmune assay (RIA, Ausria I, Abbott Laboratories) simultaneously for each mosquito species. Pools of 10 mosquitoes were triturated in 0.5 ml of 0.01 M Tris buffered saline pH 7.4 and centrifuged at 2000 rpm; 0.1 ml of the supernatant solution was placed in each of two Ausria tubes. Following this the test was run according to the directions provided with the Ausria kit. Included in each experiment was a pool of 10 unengorged mosquitoes of each species. Also one mosquito species, Aedes aegypti, was allowed to bite a non-antigenemic individual. These mosquitoes were followed in the same way.

PROGRESS: Seven mosquito species, Aedes aegypti, Aedes albopictus, Anopheles balabacensis, Anopheles maculatus, Anopheles minimus, Armigeres subalbatus, and Culex quinquefasciatus, were tested in the above manner (Figure 1). RIA results of all unengorged mosquito controls fell within one standard deviation of the mean of the negative sera controls. Further, all samples of Aedes aegypti fed on a non-antigenemic individual also were found to fall within one standard deviation of the negative control mean. For all seven

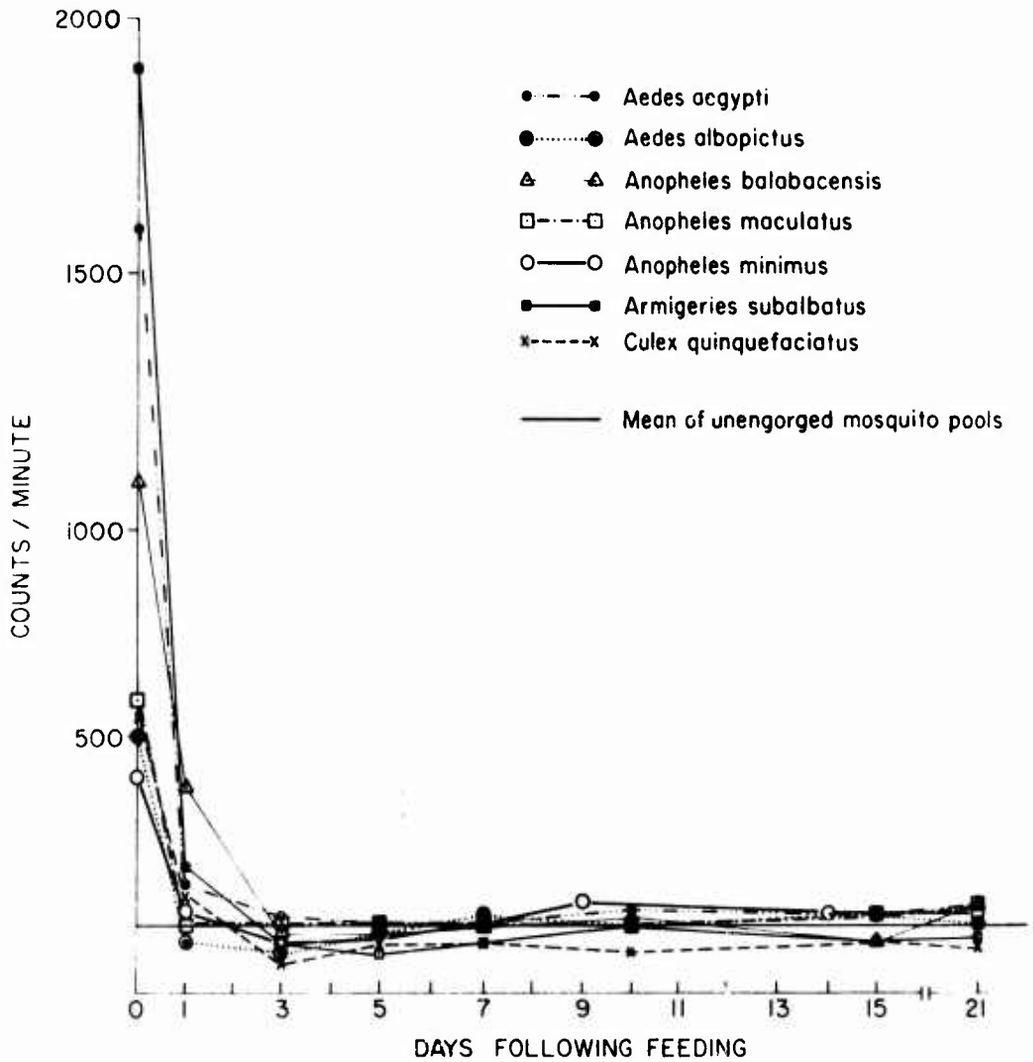

Figure 1 Radioimmuno assay for HB_SAg (Ausria I) on pools of 10 mosquitoes collected following feeding on an infectious HB_SAg positive volunteer.

species the first pool of ten was taken immediately after feeding on the HB_sAg positive volunteer. HB_sAg was detected in all mosquito species in the first sample. However, HB_sAg as determined by RIA, had disappeared by 24-72 hours after feeding. All mosquito species were followed for 21 days or longer. In all species HB_sAg did not reappear; the RIA counts per minute on these mosquito pools remained within the limits of the unfed mosquitoes.

DISCUSSION: These data indicate that disappearance of HB_sAg from the mosquito pools was simultaneous with the digestion and elimination of the blood meal. In the seven mosquito species followed for 21 or more days HB_sAg did not reappear after its initial disappearance; however, the presence of antigen in mosquitoes for 24-72 hours might allow them to serve as mechanical vectors if they refeed within this period of time.

The laboratory work on this study is now complete and the data is being analysed in preparation for publication.

C. TICK-BORNE VIRUSES

1. Tentative Identification of Langat Virus

OBJECTIVE: To identify a group B arbovirus (T-1674) isolated from ticks in Khao Yai National Park.

BACKGROUND: Three unidentified viruses have been isolated from ticks collected in Thailand (1). One of the viruses, T-1674, was shown to pass through a 50 nm filter. Growth was inhibited by either exposure to ether or pH 3.0 but not by a DNA inhibitor, 5-Bromo 2' deoxyuridine. In LLC-MK_2 cells, T-1674 produced plaques of varying size. Complement fixation (CF), hemagglutination inhibition (HI) and plaque reduction neutralization tests (PRNT) indicated T-1674 was antigenically related to the group B arboviruses. Tick-borne group B arboviruses have not been previously identified in Thailand. Since some members of this group cause severe encephalitis in man, identification of T-1674 was given first priority.

DESCRIPTION: Identification of T-1674 was accomplished by comparing its antigenicity to that of nine other group B arboviruses including a prototype strain of Langat, TP-21.

Hyperimmune mouse ascitic fluid (HMAF) was used in all serological tests. HMAF was made to T-1674 and the prototype Langat TP-21 (27). Additional HMAF to TP-21 was kindly provided by Dr. Hazel Wallace (Arbovirus Research Unit, University of Malaya, Kuala Lumpur, Malaysia).

Table 1. Comparative Reciprocal CF Antibody Titers to Four Units of Antigen

Antigen	Hyperimmune Mouse Ascitic Fluid*									
	T-1674	Langat	Dengue1	Dengue2	Dengue3	Dengue4	Japanese Encephalitis	Wesselsbron	Tembusu	West Nile
T-1674	32	256	2	<2	<2	<2	4	<2	<2	<2
Langat (TP 21)	32	256								
Dengue 1	<2		512							
Dengue 2	<2			1024						
Dengue 3	<2				512					
Dengue 4	<2					512				
Japanese Encephalitis	<2						512			
Wesselsbron	<2							256		
Tembusu	<2								8	

* HMAF were not absorbed with normal mouse brain.

Table 2. Comparative Reciprocal PRNT Antibody Titers to 95 Pfu of Virus

Virus	Hyperimmune Mouse Ascitic Fluid							
	T-1674	Langat (TP21)	Langat (University of Malaysia)	Dengue 2	Japanese Encephalitis	Wesselsbron	Tembusu	West Nile
T-1674	100	600	500	<10	<10	<10	<10	<10
Langat (TP21)	110	2000	1000					

CF and HI antigen was prepared by sucrose acetone extraction of suckling mouse brain (SMB) (28). Hemagglutin activity was optimal at pH 6.7 (range 6.4 - 7.0) at 22°C. PRNT using a constant amount of virus and dilutions of HMAF were used to determine the dilutions of antibody giving 50% plaque reduction (29).

PROGRESS: Low passage seed virus sent to the Yale Arbovirus Research Unit, New Haven, Conn., was tentatively identified as Langat virus by comparative CF testing (30). Similar testing in this laboratory supported this conclusion (Table 1). In addition, PRNT showed T-1674 was neutralized only by Langat antibody (Table 2). Although the Langat HMAF gave two to four fold higher antibody titers to TP-21 than to T-1674, it is concluded that there is sufficient similarity to consider T-1674 to be a new strain of Langat virus.

SUMMARY: Identification tests of a group B tick-borne virus (T-1674) from Khao Yai National Park suggest it is a new strain of Langat virus. Conclusive identification awaits interpretation of the results by the Yale Arbovirus Research Unit.

2. Experimental Infection of Gibbons with a Group B Arbovirus (T-1674)

OBJECTIVE: 1) To determine if a group B tick-borne arbovirus (T-1674) was infectious for gibbons and, if so, 2) to identify any evidence of illness that might also occur in man.

BACKGROUND: Tick-borne group B arbovirus infections of man may be asymptomatic or cause mild to severe encephalitis. A new strain of group B arbovirus, T-1674, which was isolated from Haemaphysalis papuana ticks in Khao Yai National Park (1) was tentatively identified as Langat virus. Langat virus, a member of this group, has been shown to be infectious for rhesus, cynomolgus and spider monkeys but not to cause disease. Experimental infection of gibbons has not been reported. Since gibbons are present in abundance in the area in which T-1674 was found, it is possible that gibbons may be a natural host for this virus.

DESCRIPTION: Three adult gibbons, Hylobates lar, which had been cared for by the Dept of Veterinary Medicine, SEATO Medical Research Laboratory for 8 to 9 years, were selected for experimental infection after determining they had no detectable antibody by hemagglutination inhibition (HI) test to Langat and 5 other group B arboviruses. Two animals (P5, B66s) were inoculated intracutaneously with 1.0 ml each of a low passage suckling mouse brain (SMB) suspension containing T-1674 at a titer of $10^{4.1}$ suckling mouse LD50/ml. One of these gibbons (B66s) had a splenectomy 7

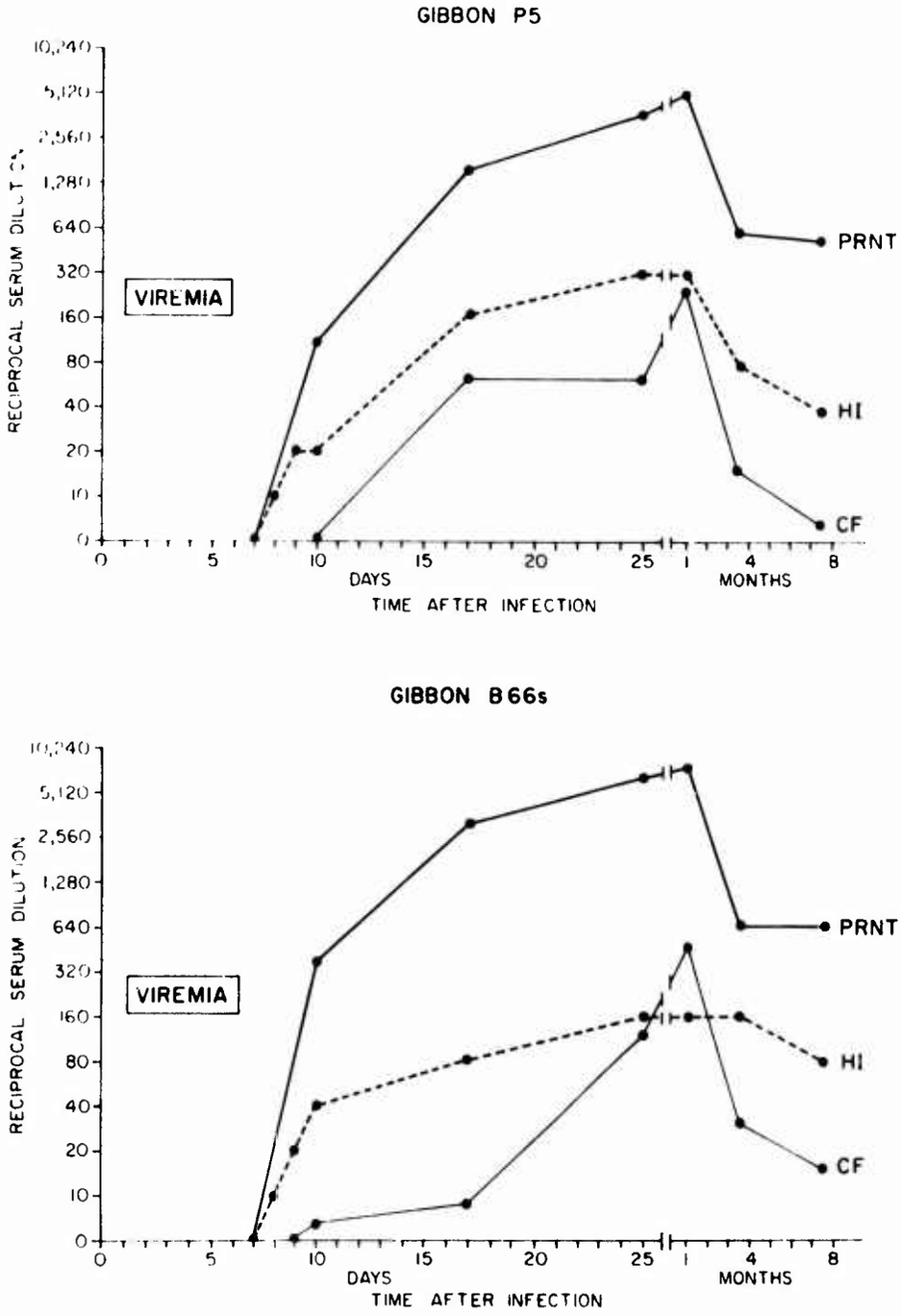

Figure 1 Response of two gibbons to intracutaneous inoculation of $10^{4.1}$ suckling mouse LD_{50} of T-1674 on day 0.

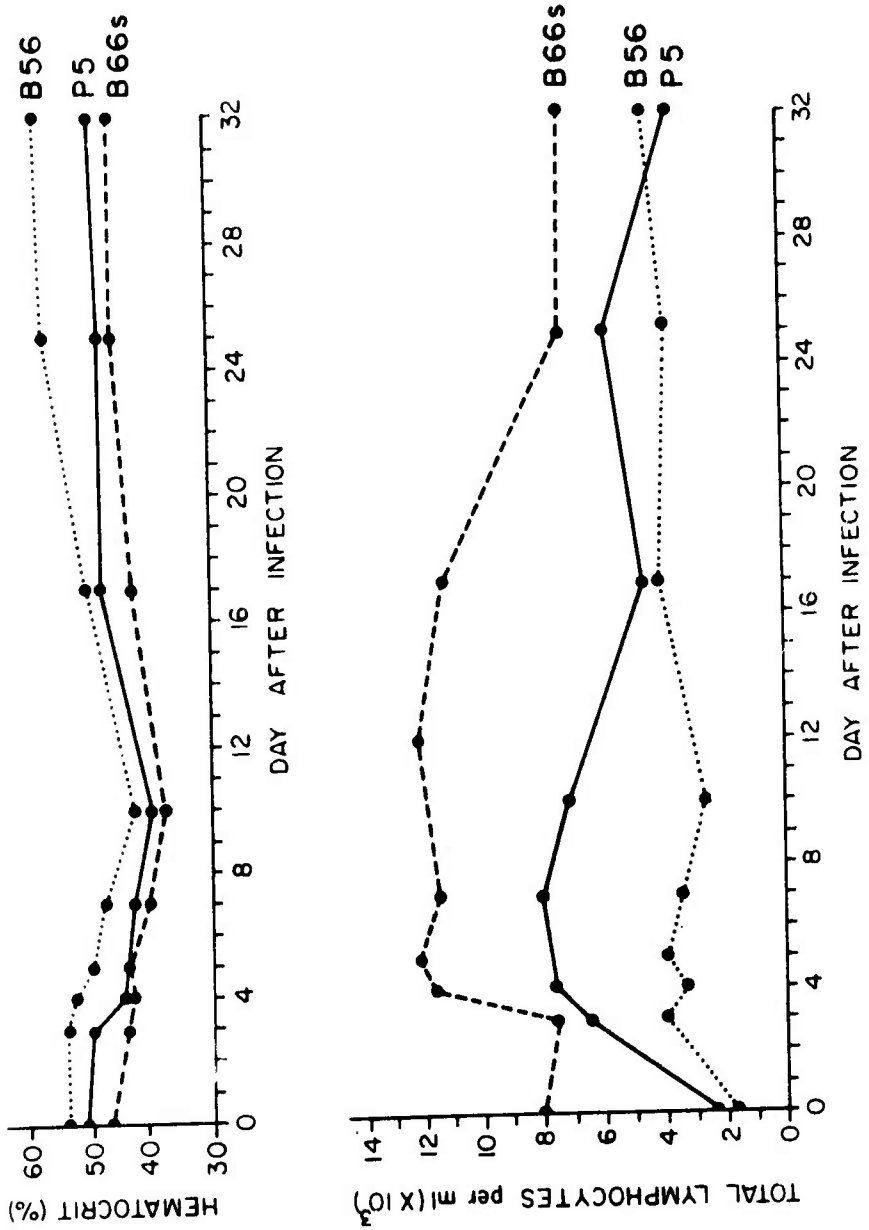

Figure 2. Serial hematocrit and total lymphocyte counts for two gibbons infected with Langat Virus (P5, B66s) and one control (B56). Infection began on day 0.

years previously. The third animal (B56) was inoculated with a placebo and housed in a cage between the infected gibbons. Each animal was observed daily for evidence of illness. Blood samples were collected daily for the first 10 days, then approximately weekly for the first month. Viremia was detected by inoculation of plasma into suckling mice.

PROGRESS: No differences were observed between the three animals with regard to food and water intake, rectal temperature and general behavior that were not within the range of expected daily variation. None of the animals developed a detectable rash, neurological abnormalities, lymph node enlargement or splenomegally.

Both gibbons who received T-1674 had viremia from days one to six after inoculation; plasma virus titers reached 10^3 suckling mouse LD_{50}/ml on day two and three, respectively. Both animals subsequently developed antibody detected by complement fixation (CF), HI and plaque reduction neutralization test (PRNT) after day 10 (Figure 1). The control gibbon did not develop viremia or detectable antibody. Routine hematological studies showed a temporary fall in hematocrit for each animal during the 10 day period of daily blood collection (Figure 2) which was attributed to frequent phlebotomy. B66s had a long previous record of leukocyte counts above 8000 wbc/ml following splenectomy. The two infected gibbons developed increased total lymphocyte levels relative to their baseline values from days 3 to 10 and 4 to 17, respectively. In these animals, mononuclear cells accounted for 58-70% and 82-88%, respectively, of all leukocytes from days four to nine after infection. In contrast, the control gibbon had 4000 lymphocytes/ml or less during the same time period and 44-63% mononuclear cells.

SUMMARY: Two adult gibbons were experimentally infected with T-1674, a group B arbovirus tentatively identified as Langat virus. Both animals developed viremia from days one to six after infection followed by antibody detected by CF, HI and PRNT. Peak antibody levels were obtained one month after infection. Both animals developed relative and absolute lymphocytosis during the first three weeks after infection but neither developed overt disease. A temporary fall in hematocrit was also seen in the infected and the control animals and was attributed to frequent phlebotomy. It was concluded that T-1674 can cause asymptomatic infections with viremia in gibbons. No additional information was learned about the potential pathogenicity of T-1674 for man.

3. A Survey for Human Antibody to T-1674

OBJECTIVE: To detect any evidence of human infection with T-1674, a group B tick-borne arbovirus.

BACKGROUND: Tick-borne arbovirus infections of humans have not been recognized to occur in Thailand. The discovery of two different viruses in ticks collected in Khao Yai National Park (KYNP) stimulated the initiation of a survey of residents of the park for antibody to these agents (1). One of the viruses, T-1674, was previously shown to be a group B arbovirus (1) and is now tentatively identified as Langat virus. Natural infections of humans with Langat is infrequent in Malaysia, but induced infections of people with neoplastic disease has caused encephalitis (31). This study sought evidence of natural human infection with T-1674 in a human population with a high incidence of mosquito-borne group B arbovirus infection.

DESCRIPTION: Human sera were collected from as many residents of Khao Yai National Park (KYNP) as was possible during visits in September 1973, February 1974 and September 1974. At the same time historical information was obtained on the length of residence in KYNP, living site, size of families, occupation, general health and exposure to ticks.

Complement fixation (CF), hemagglutination inhibition (HI) and plaque reduction neutralization tests (PRNT) were used to detect antibody (1). CF antigen was standardized by block titration against homologous HMAF. The highest dilution of a sucrose acetone extract of suckling mouse brain (SMB) giving 50% hemolysis was considered one unit of antigen. A four-fold lower dilution of antigen (4 units) was used in routine CF tests. All sera were heated to 56°C for 30 minutes before testing. A serum titer of 1:4 or greater was considered positive by CF. Eight units of sucrose acetone extracted SMB were use as antigen in HI tests. Positive serum titers by HI were 1:10 or greater. PRNT titers were based on 50% reduction of the mean number of control plaques by a serum dilution of 1:10 or greater.

PROGRESS: Residents of KYNP included employees of the Forestry Department, Highway Department and the Tourist Organization of Thailand (TOT) and their dependent relatives. Between September 1973 and September 1974, serum was collected from 497 individuals representing 80% of the total population estimated from work rosters and interviews and multiple sera were obtained from 39% (Table 1). The median age of the people sampled was 22 years compared to 21 years for the entire population. The ages of people sampled ranged from 4 months to 60 years. The median length of residence in the park was 3 years and ranged from one day to 43 years. The ratio of males to females in the sample was 1.56 compared to 1.39 for the whole population.

The questionnaire survey yielded little evidence of illness. Between 12-55% of the residents experienced one or more of 11 specific symptoms, but the responses did not correlate with the presence or absence of group B arbovirus HI antibody. In September 1973, few people reported ever being bitten by ticks; however, in February 1974 over 50% of the residents admitted to tick bites and many said ticks were abundant at that time. It appeared that human exposure to ticks was common and probably seasonal.

HI tests were done on at least one blood sample from 488 individuals with adequate demographic information. Of the entire sample, 246 people provided serial blood specimens. The age distribution of the follow-up group was representative of the larger group (Figure 1). Similarly, the age specific prevalence of arbovirus HI antibody for the follow-up group (Figure 2) was representative of all of the park residents. HI antibody to dengue virus type 2 (D2) and Japanese encephalitis virus (JE) was found in over 65% of persons aged 10-15 years and over 90% after age 20 years. Antibody to T-1674 tended to appear later than that to the other group B arboviruses, was not found in more than 87% of any age group and declined in the oldest age group. Chikungunya antibody was found in only two of 66 persons under 15 years of age but thereafter increased steadily to age 50 years.

Of the 246 residents of KYNP from whom two or three serum samples were obtained, 24 (10.2%) demonstrated a four-fold rise in HI antibody titer to one or more arbovirus antigens (Table 2). A rise in antibody to JE antigen was more frequent than to any other type and was found in individuals ranging in age from 16 months to 54 years. Two people showed a four-fold rise in antibody to T-1674 but both had pre-existing group B antibody. No one developed a higher titer of antibody to T-1674 than to either D2 or JE. The people with multiple serum samples were screened for PRNT antibody at a 1:10 dilution. The highest titer of neutralizing antibody to T-1674 in any resident was 1:10. Since the PRNT is considered to be more specific than the HI test the evidence suggests the HI reactivity to T-1674 was due to cross reactive antibody to other group B arboviruses. The constant presence of mosquito-borne group B arboviruses in KYNP, the low level and infrequent rises in HI antibody to T-1674 and the absence of high levels of PRNT antibody to T-1674 indicate that infection of residents with T-1674 virus in quite infrequent if it occurs at all.

SUMMARY: A prospective survey of arbovirus antibody was made from September 1973 to September 1974 of all residents of Khao Yai National Park. Although evidence was found of near uniform exposure to Dengue 2, Japanese encephalitis and Chikungunya, there was no conclusive evidence of any natural infection with T-1674

Table 1. KYNP Residents: Serological Sampling
Sept 1973 - Sept 1974

Dept Group	Residents No.	(%)	Sex Male	Female	M/F	Age* Median	(Range)	Length of Residence* Median	(Range)
TOT									
Bled	230	(86)	123	107	1.15	21	(7/12-48)	3	(2 day-10)
Missed	38		18	20	0.90	3	(1/12-48)		N.D.
Combined	268		141	127	1.11	20	(1/12-48)		
Forestry									
Bled	170	(82)	117	53	2.21	22	(9/12-60)	2	(1 day-13)
Missed	37		18	19	0.95	11	(9 day-59)		N.D.
Combined	207		135	72	1.88	21	(9 day-60)		
Highway									
Bled	97	(65)	63	34	1.85	26	(4/12-54)	3	(3 day-43)
Missed	53		24	29	0.83	10	(1/12-75)		N.D.
Combined	150		87	63	1.38	21	(1/12-75)		
All Depts									
Bled	497	(80)	303	194	1.56	22	(4/12-60)	3	(1 day-43)
Missed	128		60	68	0.88	9	(9 day-75)		
Combined	625		363	262	1.39	21	(9 day-75)		

* Time is in years unless otherwise indicated.

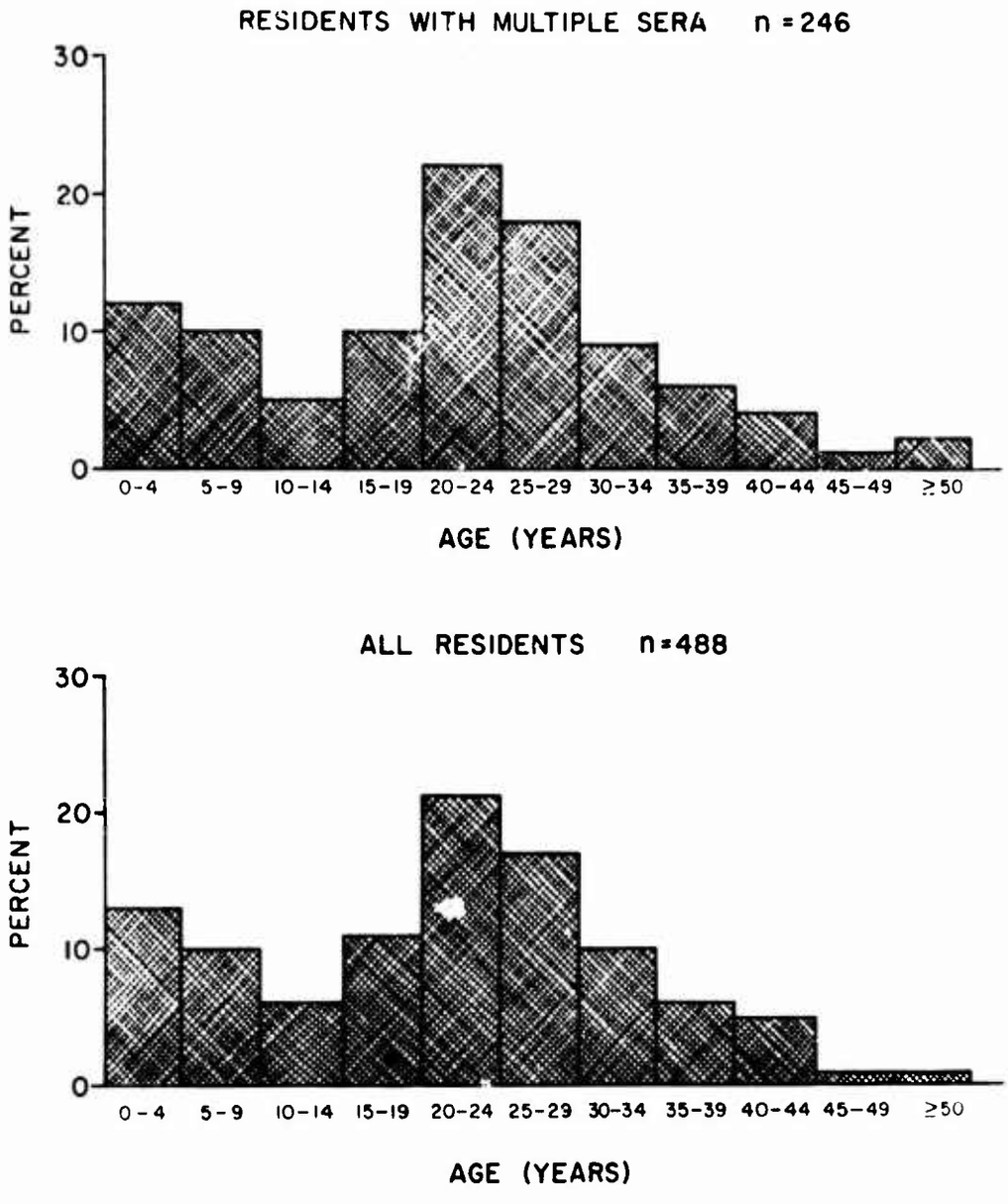

Figure 1. Percent age distribution of residents of Khao Yai National Park

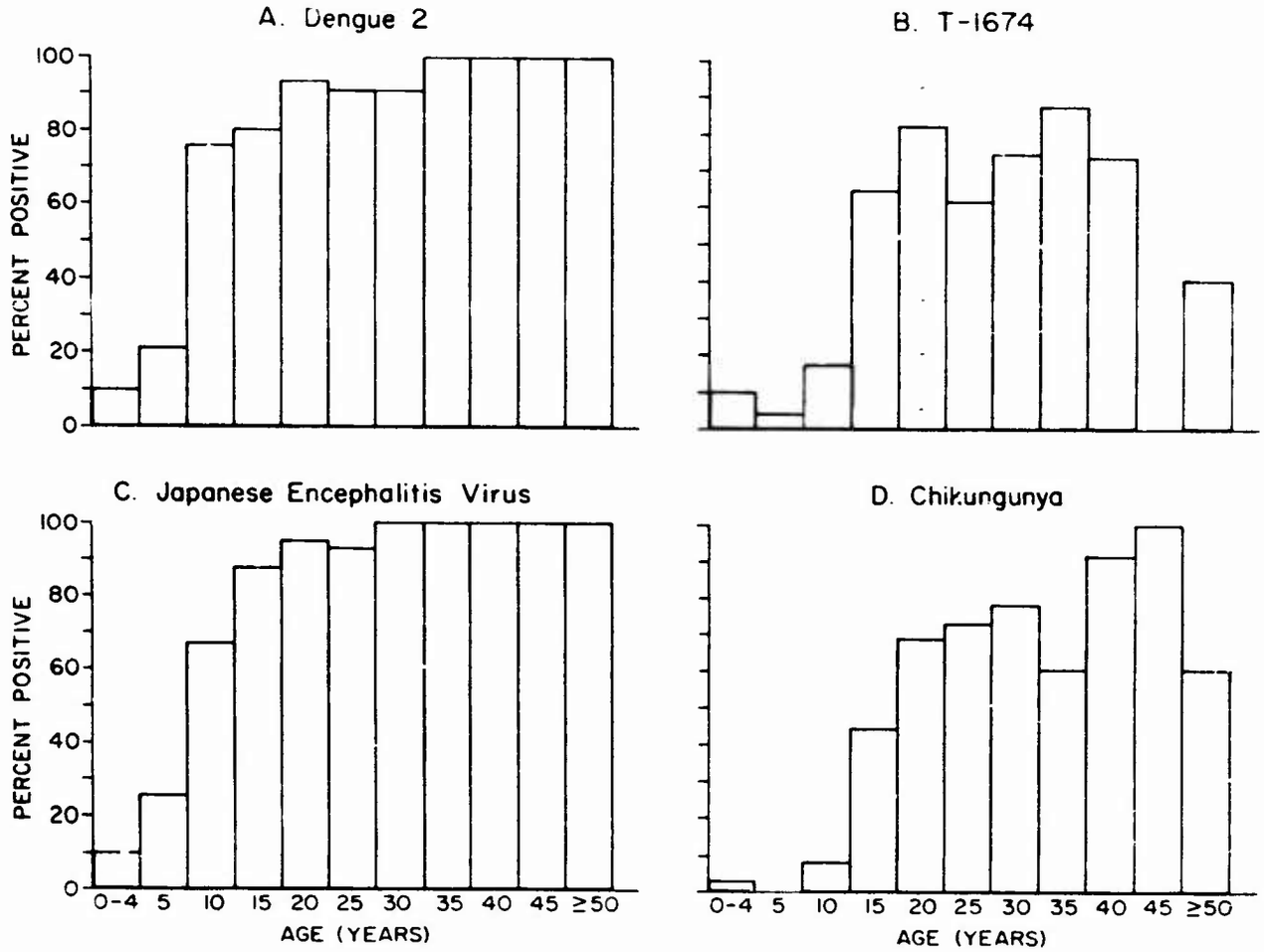

Figure 2 Age specific prevalence of HI antibody to A Dengue type 2; B. T-1674; C Japanese encephalitis Virus, and D Chikungunya in 246 residents of Khao Yai National Park

during that time period. It is concluded that HI reactivity to T-1674 antigen is probably due to the presence of cross reactive antibody produced to other group B arboviruses. There appears to be little or no risk of human infection with T-1674 in the park.

Table 2. Frequency of HI Antibody Rise to Arboviruses in 246 Residents of KYNP

Antigen	Four-fold Increase No.	(%)	Eight-fold Increase No.	(%)
Group A				
Chikungunya	6	(2.4)	0	(0.0)
Group B				
T-1674	7	(2.8)	2	(0.8)
Dengue 2	12	(4.9)	4	(1.6)
Japanese Encephalitis	15	(6.1)	7	(2.8)
Any Group B	19*	(7.7)	7	(2.8)

*Group B arbovirus incidence rate = 19/246/yr = 77.2/1000/yr

D. INFLUENZA

1. Epidemic Influenza in a Hill Tribe in Northwest Thailand

OBJECTIVE: To investigate an epidemic of respiratory disease in the Karen hill tribes of northwest Thailand.

BACKGROUND: The town of Mae Sariang is located on the banks of the Yuam River in a mountainous region of northwestern Thailand (97° 52' longitude, 18° 10' north latitude, at 350 meters above sea level). From Mae Sariang a partially paved road runs north along the river 140 Km to the provincial capital of Mae Hong Sorn. Another road runs 193 Km through the mountains east from Mae Sariang to the city of Chiang Mai (Figure 1).

The people of this region are largely Karen. They live in small isolated hillside villages of 10-500 houses and are subsistence

farmers. Due to the isolation of the villages, travel is largely by foot; it is usually limited to occasional visits to local villages and rarely, in an emergency, to nearby towns. Educational opportunities are rare and there is little understanding of simple health measures. Malnutrition, vitamin deficiency and parasitic infestations are common problems The climate of this area is influenced by the southern monsoon winds, with the wet season from May to October and the dry season from November to March. The Christian Medical Unit (CMU) of the American Baptist Mission is located in Mae Sariang. It is a ten-bed hospital with one full-time physician (BES) and it provides medical service to an estimated 20,000 people who live within a six days walk. Since 1973 the hospital has used a mobile medical unit to make visits every six weeks to hill tribe villages up to three days walk from the road.

In the third week of March 1974, an increase in respiratory disease was reported in Karen villages. The onset of the outbreak was temporally related to a two day meeting of the Karen Baptist Association (KBA) which was attended by an estimated 300 residents of Karen villages. The meeting was held in the village of Mae Hae, located approximately 38 Km northeast of Mae Sariang, 10 hours on foot from the nearest road (Figure 2). This village is composed of 40-50 houses with an estimated population of approximately 280 residents. At the time of the meeting 60-75 residents (23-27%) of the village were acutely ill with respiratory symptoms. Many people in surrounding villages also had acute respiratory disease and one village reported seven deaths. Over the two weeks following this meeting 237 patients were seen by the CMU in villages north of Mae Sariang. Many of these were people who had been present at the KBA meeting including one of the CMU staff.

On 5 April the SEATO Medical Research Laboratory was requested by the staff of the CMU to help determine the etiology of this epidemic.

DESCRIPTION: From 7-9 April 1974 a field team was deployed from the SEATO Medical Research Laboratory to substantiate reports of increased respiratory disease among the Karen people.

Clinical studies: People were examined in villages selected along migration routes so as to monitor past and current disease along these routes. Four villages were selected to the north and three villages to the south of the Mae Sariang - Chiang Mai road (Figure 2). The clinical presentation of the illness was determined by interviewing and examining sick patients with respiratory symptoms. Clinical samples were taken from all people examined and blood was obtained for serology.

Laboratory studies: Throat washes or swabs were obtained on all patients for virus isolation. The techniques for isolation and identification of influenza viruses have appeared elsewhere (32). Briefly, viruses were isolated in embryonated chicken eggs and primary monkey kidney (MK) tissue culture (Macaca irus). The presence of virus was recognized by hemagglutination or hemadsorption of guinea pig red blood cells. Prototype strains of previously isolated influenza viruses were obtained from Dr. Franklin H. Top, Jr., Walter Reed Army Institute of Research, Washington, D.C. Specific antisera to both the isolates and the prototype strains were prepared in roosters. Isolates were identified by hemagglutination inhibition (HI) using eight hemagglutinating units of antigen and homologous and heterologous rooster antisera. Neutralization tests used 100 $TCID_{50}$ of virus and quantitative neutralization were done using 10 fold dilutions of each virus against dilutions of rooster antisera (33).

Blood was obtained for serology on all patients; sera were tested for antibody to influenza by an HI test. Blood smears were obtained on older patients for estimation of the white blood count (WBC) and differential counts. Blood cultures were taken when indicated and throat swabs were obtained from all patients for bacteriological culture.

PROGRESS:

Distribution of cases: Among villages surveyed, disease was evident only in those to the north of the road between Mae Sariang and Chiang Mai. The inhabitants of these villages were largely Christian. Residents of each had attended the KBA meeting and many had developed respiratory disease during the meeting or shortly after returning to their homes. At the time of the survey there was a marked increase in respiratory disease among infants and children; however, respiratory disease among older people was reported to have occurred approximately two weeks earlier. Village headmen estimated that between 10 and 40% of the people in four villages had recently been sick. People in the three villages south of the road were not Christians. No one from these villages had attended the KBA meeting and there was no respiratory disease seen or reported for several months prior to the time of the survey in these villages.

Characteristics of illness: The clinical presentation of the illness was determined by interviewing and examining 25 patients. People of all ages were sick (Table 1). Twelve of the 25 were less than 10 years old and the oldest was 46 years old. Patients seen were said to have been ill from one to 17 days. All had a history of fever and the older ones complained of headache, malaise and

prostration. All developed a characteristic hacking cough which in some cases was productive of sputum. The majority had hyperemic throats, and one child had a mild exudative tonsillitis. Eight of the 25 patients had chest findings ranging from scattered ronchi to evidence of consolidation. In 13 patients studied, white blood counts of 10,000 or less were found in nine and differentials showed an absolute lymphocytosis (35-84%) in eight.

Bacterial cultures: Bacterial cultures were obtained from three patients; pneumococcus was recovered from one of these. This individual had first developed illness about one week prior to being seen and had an acute exacerbation of his symptoms eight hours prior to examination. No other bacterial pathogens including beta hemolytic steptococcus were identified in either the blood or the throat cultures.

Virus isolation and identification: Despite difficulties in transportation and storage, nine virus strains were isolated from the pharyngeal secretions of the 25 patients (36%) (Table 1). In one village, isolations were made in six of the eight patients examined. Isolates were easily passed in MK cells or embryonated eggs. No evidence of cytopathogenic effect was noted in the MK cells after as long as 14 days of incubation.

Table 1. Age Distribution of Respiratory Disease and Influenza Isolates of 25 Patients Examined in Northwest Thailand

Age (Years)	Patients Examined	Influenza Isolates
0-9	12	5
10-19	1	0
20-29	3	1
30-39	5	1
≥ 40	4	2
TOTAL	25	9 (36%)

Antisera prepared in roosters against two of the isolates had an HI titer of 1:320 when tested against the homologous antigens and titered within a two-fold dilution when tested against the other strains (Table 2). These results indicate that there were no significant antigenic differences among the isolates from this epidemic.

To determine the extent of the differences in antigenic configuration between the current strain (Mae Sariang/74) and earlier isolated strains, rooster antisera prepared against the isolates and prototype influenza strains were tested against homologous and heterologous viruses. The HI test demonstrated a close relationship between the current strain and prototype A/Port Chalmers/1/73 (Table 3).

It has been suggested that the neutralization test is more sensitive to antigenic variation than is the HI test (33). When antisera were tested by neutralization, a disparity was revealed in the antibody activity of antisera prepared against these strains. Antisera to A/Port Chalmers/1/73 equally neutralized at high titers both the homologous and the current strains. However, when antisera to the current strains were used, neutralization of 100 $TCID_{50}$ of A/Port Chalmers/1/73 repeatedly required 8 fold more antisera than did the current strains (Table 4). The degree of disparity

Table 2. Hemagglutination Inhibition Tests on Nine Virus Strains Isolated from Patients in Mae Sariang Using Rooster Antisera Prepared to Two of Them

Antiserum / Antigen	Reciprocal Hemagglutination Titers	
	MS/868/74	MS/913/74
MS/862/74	320	320
MS/868/74	320	320
MS/871/74	320	320
MS/872/74	320	320
MS/874/74	320	320
MS/877/74	320	320
MS/878/74	320	320
MS/883/74	320	320
MS/886/74	320	320
MS/913/74	320	320
MS/916/74	640	640

was not sufficient to differentiate a new influenza strain when analyzed by the method of Archetti and Horsfall (34). These findings, however, do suggest minor antigenic differences between the prototype strain and the present isolate. Quantitative neutralization tests using three viruses against antisera prepared against them substantiated these minor differences (Figures 3 & 4).

HI antibody response: Serum samples were obtained from 25 patients. Unfortunately due to the remoteness of the area convalescent samples were not available. In all the individuals from whom virus was isolated the titers were $<$1:10. Antibody was present in 14 of the remaining 16 people.

DISCUSSION: That this epidemic was an outbreak of influenza has been amply demonstrated. Influenza virus, closely resembling A/Port Chalmers/1/73 was isolated from 36% (9/25) of the throat secretions collected from acutely ill patients.

The magnitude and extent of the epidemic and the incidence of disease could not be accurately assessed. The population of the hill tribes can only be roughly estimated and the number and distribution of the villages affected is unknown. An incidence of influenza might be inferred from the attack rates reported for the people of Christian sentinel villages, where 10-15% of the population was said to have been involved.

In Thailand, over the past several years, influenza has usually appeared during September, October or November in Bangkok or at the Royal Thai Air Force Bases. It occurred at a time when resurgence of disease was occurring in other parts of Asia, Europe and North America and was probably introduced into Thailand from these areas. Two epidemics have been studied in the spring when the incidence of influenza was low elsewhere. Both of these were noted first in rural areas; one in Korat in April 1971 and this one in Mae Sariang. We have no information as to the source of this epidemic. The virus may have been introduced into the hills from the central valley of Thailand; a mild outbreak of influenza occurred in Bangkok in October and November of 1973, from which a virus similar to the A/Port Chalmers/1/73 strain was isolated. Alternately, the virus may have spread south through the hills from Burma, Laos or China. Consistant with this hypothesis is the occurrence of respiratory illness in hill tribe villages near Fang, 200 Km to the north of Mae Sariang in February 1974 (personal communication: Prince Pisadej Rachanee, Director, His Majesty's Hill Tribe Project, Chiang Mai, Thailand).

Influenza may have resulted in a recognizable epidemic through a series of unusual and fortuitous circumstances. Rare in itself

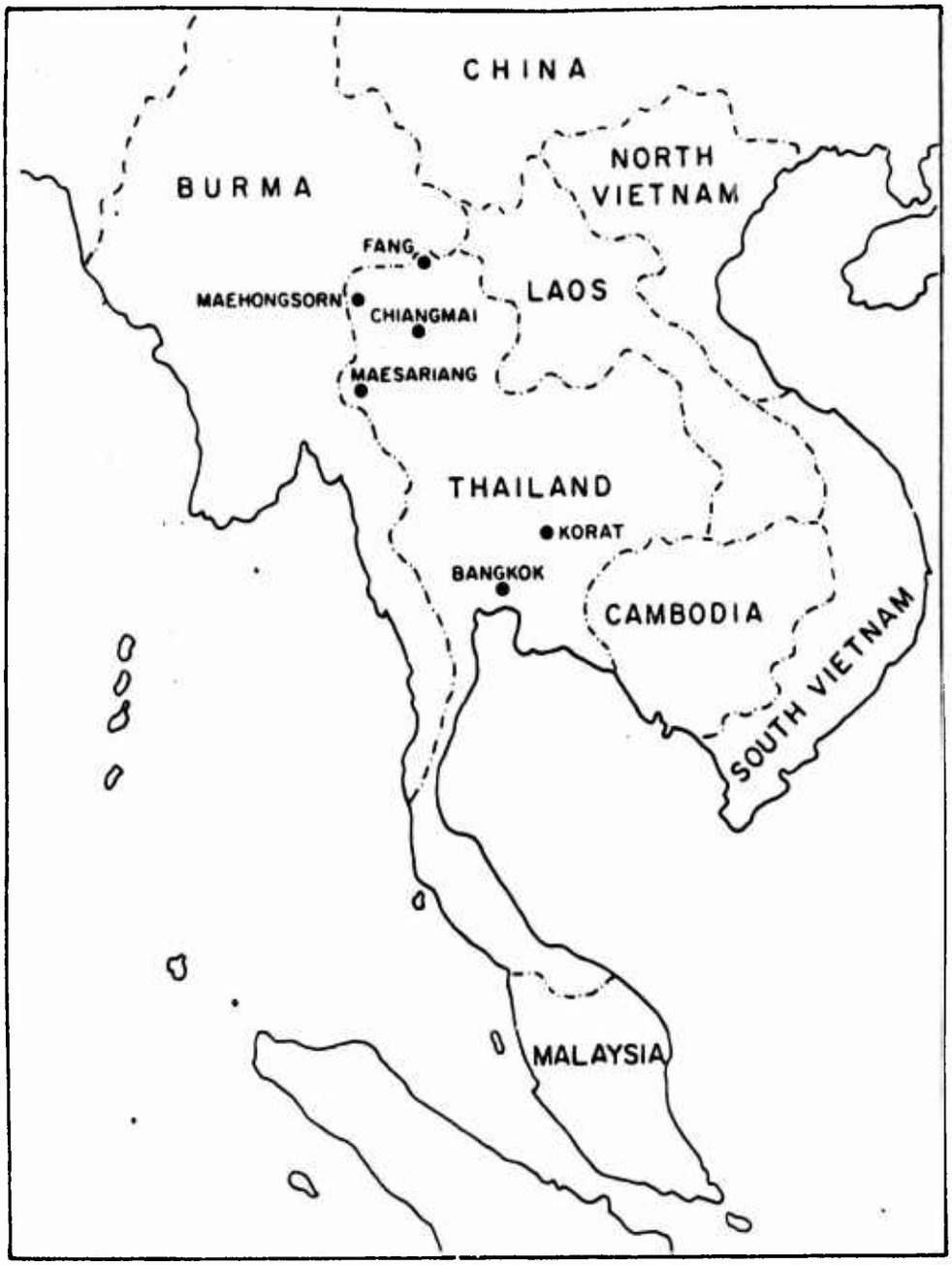

FIGURE 1. MAP OF THAILAND SHOWING THE TOWN OF MAE SARIANG IN RELATION TO CHIENG MAI, MAE HONG SORN, FANG AND BANGKOK.

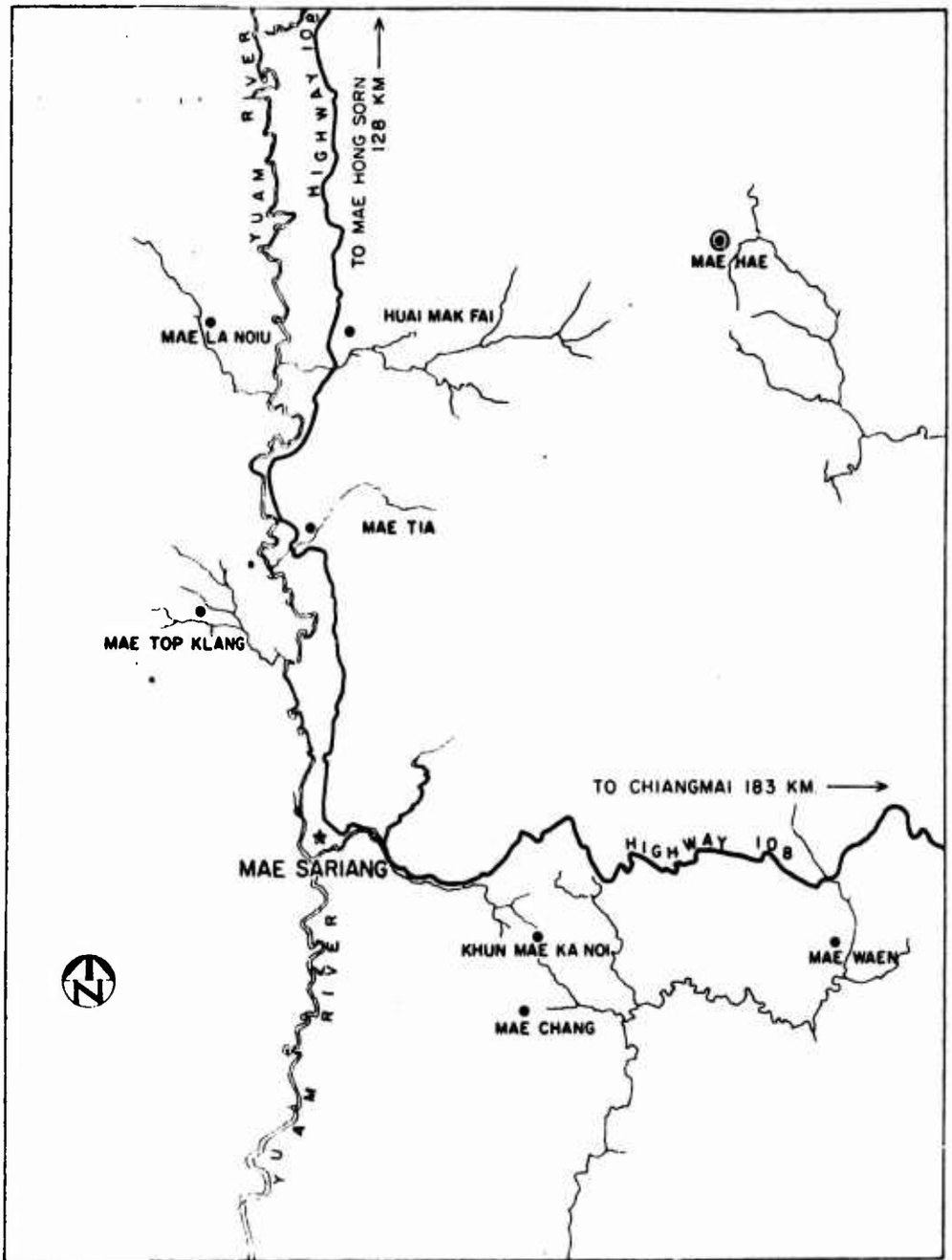

FIGURE 2. LOCATION OF THE TOWN MAE SARIANG (★), IN RELATIONSHIP TO THE YUAM RIVER (═), THE HIGHWAY (▬), THE SENTINEL VILLAGES (●) AND THE SITE OF THE MEETING OF THE KAREN BAPTIST ASSOCIATION AT BAN MAE HAE (◉).

Table 3. Comparison by Cross Hemagglutination Inhibition of Current Influenza Strains with Prototype Strains of Previously Isolated Influenza Viruses

Antisera[a] / Antigen[b]	Reciprocal Hemagglutination Inhibition Titers					
	A/MS/868/74	A/P.Chal/1/73	A/Eng/42/72	A/H.K./1/68	A/Jap/305/57	B/Lee/40
A/MS/868/74	320	320	160	80	<10	<10
A/P.Chal/1/73	320	320	320	160	<10	<10
A/Eng/42/72	80	160	160	160	<10	<10
A/H.K./1/68	80	80	80	320	10	<10
A/Jap/305/57	40	20	80	80	160	<10
B/Lee/40	<10	<10	<10	<10	<10	1280

[a] Specific rooster antisera

[b] Hemagglutination inhibition test used 8 hemagglutinating units.

Table 4. Comparison by Cross Neutralization of Current Influenza Strains with A Influenza/H_3N_2/viruses

Antisera[a] / Antigen[b]	Reciprocal of Neutralizing Antibody Titer			
	A/MS/868/74	A/P.Chal/1/73	A/Eng/42/72	A/H.K./1/68
A/MS/868/74	<u>160</u>	160	20	20
A/P.Chal/1/73	20	<u>160</u>	20	20
A/Eng/42/72	20	80	<u>160</u>	20
A/H.K./1/68	20	40	40	<u>160</u>

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[a] Specific rooster antisera

[b] Neutralization tests used 100 $TCID_{50}$ of the appropriate virus.

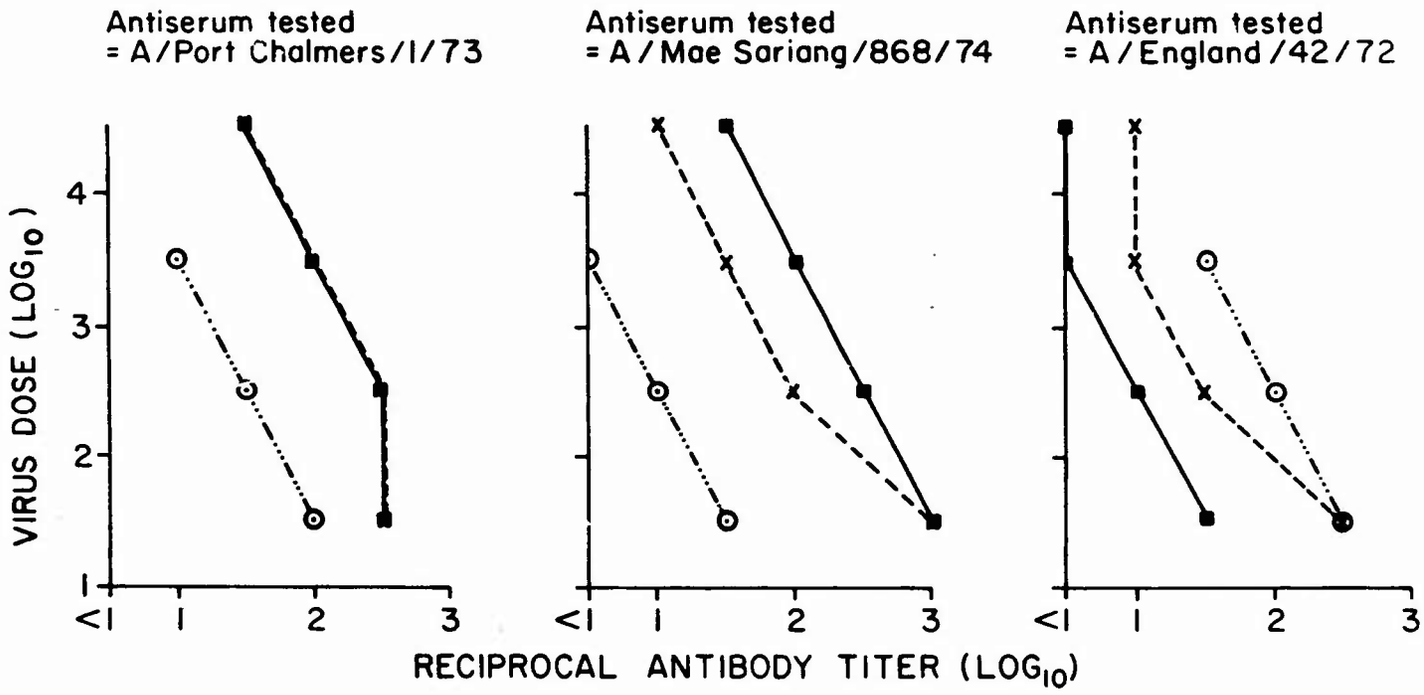

Figure 3. Quantitative relationships between recently isolated A/Influenza/H_3N_2/strains. Comparisons of neutralization reactions of A/Port Chalmers/1/73, A/Mae Sariang/868/74 and A/England/42/72 showing dissimilar patterns of neutralization. Viruses tested x----x A/Port Chalmers/1/73, ■——■ A/Mae Sariang/868/74, ⊙–···–··· ⊙ A/England/42/72

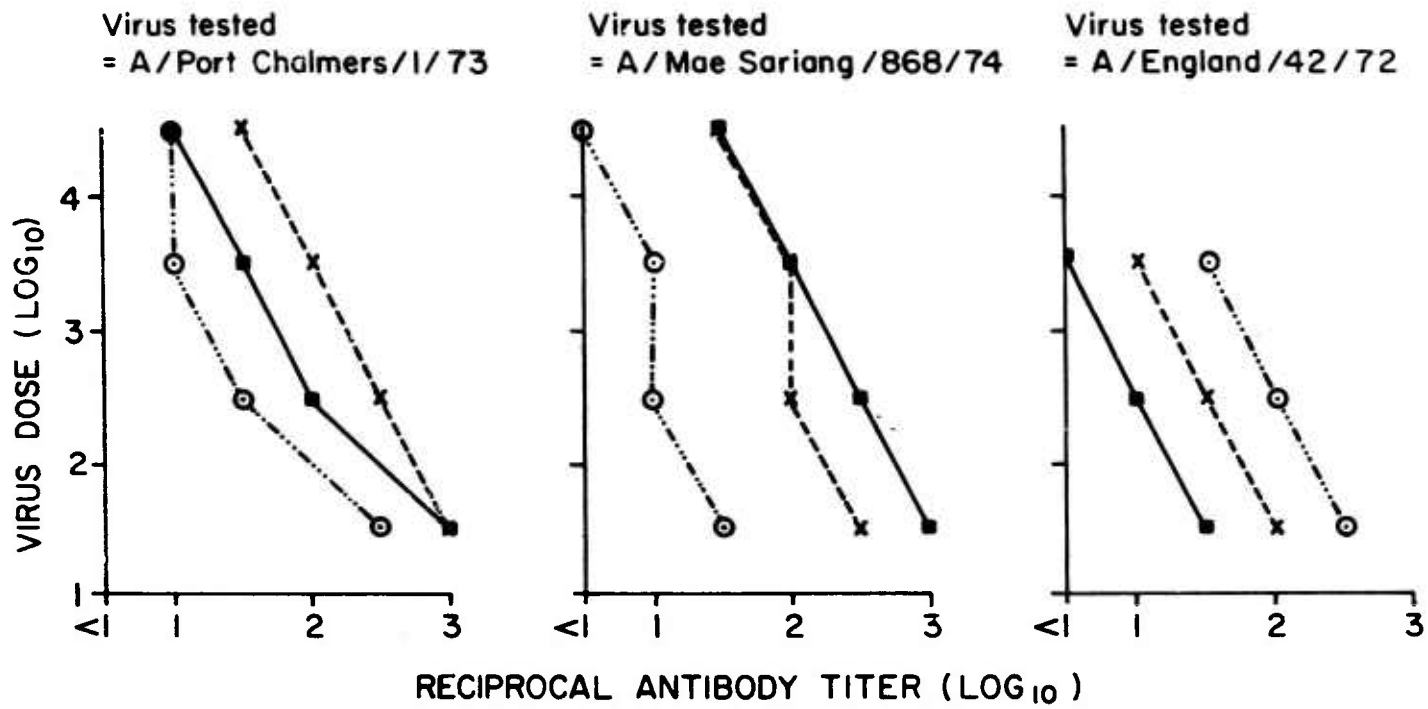

Figure 4. Quantitative relationships between recently isolated A/Influenza/H_3N_2/ strains. Comparisons of neutralization reactions of A/Port Chalmers/1/73, A/Mae Sariang/868/74 and A/England/42/72 showing dissimilar patterns of neutralization. Antisera tested x----x A/Port Chalmers/1/73, ■——■ A/Mae Sariang/868/74, ⊙ –·–·–⊙ A/England/42/72

was the gathering of individuals from many villages at the KBA meeting. The almost exclusive involvement of Christian villages, as opposed to non-Christian villages, implicate this meeting as a point source for the local epidemic. This led to the infection of people from widely scattered villages and ultimately to a simultaneous increase in disease over a large area. The epidemic probably would not have been recognized were it not for the activities of the CMU mobile unit with its program of medical service to isolated villages.

The data collection for this study is complete. We are awaiting the final identification of strains of influenza virus isolated in Bangkok during the summers of 1973 and 1974. Upon receipt of this information this work will be analysed and prepared for publication.

E. RABIES

1. Rabies Exposure During Pregnancy

OBJECTIVE: To study transfer of rabies virus and antibody across the human placenta.

BACKGROUND: Rabies virus has been shown to cross the placenta in experimental infections in many species (35-37). Transplacental transmission of this virus has also been reported following a naturally acquired infection in a pregnant cow (38). This phenomenon has not been reported to occur in man. We have recently seen two patients with rabies exposure during the third trimester of pregnancy.

PROGRESS: The first patient, a 43 year old Thai female, was approximately eight months pregnant when she was bitten on the leg by a stray dog. The patient cleansed the wound with soap and water and sought medical advice. No specific antirabies therapy was initiated, presumably because she was pregnant.

On 6 December 1972 the patient delivered a healthy male infant, without complications. On 8 December 1972, the patient's second postpartum day, she developed symptoms of encephalitis. Her condition progressively deteriorated and she died on 12 December 1972. Corneal impressions and a blood sample were obtained two hours prior to her death. An autopsy was performed. The corneal impressions and tissues obtained at autopsy were fixed and stained with fluorescein labelled anti-rabies globulin (39). The fluorescent antibody stained antemortem and postmortem corneal impressions were positive, while skin taken from the wound site was negative. Positive results were also obtained from the hippocampus, cerebrum, cornea and lacrimal gland. The serum sample contained no

neutralizing antibodies to rabies (titer <1:5) by mouse serum neutralizing antibody test. Serum specimens from the infant were also negative for rabies neutralizing antibodies in the neonatal period and again one year later. The child is alive and well at two years of age.

Case 2 was a 29 year old Caucasian female. At 35 weeks gestation the patient was bitten on the hand and arm by a pet cat subsequently found to be rabid in our laboratory. The wounds were cleansed with soap and water and 4000 U of antirabies hyperimmune serum was administered intramuscularly. Immunization with killed rabies virus vaccine (Duck Embryo Origin) was initiated. The immunization schedule was single, daily injections for fourteen days followed by three boosters on days 24, 34, and 54. On 18 April 1974, at 39 weeks gestation, the patient's labor was induced and she delivered a healthy male infant without difficulty. A maternal serum sample was obtained prior to induction, and cord blood was collected at delivery. Serum samples were also obtained from the patient and her infant at three and six weeks postpartum. All serum samples were evaluated for the presence of rabies neutralizing antibodies. The results of these tests are indicated in Table 1. The patient received a booster immunization at four weeks postpartum. Both the patient and her infant are alive and well nine months after delivery.

Table 1. Serum Rabies Neutralizing Antibody Titers Obtained from Patient 2 and her Infant

SAMPLE	TITER
Preinduction	
Maternal blood	1:70
Cord blood	1:30
Three weeks postpartum	
Maternal blood	1:40
Infant blood	1:5
Six weeks postpartum	
Maternal blood	1:80
Infant blood	< 1:5

DISCUSSION: The infant of the first patient demonstrated no evidence of acquiring his mother's infection. Additionally he had no detectable immune response to rabies virus. These findings suggest that the virus did not cross the placenta.

The second patient demonstrated a good immunologic response to antiserum and vaccine. The presence of antibodies in her infant's serum that rapidly decreased in titer with time suggests that these antibodies crossed the placenta resulting in passive immunization. Antibody titers obtained from the mother at the time of induction and from the cord blood are probably different. It may be speculated that this difference arises from an IgM contribution to the maternal titer that did not cross the placenta.

It is not our opinion that in our single case report we have established the safety of antirabies immunization during pregnancy. We do feel, however, that pregnancy is no contraindication to rabies immunization.

2. Animal Rabies in Thailand: Rabies Diagnostic Laboratory Services

OBJECTIVE: To provide rabies diagnostic services to U.S. military personnel in Southeast Asia and the Western Pacific.

DESCRIPTION: Every brain submitted to this laboratory was examined by both the fluorescent antibody test and by mouse inoculation. Agreement between the two tests was 99.87%.

PROGRESS: Of 758 brain specimens examined, 253 (33.4%) were positive (Table 1). The prevalence of rabies in the dog (42.2%) and in the cat (11.5%) was slightly less than in recent years.

F. MISCELLANEOUS

1. Prevalence of Some Viral Infections in the Residents of Phnom-Penh

OBJECTIVE: To survey the experience of residents of Phnom-Penh with polioviruses, mosquito-borne arboviruses and hepatitis B virus (HBV).

BACKGROUND: The question of transmission of hepatitis B virus remains an enigma. It has been shown that virus may be passed by the parenteral route, and contaminated needles or parenteral preparations were implicated in the transmission of disease. Recently the question of arthropod transmission has been raised as a mode of transmission in the tropics. Non-parenteral

Table 1. Summary of Rabies Diagnoses - 1 April 1974-31 March 1975

Species	Number of Specimens	Number Positive	Percent Positive
Canine	555	234	42.2
Feline	122	14	11.5
Human	2	2	100.0
Rodent	24	2 (a)	8.3
Primate	21	1 (b)	4.8
Rabbit	11	0	0
Bat	10	0	0
Squirrel	5	0	0
Other (c)	8	0	0
Total	758	253	33.4

(a) Two rats from the Republic of the Philippines

(b) Pet gibbon from Nakorn Panom

(c) 3 civets, 1 goat, 1 mole, and 3 guinea pigs

transmission has also been demonstrated experimentally. Epidemiological investigations have implicated this mode of transmission in tropical areas.

Sera from a Cambodian population were collected by the National Blood Transfusion Center and the Institute of Biology, Phnom-Penh, in a pre-polio immunization study to determine the optimum age of polio immunization in this population. This population resided in a tropical area with generally poor sanitation, and no known recent polio immunization. Biting arthropods are prevalent in Phnom-Penh and many of these are known to be virus vectors. Prior studies had indicated that this population should have a high prevalence of hepatitis B surface antigen (HB_sAg) carriers (1). This information suggested that a comparison of the experience with polio, arboviruses and hepatitis B infections in this population might shed some light on the mode of transmission of HBV in the tropics.

DESCRIPTION: This study was done in collaboration with the National Blood Transfusion Center and the Institute of Biology, Phnom-Penh at the request of Dr. Rene Sansonnens, a WHO representative. Questionnaires were completed by 370 Cambodians presenting themselves for immunization against polio. Sera were obtained from all 370 people prior to immunization.

Sera were screened at a dilution of 1:10 for antibody against polio types one, two and three using a metabolic inhibition technique with LLC-MK_2 cells (40). Antibody to mosquito-borne arboviruses found in Southeast Asia were detected by a hemagglutination inhibition test (40) using Chikungunya (Ross) virus as representative of group A arboviruses, Dengue 2 (New Guinea) and Japanese Encephalitis (Nakayama) viruses as representative of group B arboviruses. Sera with no antibody detectable at a 1:10 dilution were considered to be negative. Hepatitis B surface antigen (HB_sAg) was detected by immunoelectrophoresis (IEOP) using high titered human antiserum. The technique of this test has been previously described (41).

Antibody to hepatitis B surface antigen will be detected by either a radioimmune assay inhibition (RIAI) or a passive hemagglutination test (PHA, Electronucleonics).

PROGRESS: Polio antibody was present in approximately 25% of children under one year of age (Table 1). Prevalence of antibody increased rapidly, approached 100% by the age of four years and remained between 90 and 100% in all older age groups studied. At least 50% of the children between the age of six to nine years had acquired antibody to all three types of polioviruses. By the age

of 15, approximately 85% of the population had antibody to type one, 92% to type two and 82% to type three. These data indicate a high incidence of orally transmitted polio infection in this population over the first few years of life.

Almost 50% of children under the age of one year had experience with group B arbovirus infections (Table 2). This figure dropped significantly in the second year of life to 38% but rose rapidly approaching 100% by the fourth year of life. Antibody to group A arboviruses rose more slowly, being present in 26% of children studied under one year of age. As with group B arboviruses, antibody prevalences fell during the second year. After that, group A antibodies rose more slowly than group B, approaching 100% by 13 years of age.

Twenty-nine individuals or 8% of this population were found to be HB_S antigenemic at the time of collection (Table 3). HB_SAg was found in 4.6% of children under four years of age and rose to about 19% by the age of 15-19. The age specific point prevalence of anti-HB_S remains to be determined.

DISCUSSION: A conclusion from this work is that vaccination against poliomyelitis in this population is indicated for children under the age of four years.

These data indicate extremely rapid transmission of both polio (acquired by the oral route) and group A and B arboviruses (acquired by the parenteral route). HB_SAg appeared in very young children but it reached a peak prevalence later than that of polio or arbovirus antibody (Figure 1).

The prevalence of antibody to HB_SAg has not yet been determined. It remains to be seen whether the transmission rate of HBV is similar to that of polio or arboviruses or occurs at a slower rate. Experience in Bangkok, with a lower socio-economic housing development suggests that the latter might be the case.

Table 1. Age specific point prevalence of polio neutralizing antibody in residents of Phnom-Penh

Age (years)	No. Tested	Negative Ab all 3 types		Positive Ab all 3 types		Positive Neutralizing Antibody to					
						Polio 1		Polio 2		Polio 3	
		No.	%	No.	%	No.	%	No.	%	No.	%
0-1	20	15	75.00	2	10.00	3	15.00	3	15.00	3	15.00
1-2	29	12	41.38	1	3.45	10	34.48	6	20.69	11	37.93
2-3	15	1	6.67	2	13.33	8	53.33	4	26.67	10	66.67
3-4	22	7	31.82	4	18.18	11	50.00	10	45.45	8	36.36
4-5	25	1	4.00	2	8.00	14	56.00	17	68.00	12	48.00
5-6	36	1	2.78	9	25.00	26	72.22	29	80.56	20	55.56
6-9	36	2	5.56	18	50.00	23	63.88	31	86.11	25	69.44
9-12	21	1	4.76	13	61.90	15	71.42	18	85.71	17	80.95
12-15	40	0	0	26	65.00	34	85.00	37	92.50	33	82.50
15-20	30	1	3.33	16	53.33	23	76.67	28	93.33	20	66.67
20-25	50	2	4.00	26	52.00	36	72.00	45	90.00	32	64.00
25-30	30	3	10.00	15	50.00	23	76.67	26	86.67	16	53.33
30-35	15	1	6.67	7	46.67	12	80.00	14	93.33	8	53.33
35-40	1	0	0	1	100.00	1	100.00	1	100.00	1	100.00

Table 2. Age specific point prevalence of HI antibody to mosquito-borne arboviruses in residents of Phnom-Penh

Age (years)	No. Tested	No Ab to both Gr. A and Gr. B		Positive Ab to both Gr. A and Gr. B		Positive Ab to Gr. A		Positive Ab to Gr. B	
		No.	%	No.	%	No.	%	No.	%
0-1	19	8	42.18	3	15.79	5	26.50	9	47.36
1-2	29	18	62.00	5	17.24	5	17.24	11	37.93
2-3	15	4	26.70	3	20.00	3	20.00	11	73.33
3-4	23	1	4.34	6	26.08	7	30.43	21	91.30
4-5	25	3	12.00	7	28.00	7	28.00	22	88.00
5-6	35	4	11.42	14	40.00	15	42.85	30	85.71
6-9	36	2	5.56	19	52.78	19	52.78	34	94.44
9-12	21	1	4.76	13	61.90	13	61.90	20	95.24
12-15	39	0	0	36	92.30	36	92.30	39	100.00
15-20	29	0	0	24	82.75	24	82.75	29	100.00
20-25	50	0	0	49	98.00	49	98.00	50	100.00
25-30	30	0	0	30	100.00	30	100.00	30	100.00
30-35	15	0	0	14	93.33	14	93.33	15	100.00
35 +	1	0	0	1	100.00	1	100.00	1	100.00

Table 3. Age specific point prevalence of HBsAg in residents of Phnom-Penh

Age (years)	Male			Female			Total		
	No. Tested	HBsAg +ve		No. Tested	HBsAg +ve		No. Tested	HBsAg +ve	
		No.	%		No.	%		No.	%
0-4	55	4	(7.3)	53	1	(1.9)	108	5	(4.6)
5-9	35	2	(5.7)	46	3	(6.5)	81	5	(6.2)
10-14	20	4	(20.0)	31	4	(12.9)	51	8	(15.7)
15-19	9	2	(22.2)	23	4	(17.4)	32	6	(18.8)
20-29	3	1	(33.3)	68	4	(5.9)	71	5	(7.0)
30-39	2	0	(0)	16	0	(0)	18	0	(0)
Total	124	13	(10.5)	237	16	(6.8)	361	29	(8.0)

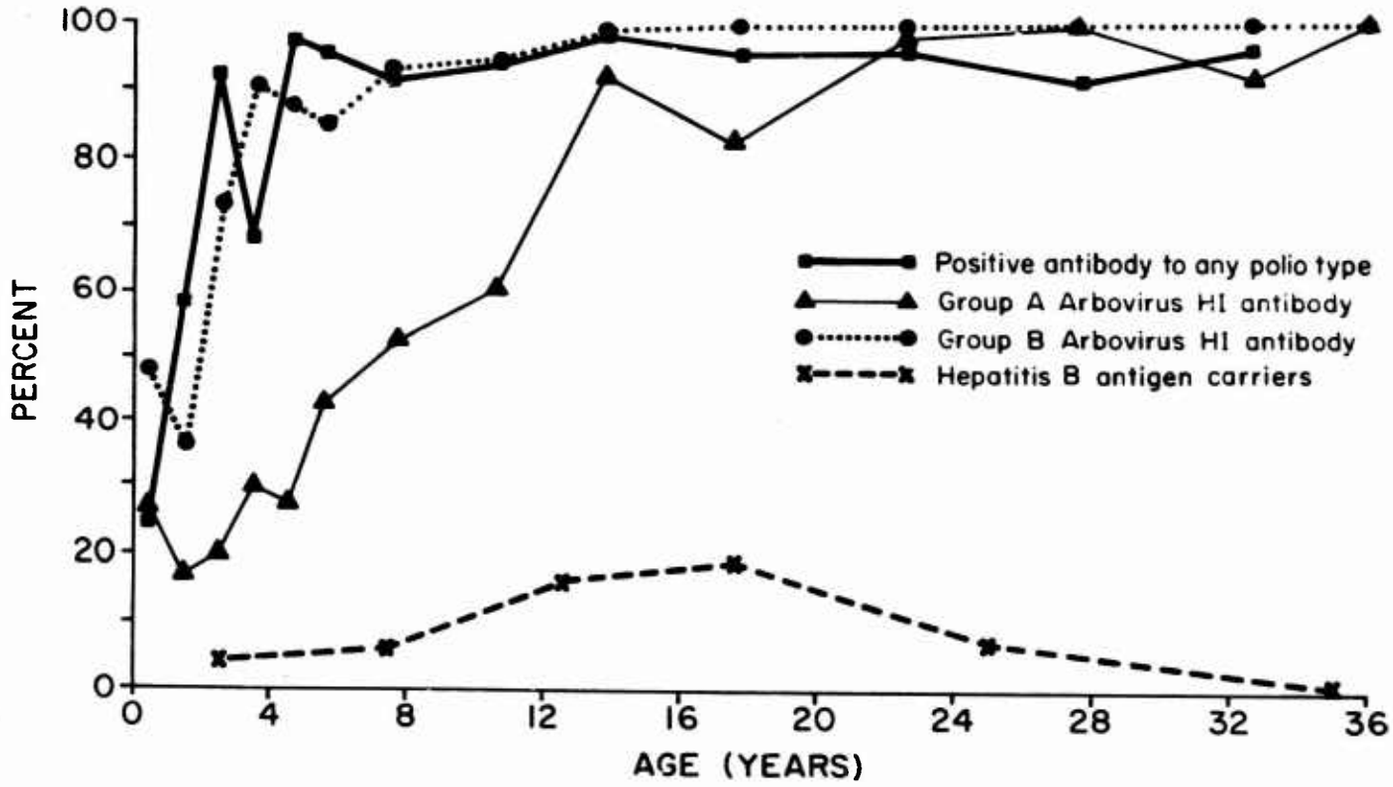

FIGURE 1. AGE SPECIFIC POINT PREVALENCE OF HI MOSQUTO-BORNE ARBOVIRUSES, POLIO NEUTRALIZING ANTIBODIES AND HEPATITIS B ANTIGEN CARRIERS IN RESIDENTS OF PHNOM-PENH (JUNE 1973)

II. BACTERIAL DISEASES OF MAN AND ANIMALS

A. GONORRHEA

1. Changing Penicillin Resistance of the Gonococcus in Thailand

OBJECTIVE: To determine if the penicillin resistance of the gonococcus in Thailand is changing.

BACKGROUND: Although the relative resistance of the gonococcus to penicillin has been shown to be decreasing in two geographical locales (42, 43), most reports indicate that the organism is becoming more resistant at a very alarming rate (44, 45).

DESCRIPTION: Test organisms consisted of all clinical isolates of N. gonorrhoeae submitted for confirmation to the Department of Microbiology, SEATO Medical Research Laboratory, Bangkok, Thailand between 1 January 1972 and 31 December 1974. Specimens were clinical isolates submitted from clinical facilities in Thailand. These facilities included: Royal Thai Army Hospital, Bangkok; US Army Hospital, Bangkok; and SEATO Medical Research Laboratory venereal disease field team. The number of isolates evaluated was: 752 in 1972; 622 in 1973; and 867 in 1974.

All organisms were confirmed to be N. gonorrhoeae by standard techniques previously reported. Organisms confirmed as N. gonorrhoeae were grown on PB plates (Proteose #3 agar + 1% Hemoblobin + 1% IsoVitaleX) overnight. The colonies were scraped off with a sterilized swab and heavy suspensions were prepared in tryptose phosphate broth. The suspensions were standardized spectrophotometrically (Coleman Jr. II, Spectrophotometer, Coleman Istruments Maywood, Ill.) with a $BaSO_4$ solution (0.5 ml 1% $BaCl_2$ + 99.5 ml 1% H_2SO_4) to a final bacterial count of 10^7 colony forming units/ml. Inoculum-replicating equipment (Div. of Instrumentation, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C.) placed 0.001-0.003 ml of inoculum in a spot on the surface of a plate containing Mueller-Hinton Agar + 5% defibrinated, chocolatized sheep blood and added 1% IsoVitaleX. Serial dilutions of penicillin G were added to the plates for penicillin minimum inhibitory concentration (MIC) determinations. The concentrations tested were: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 1.8 and 2.0 units/ml. One additional plate to which no penicillin was added was also evaluated. A Staphylococcus strain, S.aureus ATTCC 6538 P was used as a control for the penicillin content of the plates. The plates were stored at

4°C after preparation and were employed within three days. Before use the plates were dried of excess moisture in the incubator. No moisture droplets were on the surface of the media or petri dish lides when inoculations were made. The inoculated plates were evaluated after overnight incubation (20-24 hrs.). The MIC was determined as the smallest concentration of antibiotic required to inhibit growth completely. In reading the plates, a barely visible haze of possible growth or a single macroscopic colony was disregarded.

Three study populations consisting of all isolates sqbmitted within a given calendar year were compared. In order to apply statistical methods in evaluating the data all isolates with MIC's of less than 0.5 units/ml or more than 2.0 units/ml were excluded. Thus the MIC data for 14 organisms in 1972, 27 in 1973 and 130 in 1974 were not used in calculating means, standard errors and significance of differences. The MIC results of 362 organisms collected between April 1972 and January 1973 have been discussed elsewhere (46). Analysis of variance was utilized to determine if between-group differences existed (47). Student's test for unpaired variates was employed to determine the significance of differences between the mean MIC's of the organisms tested within a given year (47).

PROGRESS: The mean penicillin minimum inhibitory concentration $\pm$ 2 standard errors for gonococci isolated in 1972, 1973 and 1974 were 0.58 $\pm$ 0.02, 0.72 $\pm$ 0.04 and 1.05 $\pm$ 0.04, respectively. Analysis of variance for between-group differences of these three populations was significant ($F = 215.5$, $df = 2067$, $P < 0.001$).

MIC data obtained in 1972 was significantly different from that of 1973 ($p < 0.001$). An identical result was found when the 1973 data was compared to 1974 ($p < 0.001$).

The data are graphically represented in Figures 1, 2 and 3. Each year the proportion of isolates with a penicillin MIC of more than 2.0 units/ml increased. The mean MIC $\pm$ 2 S.E.'s for each of the three years is displayed in Figure 4. Calculations of means and standard errors in Figure 4 were performed excluding the data extremes as explained above.

SUMMARY: Penicillin minimum inhibitory concentrations (MIC) were determined for 2241 gonococcal isolates submitted to the SEATO Medical Research Laboratory between 1 January 1972 and 31 December 1974. Isolates were separated into three groups, determined by the calendar year in which they were submitted.

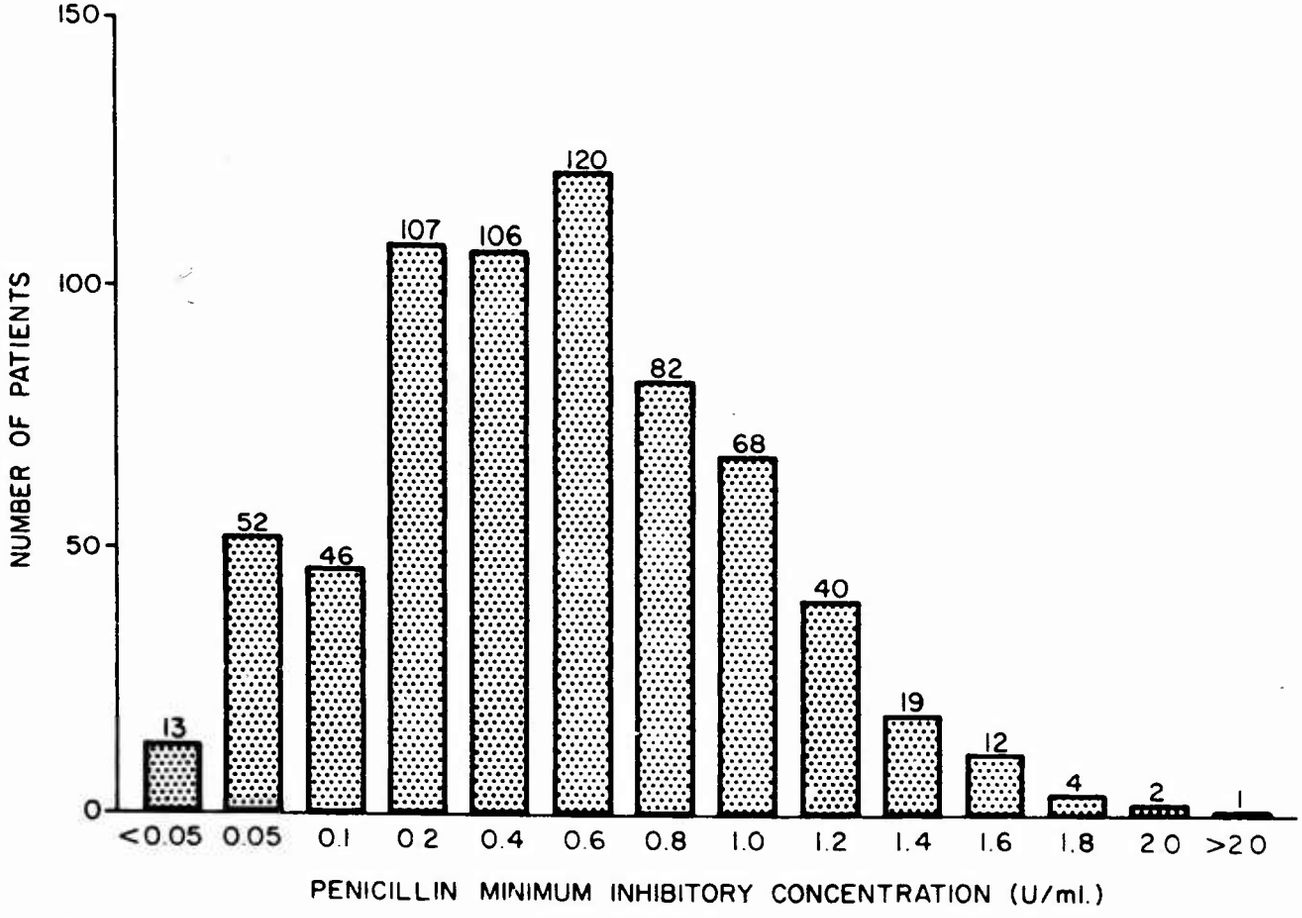

FIGURE 1. PENICILLIN MINIMUM INHIBITORY CONCENTRATION FOR NEISSERIA GONORRHOEAE ISOLATED IN 1972. DATA EXTREMES (0.05U/ml. >MIC >2.0U/ml.) WERE NOT INCLUDED IN CALCULATIONS OF MEAN AND STANDARD ERROR

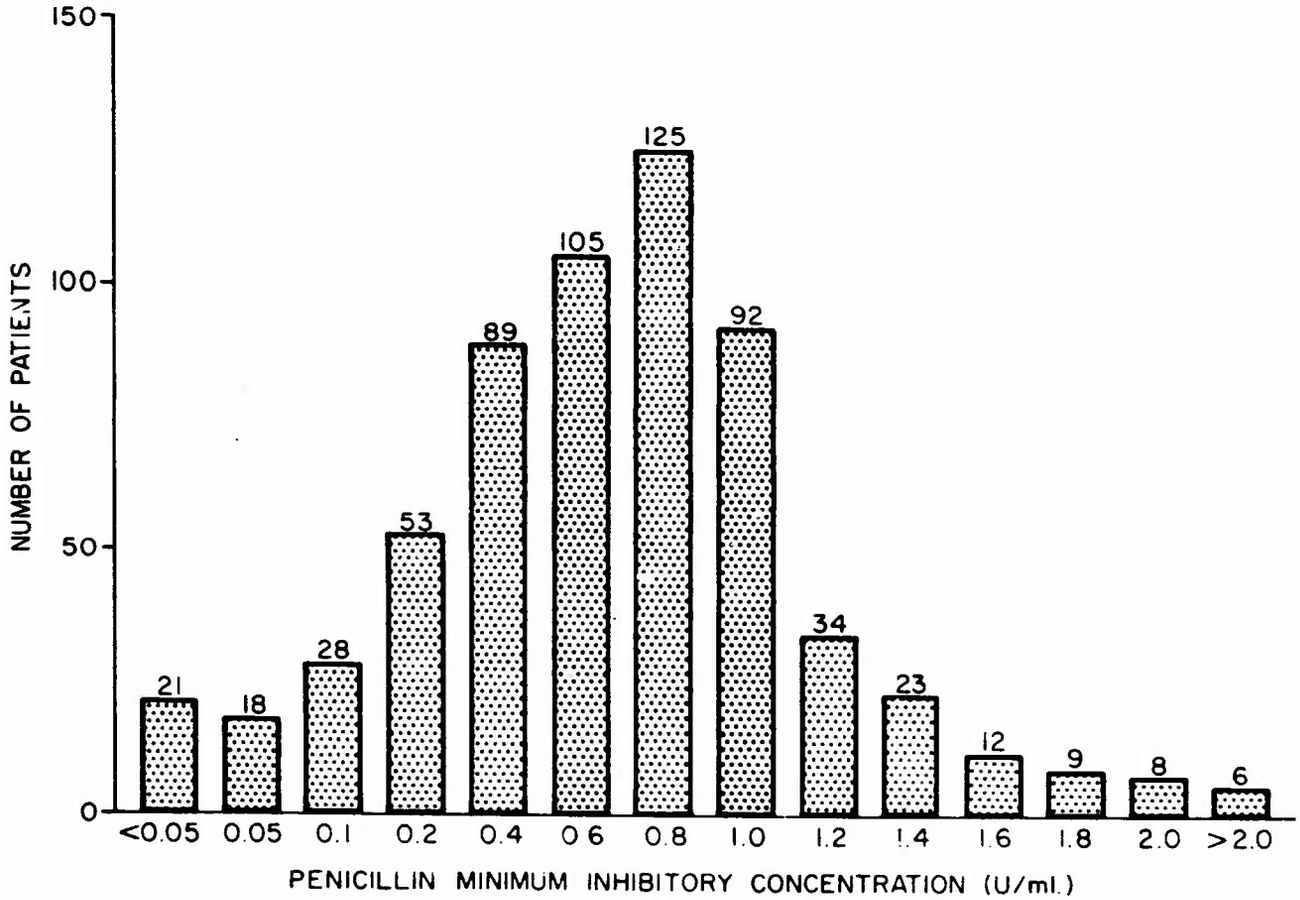

FIGURE 2 PENICILLIN MINIMUM INHIBITORY CONCENTRATION FOR NEISSERIA GONORRHOEAE ISOLATED IN 1973. DATA EXTREMES (0.05 U/ml. >MIC >2.0 U/ml.) WERE NOT INCLUDED IN CALCULATIONS OF MEAN AND STANDARD ERROR

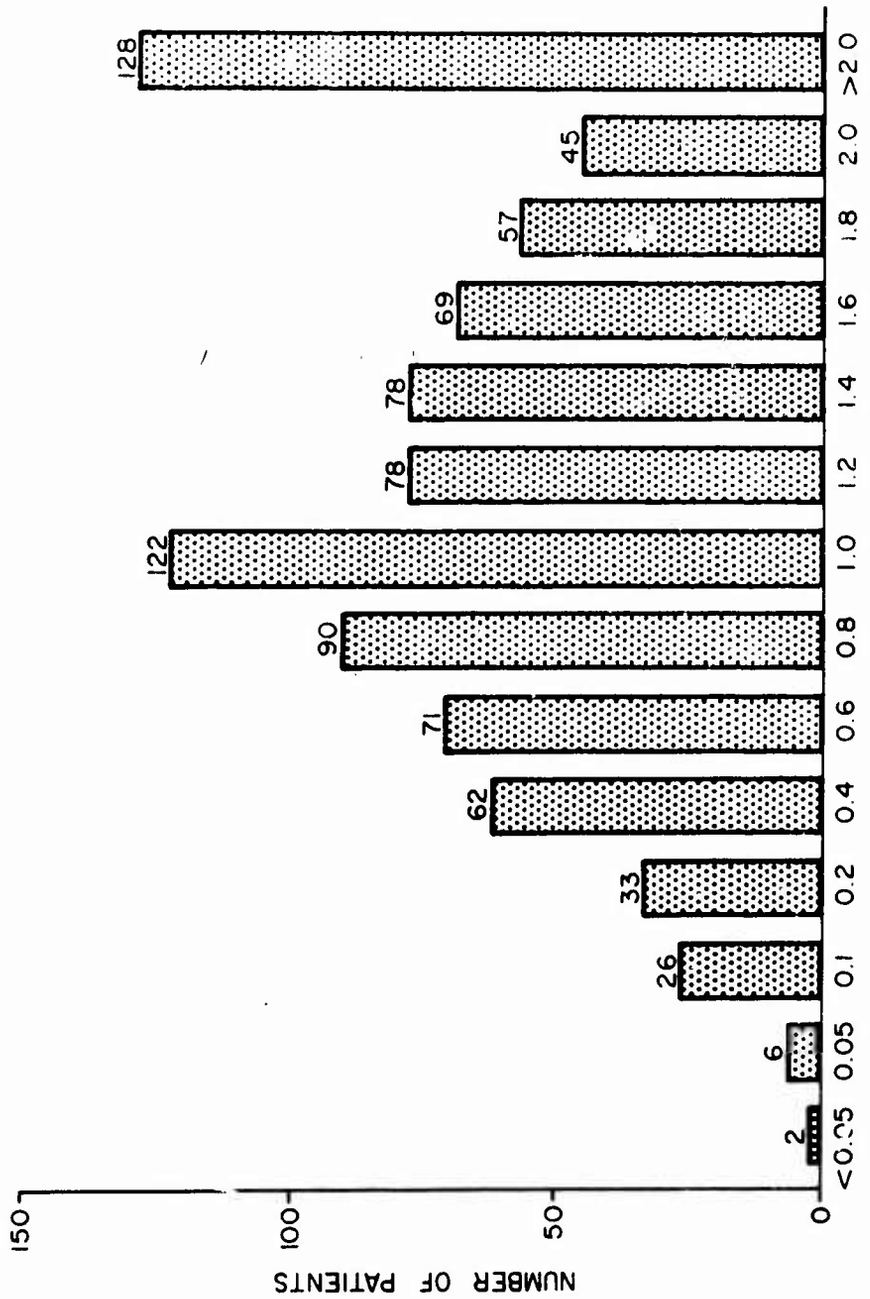

FIGURE 3. PENICILLIN MINIMUM INHIBITORY CONCENTRATION FOR NEISSERIA GONORRHOEAE ISOLATED IN 1974. DATA EXTREMES (0.05 U/ml.>MIC>2.0 U/ml.) WERE NOT INCLUDED IN CALCULATIONS OF MEAN AND STANDARD ERROR

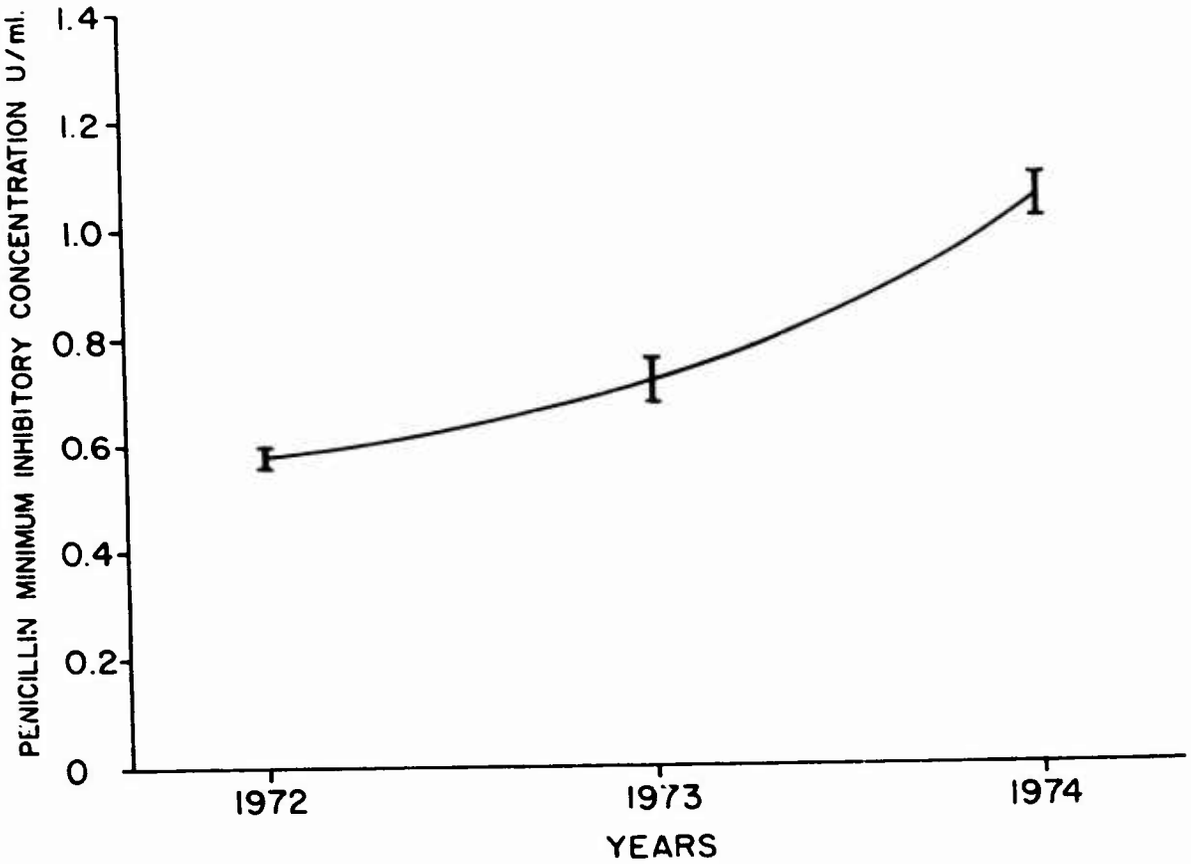

FIGURE 4. MEAN PENICILLIN MINIMUM INHIBITORY CONCENTRATION ± 2 S.E. FOR NEISSERIA GONORRHOEAE ISOLATED IN 1972, 1973 AND 1974. DATA EXTREMES (0.05U/ml. >MIC >2.0U/ml.) WERE DELETED IN MEAN AND STANDARD ERROR CALCULATIONS

The mean penicillin MIC ± 2 standard errors was calculated for each of the three groups. These values were 0.58 ± 0.02 in 1972, 0.72 ± 0.04 in 1973, and 1.05 ± 0.04 in 1974. These means were found to be significantly different ($p < 0.001$).

2. Oropharyngeal Gonorrhea During Pregnancy

OBJECTIVE: To compare the prevalence of Neisseria gonorrhoeae infections in a prenatal population of U.S. military dependents to a prenatal population of Thai civilian and a non-pregnant population of female military dependents.

BACKGROUND: Neisseria gonorrhoeae was isolated in 16% of 150 pregnant U.S. military dependents (1). of the 24 positive cultures 23 were obtained from the oropharynx. In view of this finding it was felt that similar studies of Thai prenatal patients and non-pregnant U.S. military dependents should be performed.

DESCRIPTION: Identical techniques to those previously described (1) were employed to obtain specimens from prenatal patients attending the Obstetrics Outpatient Clinic of Women's Hospital, Bangkok, Thailand during the first three months of 1974. A culture of the oropharynx was obtained from all females between the ages of 15 and 24 years who attended the U.S. Army Dental Clinic, Bangkok from 1 January 1974 to 30 March 1974. N. gonorrhoeae was isolated and confirmed by methods discussed previously (1).

PROGRESS: Positive cultures were obtained in 19 of 160 (11.9%) Thai patients attending Women's Hospital. No positive cultures were obtained from the oropharynx in this group. Two of 114 patients (1.8%) attending the Dental Clinic had the gonococcus cultured from their oropharynx.

DISCUSSION: No oropharyngeal infections were found in the patients attending the prenatal clinic of Women's Hospital. These patients were predominately housewives and did not admit to the practice of fellatio. The practice of fellatio is not generally accepted in the culture of Thailand; therefore, one would not expect to routinely find the infection in the oropharynx if the mode of transmission is primarily genital to oral.

The fact that oropharyngeal infections of N. gonorrhoeae were detected in women visiting a Dental Clinic on a routine visit suggests the possibility of this being a means of identifying asymptomatic infections. The low proportion of positive subjects

may partially be attributed to a slightly different subject population in this group when compared to the other two groups.

None of the single women admitted to sexual activity, and although the older patients (35 and over) were still capable of childbearing, it is possible that they had reached an age where the mental attitude, in general, is toward not bearing children, particularly if they are multiparous. Because of this possible change in attitude, the exposure of older women may be less frequent and of a different nature than may occur in the younger age group. Another possible cause for the observed difference between the two U.S. military dependent groups may be related to dental care. Many individuals may have brushed their teeth or used some form of cleanser for their mouth and throat just prior to the sample being taken, thereby making the organism more difficult to recover for culture purposes.

3. Effect of a Copper-containing Intrauterine Device on Neisseria Gonorrhoeae in Vitro

OBJECTIVE: To determine if a copper containing intrauterine device (IUD) inhibits the growth of Neisseria gonorrhoeae in vitro.

BACKGROUND: Copper possesses antifertility properties (48). It also inhibits both adult and fetal cell growth in tissue culture (49). Metallic copper and cupric ion either kill or inhibit the growth of N. gonorrhoeae in vitro (50).

DESCRIPTION: Thayer-Martin selective media (G.C. Agar Base, hemoglobin VCN and IsoVtaleX, Baltimore Biological Laboratories, Cockeysville, Maryland 21030 U.S.A.) was used for isolation. Determinations of growth inhibition by copper were made on a typing medium developed by Kellogg (51). All plates were incubated at 36°C for 24 or 48 hours in candle jars in an increased CO_2 atmosphere. Electrolytic copper wire, 0.5 mm in diameter, was cut to one inch lengths and sterilized by autoclaving. An electrolytic copper plate was cut into discs approximately 1.5 cm in diameter and sterilized by autoclaving. The copper-containing IUD (Gravigard) was furnished by G.D. Searle (Thailand) Ltd. and was aseptically prepackaged. The strain of N. gonorrhoeae used was isolated from the urethral exudate of a male with acute urethritis. The exudate was streaked directly on freshly prepared Thayer-Martin media which was then incubated at 36°C in a candle extinction jar. The culture was inspected at 24 and 48 hour intervals and those colonies with gross morphology resembling Neisseria were subjected to Gram staining and tested with

Figure 1. Inhibition of the growth of gonococci by a copper-containing intrauterine device (Gravigard). A zone of inhibition indicated by arrows at the margins may be seen surrounding the copper-containing portion of the IUD

Figure 2. Inhibition of the growth of gonococci by a copper-containing intrauterine device (Gravigard). The IUD has been removed to better demonstrate the zone of inhibition. Although growth has been inhibited in the area where the plastic was present (arrows), it is believed that this is not due to any peculiar characteristic of the plastic, rather to the media being covered as discussed in the text

oxidase reagent. All colonies of Gram negative diplococci that gave positive tests with oxidase reagent were verified as N. gonorrhoeae by sugar fermentation.

The copper wires, discs and copper-containing IUD were placed on Kellogg's typing medium previously streaked with gonococci and incubated at 36°C for 48 hours under an increased CO_2 atmosphere.

PROGRESS: The copper wires and discs inhibited the growth of N. gonorrhoeae as previously described (50). In addition, the copper coil portion of the Gravigard IUD also inhibited the growth of the organism (Figure 1). When the IUD was removed from the medium (Figure 2), it was observed that the presence of the plastic portion of the IUD had also prevented growth; however, we believe that this was not due to the plastic, but to the fact that the medium had been covered as no zone of inhibition was observed in these areas. A similar absence of growth has also been observed when plastic tubing, an inert material, was placed on the medium. The area surrounding the copper coil (approximately 2 mm on either side of the IUD), however, did not have bacterial growth.

SUMMARY: This preliminary study suggests that the copper-containing IUD may play a useful role in preventing gonorrhea. The solubilization of copper has been estimated at one microgram of copper per day (50) which is within the known bactericidal range in vitro suggesting that growth of N. gonorrhoeae may be inhibited for a period of time after the IUD has been inserted in the uterus.

4. Microbial Flora Present in the Anterior Urethra of Venereal Disease Patients

OBJECTIVE: It is the purpose of this study to determine the microorganisms present in the anterior urethra of males attending a Venereal Disease Clinic.

BACKGROUND: Microorganisms isolated from patients with urethritis may be clearly pathogenic or they may be organisms which are commonly associated with normal flora of the skin. Gram positive cocci have frequently been implicated in non-specific urethritis (NSU) and urinary tract infections (52, 53, 54). Anaerobic organisms have been reported by various investigators as pathogens of the genito-urinary tract (55, 56).

DESCRIPTION: The patient population consisted of 72 men attending the Venereal Disease Clinic of the Royal Thai Army Hospital,

Bangkok, Thailand. Patient's ages ranged from 17-24 years (average 22.8). The patients were separated into two groups. Group I consisted of all patients presenting with symptoms of urethritis. Group II were those patients with venereal disease other than urethritis. Both groups were sexually active, and of the same age and socioeconomic position. Specimens for culture were obtained from the anterior urethra of all patients using a calcium alignate naso-pharyngeal swab. The swab was roll-streaked on various plated media immediately after it was obtained. A duplicate specimen was obtained from the anterior urethra for the culture of anaerobic organisms. All clinical specimens were inoculated into three basic media: modified Thayer-Martin (TM) media (57), 5% sheep blood agar plates (BAP), and anaerobic broth media. The anaerobic media consisted of a pre-reduced broth (Basal Medium-PY-Peptone Yeast) which was used as a transport medium until the sample could be transferred to chopped meat media (58). The cultures were transferred to the laboratory at the close of the clinic (normally less than two hours after collection) where they were streaked for isolation.

The plate media were incubated at 35°C under increased CO_2 tension (candle extinction jar). The samples for anaerobic culture were removed from the basal media by piercing the rubber stopper with a sterile needle and aspirating the fluid with a syringe. The specimen was then inoculated into chopped meat broth media under a stream of CO_2 gas rendered free of trace amounts of oxygen by passing through a heated copper oven (Sergen Welch Scientific Co.). The tubes were sealed with rubber stoppers and shrinkable cellulose sealing bands (Pharmaceutical Laboratory, Perry Point, Md.). Simultaneously a 0.5 ml portion of the basal media was inoculated onto duplicate BAP's and streaked for isolation. The plates were incubated in an oxygen free atmosphere using Gas-Paks (Baltimore Biological Laboratories, Cockysville, Maryland) at 37°C for 48 hours. When colonies developed they were subcultured to biochemicals for identification. The chopped meat broth cultures were observed for a maximum of two weeks and then discarded if bacterial growth was not detected by Gram stain. If growth developed they were subcultured to BAP's and incubated anaerobically at 37°C for 24-48 hours. Colonies isolated on BAP's were further subcultured to biochemicals for identification. All biochemical subcultures were observed for a maximum of 14 days before discarding.

All aerobic plates were examined after 24 hours of incubation for the growth of colonies with morphology resembling that of Neisseria sp. In some cultures it was necessary to incubate the

Table 1. Microorganisms Found in the Anterior Urethra of Urethritis Patients Compared with Those Having Venereal Disease Other Than Urethritis

Organisms Found	V.D. Other Than Urethritis (35 Patients)	Urethritis (37 Patients)
Gram-positive Cocci	29 (83%)	32 (87%)
Anaerobic Organisms	21 (60%)	26 (70%)
Anaerobes and Gram-positive Cocci	18 (51%)	22 (59%)
Neisseria gonorrhoeae	2 (5.7%)	15 (41%)

Table 2. Microorganisms Found in the Anterior Urethra of Gonococcal (GC) Urethritis and Non-gonococcal Urethritis Patients

Organisms Found	G.C. Urethritis (15 Patients)	Non-G.C. Urethritis (22 Patients)
Gram-positive	4 (27%)	5 (23%)
Anaerobes	1 (6.7%)	2 (9.1%)
Anaerobes and Gram-positive Cocci	9 (60%)	12 (55%)
Neisseria gonorrhoeae only	1 (6.7%)	0
Fecal Organisms	0	3 (14%)

plates an additional 24 hours before growth was adequate for evaluation. Suspect colonies were identified as N. gonorrhoeae on the basis of morphology, oxidase reaction, Gram stain, and sugar fermentations. Organisms which were inhibited on TM media were evaluated using BAP's. Subsequent identification employed tubed biochemical media. Significance of differences between groups was determined by Chi-square testing employing Yates correction (59).

PROGRESS: There were no significant differences in the recovery of Gram positive cocci between the two groups. Anaerobic organisms were found only slightly less frequently than the Gram positive cocci. They were isolated 10% more often in patients with urethritis as compared to those with other venereal diseases (Table 1). This difference is not significant.

Neisseria gonorrhoeae was found in 40.5% of the patients in Group I. In Group II patients, 5.7% were found to harbour N. gonorrhoeae, thus representing the asymptomatic carrier (Table 1).

Two patients were found to have Staphylococcus aureus and three had fecal organisms. None of these occurred along, but were found concomitantly with other Gram positive cocci and anaerobes. There were no significant differences in the isolation of anaerobes and Gram positive cocci in patients with gonococcal urethritis and non-gonococcal urethritis (Table 2).

DISCUSSION: Non-specific urethritis is frequently a complaint of males attending military health clinics. The cause of this condition is currently unknown. Gram positive cocci, diphtheroid bacilli, chlamydia, and the T - strain of mycoplasma have all been alleged to be causative agents (57). Gram positive cocci have been found by some observers to be the cause of NSU. However, we found these organisms present with nearly identical frequencies in both of our study groups. It may be inferred from this observation that while the Gram positive cocci may be pathogenic they are frequently present as opportunist commensals. The susceptibility of the individual may in some way lead to the disease state, but this is unclear at this time.

Anaerobes were found in 60% of our nonurethritis patients and 70% of those with urethritis. In another sample population (unpublished observations) consisting of 17 normal healthy males, we found 15 (88%) harbouring anaerobes in the urethra. Anaerobes have been found in the urethra of both symptomatic and asymptomatic males attending a venereal disease clinic. These findings confirm the presence of anaerobes in the male anterior urethra.

Swenson, et al found anaerobic bacteria causative in 80% of female genital tract infections (60). These organisms have also been found in normal vaginal secretions (61). Therefore, they should be considered as part of the normal microbial flora in both the male and female genital tract. They may serve as sources of genito-urinary infections in susceptible sexually active males.

5. An Epidemiological Survey of Males with Urethritis Attending a Military V.D. Clinic in Thailand

OBJECTIVE: To review data obtained from an informal questionnaire of male patients with a urethral discharge and to correlate this information with their smear and culture results.

BACKGROUND: A Venereal Disease Workshop (SEATO Medical Research Laboratory, June 1974) revealed an increasing frequency of venereal dise-se among American military forces in Thailand. Little is known of factors influencing this increase. It is the purpose of this study to determine if the sexual and social habits of patients correlate with their smear and culture results.

DESCRIPTION: Data from 139 males presenting to the U.S. Army Dispensary, Camp Samaesan, Thailand, were studied. An informal questionnaire was verbally administered to each patient by a technician.

For all patients a smear of the urethral discharge was prepared, Gram stained and microscopically examined for the presence of Gram negative intracellular and ext-acellular diplococci morphologically resembling Neisseria gonorrhoeae. A culture was obtained by introducing a calcium alginate nasopharyngeal swab into the anterior urethra. Appropriate media were streaked for the isolation of anaerobes, aerobes and N. gonorrhoeae.

PROGRESS: The information obtained by questionnaire is currently being correlated with the laboratory results.

6. Resistance of Gibbons (Hylobates lar) to Gonococcal Infection

OBJECTIVE: To determine if the white-handed gibbon (Hylobates lar) would serve as a satisfactory host for experimental infection with Neisseria gonorrhoeae.

BACKGROUND: Attempts to produce N. gonorrhoeae infection in gibbons were previously unsuccessful at SEATO Medical Research Laboratory (1). This study is a continuation of that investigation.

DESCRIPTION: Adult male gibbons were inoculated intra-urethrally with 5×10^6 gonococcal colony forming units (CFU) from urethral exudate of ten male patients. The animals were observed for 30 days with daily urethral cultures obtained throughout the testing period.

Urethral exudate gonococci (GC) were incubated at both 39°C (gibbon body temperature) and at 36°C. After incubation CFU's were determined for both incubation temperatures. For comparison, cultures of type one GC (F 62) were also incubated at 39°C and 36°C and CFU's observed.

The gonococcocidal activity of gibbon blood leukocytes was measured. This was accomplished by incubating a mixture of type one GC and leukocytes at 39°C in the absence of serum.

RESULTS: All four gibbons inoculated with urethral exudate showed elevated leukocyte counts and developed a clear discharge, but yielded negative results for urethral smears and cultures for N. gonorrhoeae throughout the 30 days of observation. Urethral exudate gonococci incubated at 39°C. Stock cultures of type one GC (F 62) produced analogous results at both temperatures. In the measurement of the gonococcocidal activity of gibbon leukocytes, it was found that killing was negligible (mean = 53%) at 60 minutes but became significant (mean = 78%) at 120 minutes of incubation.

PROGRESS: We previously reported that type one GC (F 62) incubated as above with human leukocytes were not significantly killed (mean = 40%) at 120 minutes of incubation. That data in combination with the present results suggests that the relatively more efficient gonococcocidal activity of gibbon leukocytes may play a role in the resistance of gibbons to gonococcal infection.

B. VIBRIO INFECTIONS

1. Clinical Observation of Vibrio parahemolyticus Infection in Thailand

OBJECTIVE: To determine the clinical pattern of V. parahemolyticus gastroenteritis in Thai patients, and to evaluate the efficacy of common antimicrobial agents in the treatment of the disease.

BACKGROUND: Studies on V. parahemolyticus infection in Thailand were initiated by SEATO Medical Research Laboratory (SMRL) in 1970. The preliminary study indicated that V. parahemolyticus was a major cause of gastroenteritis in adults in Bangkok (41). At the Bumrasnaradura Infectious Disease Hospital, located in Nonthaburi, this organism was isolated from approximately 25% of the diarrheal patients. Throughout the year the marine environment, (sea water, fish, crabs, oysters) was found heavily contaminated with this halophil bacillus; therefore, sea foods are implicated as the major source of the V. parahemolyticus diarrheal outbreaks in this community. The detailed clinical picture of this disease, its mode of transmission in Thailand, and the efficacy of antimicrobial therapy has not previously been described.

DESCRIPTION: All patients admitted to the Infectious Disease Hospital, Nonthaburi, with symptoms of acute gastroenteritis between September 1973 and August 1974 were included in the study. Rectal swabs for bacterial cultures were obtained daily for three consecutive days. Those patients with positive stool cultures for V. parahemolyticus were selected for the study.

A detailed history of the illness, and clinical findings were recorded. Blood cultures, leukocyte counts, urinalysis, and serum electrolyte determinations were made on the first day of admission, and subsequently as indicated.

Patients were randomly divided into one control group and two test groups. The control group received a placebo plus symptomatic and supportive therapy. One test group received co-trimoxazole (two adult tablets two times a day for five days) plus symptomatic and supportive therapy..The remaining group received oral tetracycline (40 mg/kg/day for five days) plus symptomatic and supportive therapy. Rectal swabs in each group were obtained and cultured daily for seven days or until cultures were given negative for V. parahemolyticus for the three consecutive days.

RESULTS: Two hundred and twenty eight patients admitted to the hospital during the study period were found to harbor V. parahemolyticus in their diarrheal stools; of these patients 133 were available for clinical evaluation.

Forty three patients were treated with co-trimoxazole, 42 with tetracycline, and 48 were given placebos as a control. All patients were characterized in terms of age, sex, and duration of illness before therapy. These variables were comparable in all three study groups (Table 1).

Table 1. Description of the 133 Patients Studied by Age, Sex, and Duration of Illness

	Placebo	Tetracycline	Co-trimoxazole
Age (Years)			
Less Than 20	8	7	10
20-40	28	22	20
40-60	9	10	10
More Than 60	3	3	3
Sex			
Male	30	30	27
Female	18	12	16
Duration of Illness before Therapy			
Less Than One Day	47	40	43
One-Two Days	1	2	-
Total Patients	48	42	43

Table 2. Clinical Findings of V. parahemolyticus Gastroenteritis in 133 Patients

Clinical Features	No. of Patients	%
Symptoms and Signs		
Abdominal Pain	132	99
Abdominal Distension	59	44
Abdominal Tenderness	6	5
Vomiting	118	89
Fever	59	44
Headache	43	32
Characteristics of the Stool		
Watery	102	76
Semisolid	30	22
Bloody	1	1
Mucus	1	1

Table 3. Sensitivities of 228 Strains of V. parahemolyticus to Eight Antimicrobial Agents

Antimicrobial Agents	Sensitive		Intermediate Sens.		Resistant	
	No. Strain	%	No. Strain	%	No. Strain	%
Co-trimoxazole	228	100	-	-	-	-
Tetracycline	197	86.4	31	13.6	-	-
Chloramphenicol	228	100	-	-	-	-
Ampicillin	2	0.9	12	5.3	214	93.8
Neomycin	98	43	128	56.1	2	0.9
Colistin	70	30.7	72	31.6	86	37.7
Streptomycin	36	15.8	141	61.8	51	22.4
Erythromycin	167	73.3	60	26.3	1	0.4

Table 4. Clinical Response to Antimicrobial Therapy in 133 Patients

Regimens	No. of Patients	No. Improved, Days after Treatment							
		1	2	3	4	5	6	7	Unknown*
Placebo	48	-	5	13	11	10	4	-	5
Tetracycline	42	1	9	10	14	2	-	2	4
Co-trimoxazole	43	1	5	15	12	7	1	-	2

* Unknown = Patients excluded from the study because of incomplete study schedule.

Table 5. Bacteriological Response to Antimicrobial Therapy in 133 Patients

Regimens	No. of Patients	No. with Positive Stool Culture, Days after Treatment						
		2	3	4	5	6	7	Unknown*
Placebo	48	7	20	14	6	-	-	1
Tetracycline	42	10	18	12	-	1	-	1
Co-trimoxazole	43	12	19	7	3	1	1	-

* Unknown = Patients excluded from the study because of incomplete study schedule.

Table 6. Laboratory Findings of V. parahemolyticus Gastroenteritis

Laboratory Findings	No. of Patients	%
Total Leukocyte Count (per cu mm.)		
Less Than 8,000	63	47.72
8,000-10,000	32	24.24
10,000-15,000	30	22.72
More Than 15,000	7	5.30
Total Number of Patients Tested	132	
Serum Sodium (MEq/L)		
Less Than 130	0	
130-150	83	79.80
More Than 150	21	20.19
Total Number of Patients Tested	104	
Serum Potassium (MEq/L)		
Less Than 3.5	12	11.53
3.5-5.5	88	84.61
More Than 5.5	4	3.84
Total Number of Patients Tested	104	
Blood Culture (No growth)	133	100.00

The disease was characterized by acute, profound diarrhea with nausea and vomiting. The predominant symptom was colicky abdominal pain. Fever and headache were observed to a lesser degree. The stool was watery or semisolid without blood or mucus in the majority of cases (Table 2).

Antimicrobial Sensitivity:

Sensitivity profiles are presented in (Table 3). Using the standardized single disc method of Bauer and Kirby (62), it was found that the majority of the isolates (78% to 100%) were sensitive to chloramphenicol, tetracycline, co-trimoxazole, neomycin, erythromycin and streptomycin. Only 6% and 62% of the vibrios tested were sensitive to ampicillin and colistin, respecitively.

Response to Antimicrobial Agents:

Antimicrobial therapy trials comparing oral tetracycline, co-trimoxazole and placebo were performed. There was no great difference in terms of clinical response (Table 4) or bacteriological response (Table 5) among the three groups studied. The majority of patients in each group had negative vibrio stool cultures after four days of therapy.

DISCUSSION: In this study only the severely ill patients requiring hospitalization are presented. The clinical syndrome which we observed in these patients may represent only the severe form of the infection. A complete clinical picture of the mild form of the disease needs to be described. Dehydration was not as severe as that seen in infection with Vibrio cholera. Intravenous fluid therapy was required only for the first few days of the illness. Localization of the infection in the lumen of the intestine is suspected due to the presence of diarrhea without bacteremia, leukocytosis or toxic symptoms (Table 6). Previous experiments on the pathogenicity of V. parahemolyticus using the infant rabbit model indicated that the organism elaborated toxic substances, presumably enterotoxins, into the intestinal fluid (41). Enterotoxins may play a major role in the pathogenesis of this disease.

C. MISCELLANEOUS

1. Detection of Specific Bacterial Antigen by Counter Immunoelectrophoresis (CIE)

OBJECTIVE: To detect specific bacterial antigen in infected body fluids by CIE and to compare the presence of antigen with bacteriological studies of the same specimens.

BACKGROUND: Pneumococcal and Hemophilus influenza b capsular antigens have been detected in the cerebrospinal fluid (CSF) of patients with purulent meningitis at Children's Hospital. H. influenza b antigen has also been detected in subdural effusions from patients with H. influenza b meningitis and in the pericardial fluid from a patient with a pericardial effusion, while pneumococcal antigen has been detected in pleural fluid obtained from patients with pneumococcal empyema.

Bacterial antigen has been detected when Gram's stain and culture results have been negative, thus providing an etiologic diagnosis despite negative bacteriological studies.

DESCRIPTION: CIE was carried out on a variety of body fluids, CSF, subdural fluid, pleural fluid and serum. CIE using commercial antisera against H. influenza b, pneumococcus and meningococcus type A-D; and antisera prepared in rabbits against Staphylococcus aureus was carried out by a previously described method (63).

PROGRESS:

Table 1. Detection of H. influenza b Antigen

Fluid Source	Positive Specimens			Antigen Duration (Mean Days)
	Gram's Stain	Culture	CIE	
CSF	8	12	16	3.8
Subdural	1	1	6	9.1
Pleural	0*	0*	4	Not done
Serum	Not done	Not done	4	4.6

* 1 Specimen: Escherichia coli

H. influenza b antigen was detected in the initial CSF of 16 patients with purulent meningitis (Table 1). Eight patients Gram's stain and cultures for H. influenza b. Four patients had negative Gram's stain and cultures. In eleven patients repeat CSF samples were studied and the mean duration of H. influenza b antigen was 3.8 days (range 0.11 days). Subdural effusions were present in six patients with H. influenza b meningitis. One was Gram's stain and culture positive for H. influenza,b, the remainder were negative. The mean duration of antigen in subdural fluid, from

the day of admission, was 9.1 days (range 1-16 days).

H. influenza b antigen was detected in pleural fluid from four patients One patient had Escherichia coli on Gram's stain and culture of the pleural fluid while the remaining patients had negative Gram's stains and cultures. One of these remaining patients had purulent meningitis and a subdural effusion also; H. influenza b antigen was detected in these fluids as well.

H. influenza b antigen was found in the sera of four meningitis patients with a mean duration of 4.6 days (range 2-6 days) from the day of admission.

Table 2. Detection of Pneumococcal Antigen

Fluid Source	Positive Specimens			Antigen Duration (Mean Days)
	Gram's Stain	Culture	CIE	
CSF	3	5	11	4.3
Subdural	2	2	2	3.5
Pleural	6	7	15	15.2
Serum	Not done	Not done	3	10.7
Pericardial	1	1	1	1

Pneumococcal antigen was detected in the initial CSF fluid of 11 patients with purulent meningitis (Table 2). Three patients had positive Gram's stain and five patients had positive cultures for pneumococci. In nine patients repeat CSF samples were studied and the mean duration of pneumococcal antigen was 4.3 days (range 0-11 days). Subdural effusions were present in two patients with pneumococcal meningitis; both were culture positive and Gram's stain positive. Antigen was detected three and four days after admission.

Pneumococcal antigen was detected in the pleural fluid from 15 patients. Six had postive Gram's stain and seven had positive cultures for pneumococci. The mean duration of antigen in pleural fluid was 15.2 days (range 0-44 days).

Pneumococcal antigen was present in the serum of two patients with meningitis and one patient with empyema for an average of 10.7 days (range 4-15 days) after admission.

One patient with a pyopericardium had pneumococcal antigen detected in the pericardial fluid, which was also Gram's stain and culture positive for pneumococci.

Table 3. Detection of Staphylococcal Antigen

Fluid Source	Positive Specimens		
	Gram's Stain	Culture	CIE
Pleural	1	9	13
CSF	0	0	1
Subdural	Not done	0	1
Abscess	1	1	1
Pericardial	1	1	1

Staphylococcal antigen was detected in the pleural fluid from 13 patients. Only one had a positive Gram's stain and nine had positive cultures. Staphylococcal antigen was detected in fluid from other sites in individual patients as well (Table 3).

DISCUSSION: Employing specific antisera to detect H. influenza b, pneumococcal and staphylococcal antigen by counter immuno-electrophoresis has been useful in providing a rapid etiologic bacterial diagnosis of cases of purulent meningitis and empyema. This technique detects bacterial antigen when Gram's stains and cultures and negative, thus it appears to be a useful adjunct to reoutine bacteriologic methods. One cross reaction of H. influenza b antisera with Escherichia coli from pleural fluid was noted, and this may limit the usefulness of the procedure should more frequent cross reactions be observed.

2. Antibiotic-Resistant Typhoid Fever

OBJECTIVE: To determine the prevalence and degree of antibiotic resistance among Salmonella typhi isolates from typhoid fever patients at Children's Hospital and to correlate the in vitro pattern of resistance with the phage types of S. typhi and the clinical response to therapy.

BACKGROUND: Antibiotic-resistance strains of S. typhi have been isolated from patients with typhoid fever at Children's Hospital.

These S. typhi strains have resistance patterns and transferable R factor similar to strains isolated from Mexico, India and Vietnam. Chloramphenicol has been ineffective therapy in patients infected with Chloramphenicol-resistant S. typhi.

DESCRIPTION: Isolates of S. typhi from patients with clinical typhoid fever at Children's Hospital were obtained and disc sensitivities performed by the Kirly-Bauer method, and minimal inhibitory concentrations (MIC) by the plate dilution technique. S. typhi isolates from other parts of Thailand, and from Cambodia and South Vietnam were similarly studied.

The presence of R factor in antibiotic resistant strains was determined by the transfer of antibiotic resistance to a sensitive strain of Escherichia coli.

Phage typing of the S. typhi strains was performed through Dr. E.S. Anderson at the Enteric Reference Laboratory, London, England.

PROGRESS: Forty-four S. typhi isolates from Children's Hospital obtained between 28 March 1974 to 11 March 1975 were studied; seventeen (39%) were resistant to Chloramphenicol.

The Kirly-Bauer Disc method showed the resistance pattern I, Chloramphenicol, Streptomycin, Sulfadiazine, and Tetracycline (C S Su T) in eight strains, and the resistance pattern II (Ampicillin C S Su T) in nine strains.

The disc sensitivities are presented in Table 1 and the minimal inhibitory concentrations to Chloramphenicol, Ampicillin and Trimethoprim/Sulfamethoxazole (TMP/SMZ) are illustrated in Figure 1. In Figure 1 the MIC for TMP/SMZ refers to the ugm/ml of TMP in a 1:20 ratio of TMP: SMZ.

Each Chloramphenicol-resistant strain of S. typhi possessed R factor capable of transfering Chloramphenicol resistance to a sensitive E. coli. The strains that were also resistant to Ampilillin transferred both Chloramphenicol and Ampicillin resistance to sensitive E. coli.

Vi phage type results are available on 53 S. typhi strains obtained from Children's Hospital since November 1973. Their distribution by Chloramphenicol sensitivity in illustrated in Table 2. Most of the S. typhi strains resistant to Chloramphenicol but sensitive to Ampicillin have been phage type 53. All of the Ampicillin and Chloramphenicol resistant strains of S. typhi have been phage type D 1 (variant).

Table 1. Antibiotic Disc Sensitivity of Salmonella typhi Strains (44)

Antibiotic	Sensitivity (Per cent)
TMP/SMZ	100%
Kanamycin	100%
Gentamicin	100%
Cephalothin	100%
Ampicillin	80%
Chloramphenicol	61%
Tetracycline	61%
Sulfadiazine	30%
Streptomycin	0%

Table 2. Distribution of Vi Phage Types by Sensitivity (53 S. typhi Strains)

Phage type	Chloramphenicol Sensitive	Chloramphenicol Resistant
A	5	
D2	5	
M1	4	1*
53	4	13*
E10	3	
Degraded Vi (7)	2	
E1	1	
E2	1	
E3	1	
D6	1	
Degraded Vi (8)	1	
Vi negative		1*
D 1 variant		10**
Total	28	25

* Pattern I: C S Su T

** Pattern II: A C S Su T

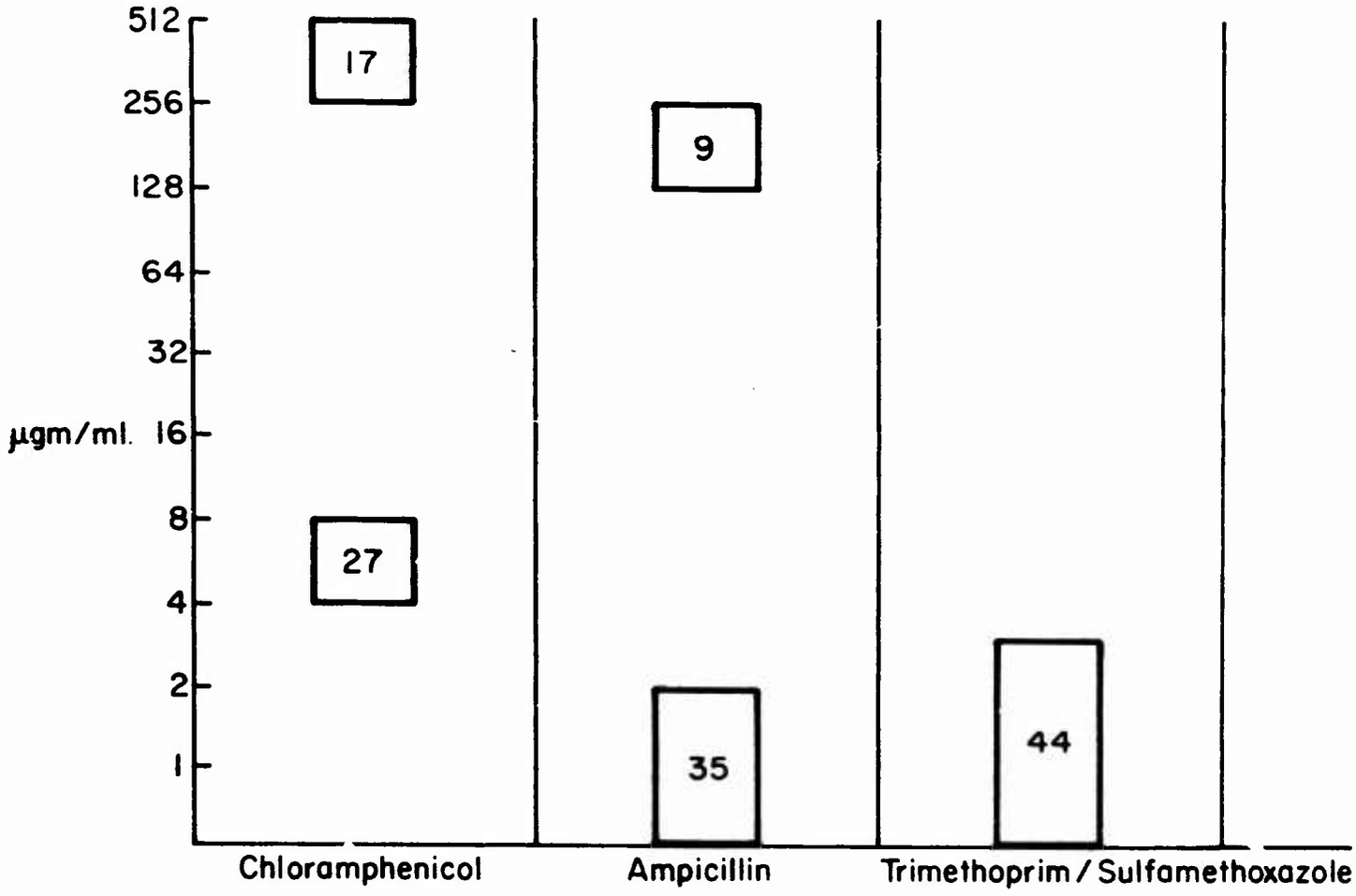

Figure 1. Range of Minimal Inhibitory Concentrations (for 44 S. typhi Strains)

The 10 patients with phage type D 1 (variant) came from scattered locales in Bangkok and nine were hospitalized during the months of May, June and July 1974. Initial therapy with Chloramphenicol or Ampicillin was ineffective in these patients· however, therapy with Trimethoprim/Sulfamethoxazole resulted in satisfactory clinical improvement.

Twenty five strains of S. typhi from Vietnam and ten strains of S. typhi from Siriraj Hospital, Bangkok, Thailand were confirmed to be resistant to Chloramphenicol by disc sensitivity and MIC. All of the strains exhibited resistance pattern I, C S Su T. No Ampicillin resistance was detected.

One S. typhi strain from Cambodia, and two from Songkla, Thailand were sensitive to Chloramphenicol. One of seven strains of S. typhi from the Bumrasnaradura Infectious Disease Hospital exhibited resistance pattern I, while the remainder were sensitive to Chloramphenicol. All of the 21 S. typhi strains from Chiangmai were sensitive to Chloramphenicol and Ampicillin.

DISCUSSION: The emergency of Chloramphenicol-resistant S. typhi strains with demonstrable in vitro and in vivo resistance which was first noted in 1973 has continued and a number of strains have been resistant to Ampicillin as well. All strains have been sensitive to TMP/SMZ and satisfactory clinical responses have been observed with this drug. The potential emergency of resistance of S. typhi to TMP/SMZ, remains; however, since resistance to TMP/SMZ is seen in other enteropathogens. Approximately 10% of Shigella isolates at SEATO Laboratory during the past year have demonstrated in vitro resistance (disc method) to TMP/SMZ. Continued surveillance of antibiotic resistance patterns of S. typhi will continue.

The association of certain phage types with antibiotic resistant strains of S. typhi suggests that certain phage types are more likely to be associated with R factor. The phage type associated with antibiotic-resistant strains of S. typhi are different than phage types noted in Mexico and in Vietnam and determination of the phage type may prove useful in determining the source of the future.

3. Frequency of Glucose-6-Phosphate Dehydrogenase Deficiency (G-6-PH) among Infectious Disease and Control Patients at Children's Hsopital

OBJECTIVE: To determine the frequency of erythrocyte G-6-Ph deficiency among patients hospitalized with various infectious

diseases and out-patients free of serious disease, seen at Children's Hospital.

BACKGROUND: A high frequency of G-6-PD deficiency has been noted among Thai children with typhoid fever at Children's Hospital. Additional children hospitalized with infectious diseases including bacterial empyema, meningitis and osteomyelitis, tuberculous meningitis and typhoid fever were studied in addition to outpatient controls to determine the frequency of G-6-PD deficiency.

PROGRESS: Children with bacteriologically confirmed diagnoses of empyema, meningitis, osteomyelitis and typhoid fever had G-6-PD determinations performed by the methemoglobin elution technique of Gall (64).

Children with the clinical diagnosis of tuberculous meningitis and supportive skin test and cerebrospinal fluid chemistries were also studied as were 100 outpatient children judged by history and physical examination to be free of serious bacterial disease.

Table 1 presents the frequency of G-6-PD deficiency according to bacterial etiology and sex of the patients.

DISCUSSION: A high frequency of G-6-PD deficiency was noted in patients with typhoid fever (45%), pneumococcal empyema and meningitis (36%), and tuberculous meningitis (26%). A smaller number of patients with Staphylococcal or Hemophilus influenzae b infections had no apparent increased prevalence of G-6-PD deficiency (0-10%). The overall frequency of G-6-PD deficiency among outpatient controls was 11%.

The association of G-6-PD deficiency with typhoid and pneumococcal infections is analogous to the association of sickle cell anemia with Salmonella osteomyelitis and pneumococcal infections. An impaired reticuloendothelial system as a result of hemolysis has been suggested as a factor in the susceptibility of sickle cell patients to these infections. Defective bacteriocidal activity of the leukocytes from patients with erythrocyte G-6-PD deficiency has been noted when the G-6-PD activity of these leukocytes is absent or markedly depressed (less than 25%). An overburdened reticuloendothelial system as a result of hemolysis or defective bacteriocidal activity of leukocytes from G-6-PD deficient patients are possible explanations for this association of erythrocyte G-6-PD deficiency and some bacterial infections.

Table 1. Frequency of G-6-PD Deficiency by Bacterial Etiology and Sex

Study subjects	Sex	No. tested	G-6-PD Deficiency		Frequency (%)
			Homozygous	Heterozygous	
Typhoid fever	M	19	7	0	37
	F	25	4	9	52
Pneumococcal infections	M	17	6	0	35
	F	8	0	3	37
Tuberculous	M	14	3	0	21
	F	9	1	2	33
Staphlococcal infections	M	4	0	0	0
	F	5	1	0	20
H. influenza b infections	M	6	0	0	0
	F	2	0	0	0
Outpatient control	M	55	8	0	15
	F	45	0	3	7

III. PARASITIC DISEASES OF MAN AND ANIMALS

A. FILARIASIS

1. Ecology of Bancroftian Filariasis

OBJECTIVE: To investigate the ecology of bancroftian filariasis in rural areas of Sangkhlaburi district, Kanchanaburi Province. Specific objectives include the following:

1. To identify the vector(s) of Wuchereria bancrofti by A) the demonstration of filariae in wild-caught mosquitoes and B) by feeding laboratory-reared strains of potential vector species on known microfilaria-carriers.

2. To determine the prevalence of human infections and the periodicity of microfilaremia, applying the techniques of direct chamber counting and membrane filtration for the isolation of microfilariae.

3. To collect information on the distribution, larval habitats and bionomics of suspected vector species and to obtain correlated series of larvae, pupae and adults of these mosquitoes for taxonomic studies.

4. To evaluate the clinical expression of human infections.

DESCRIPTION: In 1970 Harinasuta and associates (65) described an endemic focus of bancroftian filariasis in rural villages located near the headwaters of the Kwai River in the Sangkhlaburi district of Kanchanaburi Province. Prevalence rates of infection with W. bancrofti up to 30 per cent were observed in some villages, and many cases of filarial hydrocoele were reported. Bancroftian filariasis is rarer in Thailand than the type caused by Brugia malayi; moreover, in this area it differed from the type usually seen in Southeast Asia in that microfilaremia was nocturnally subperiodic, with peaks near 2000 hours, but with microfilariae present in significant numbers in the peripheral blood during daylight hours. Infective stage larvae of W. bancrofti were found in wild-caught mosquitoes belonging to the Aedes (Finlaya) niveus complex. The females of A. niveus and possibly 20 other closely related species are so similar that they cannot be differentiated with certainty at the present time; these mosquitoes are among the most common diurnal man-biting mosquitoes in the forested areas of Southeast Asia. Harinasuta et al also reported finding Aedes (Finlaya) harveyi, Anopheles maculatus, Anopheles minimus and Anopheles vagus infected with immature filarial larvae.

Subperiodic W. bancrofti infections transmitted by Anopheles minimus flavirostris and species of the Aedes niveus complex have also been reported from the Philippines by Cabrera and Rozeboom (66) and Anopheles leucosphyrus was identified as the vector of W. bancrofti in Sarawak (67).

The detection of microfilaremia, most often by examination of thick films prepared from 20 to 40 c.mm. of blood obtained from the finger, has been commonly relied upon to determine filariasis prevalence rates. The thick film technique has the advantage of being easy to use in the field; however, in recent years it has been shown that prevalence rates and the apparent age distribution of microfilaremia, based upon this survey method, have been imprecise (68). A relatively new technique, that has been shown to be as sensitive as the thick film and as easy to perform under field conditions, is that of direct counts of microfilariae in specially constructed chambered slides (69). Another new survey procedure, the isolation of microfilariae by filtration of blood through Millipore (70) or Nucleopore filters (71), has yielded higher positivity rates, in areas of nocturnally periodic infections, from daytime bloods than the standard thick blood film taken at night during microfilaremia peaks (72). The value of this technique in identifying microfilaremia carriers with low density infections is obvious. These newer survey techniques should prove valuable in clinical practice, in the evaluation of control and treatment schemes and in attaining a better understanding of the mechanisms of filarial transmission.

During the previous reporting period seven villages located in semiforested areas of Sangkhlaburi district were surveyed for microfilaremia. Five of these villages - Kupadu, Lawa, Nithae, Nong Padong and Wang Kalang - were selected as sites for further studies because their high microfilaremia rates and/or accessibility.

PROGRESS: Between July and December 1974, 6169 mosquitoes were caught in 1832 man-hours of collections from human hosts, made during both daylight and evening hours, in the five study sites. Eight genera of mosquitoes, comprising 87 species, were represented in these collections, however, Aedes albopictus, Armigeres annulitarsis and members of the Aedes niveus complex together formed the major portion of those collected. A total of 5141 mosquitoes were dissected, and 45 mosquitoes, belonging to six species (Aedes niveus species "A", Aedes desmotes, A. mediopunctatus, A. gardneri, A. imprimens and Armigeres annulitarsis), were found naturally infected with filarial larvae (Table 1). Identifications of the mature larvae are not yet

complete, but some are characteristic of W. bancrofti while others appear to be species of Setaria, Diptelonema and/or Breinlia.

Surveys for larval mosquitoes were made in the five study sites between July 1974 and March 1975. Larvae of 82 species, belonging to 14 genera of mosquitoes, were collected from a variety of habitats within the vicinity of the five villages. Dense thickets of bamboo are present around Kupadu, Lawa and Nong Padong, and mosquitoes which breed in bamboo nodes, such as Aedes (Stegomyia), Aedes (Finlaya) and Armigeres (Leicesteria), were especially numerous there. This accounts for the abundance of adults of A. albopictus, Armigeres annulitarsis and members of the Aedes niveus complex caught in biting collections made during this period in those villages. During the rainy season (July-November), larvae of Anopheles balabacensis, the principal malaria vector in Thailand, were present in ground pools in all five of the villages. The domestic mosquitoes, Aedes aegypti and Culex quinquefasciatus, were not present in the more isolated villages of Kupadu, Lawa and Nong Padong, which, incidentally, had the highest microfilaremia rates of the five study sites. On the other hand, larvae of A. aegypti and C. quinquefasciatus were found breeding on the premises of 72% and 24%, respectively, of the houses in Nithae, which is the most urbanized and had the lowest microfilaremia rate of the five villages.

Colonized strains of Aedes aegypti, A. albopictus, A. togoi, Armigeres annulitarsis, and Culex quinquefasciatus were fed upon known microfilaria carriers from the Sangkhlaburi district during this period. Of these five species, only Aedes togoi and Culex quinquefasciatus developed infections with W. bancrofti (Table 2).

Between August 1974 and March 1975, a comparison of the relative sensitivity of the 20 c.mm. thick film, 20 c.mm. counting chamber and the 1.0 ml. membrane filtration techniques for detecting microbilaremia was made in the villages of Kupadu and Nong Padong. Blood specimens were obtained from 117 individuals for comparison of the three techniques; the results are shown in Table 3. Microfilaremia rates by age and sex, obtained by the membrane filtration technique, are shown in Tables 4 and 5. A further evaluation of the three techniques was made in a study of the periodicity of microfilaremia in 10 villagers found positive during the earlier survey. Blood was taken at two hour intervals over a 24 hour period, and the microfilaremia densities at each interval, as measured by each of the three techniques, are given in Tables 6-8. The mean values of microfilaremia periodicity determined by membrane filtration for the 10 patients are shown in Figure 1.

Table 1. Mosquitoes Found Infected with Filarial Larvae - Sangkhlaburi, 1974.

Species	Kupa Du		Nong Pa Dong		Lawa		Nithae		Wong Ka Lang		Totals	
	No. Coll.	No. Pos.	No. Coll.	No. Pos.	No. Coll.	No. Pos.	No. Coll.	No. Pos.	No. Coll.	No. Pos.	No. Coll.	No. Pos.
Aedes (S) gardnerii	19	1	29	1	45	11	10	1	19	3	122	17
Aedes (S) desmotes	55	2	37	0	42	3	8	0	3	0	145	5
Aedes (S) mediapunctatus	45	1	25	3	23	0	3	1	2	0	98	5
Aedes (F) niveus "A"	421	2	83	1	134	3	6	0	21	0	665	6
Aedes (E) imprimens	63	0	51	2	12	0	0	0	1	0	127	2
Armigeres (L) annulitarsis	333	1	125	3	122	1	111	1	5	0	696	6
Armigeres (L) flavus	7	0	0	0	9	1	6	0	0	0	22	1
Mansonia (M) dives	11	0	15	0	1	0	4	0	32	3	63	3
Totals	954	7	365	10	388	19	148	3	83	6	1938	45

Table 2. Mosquitoes Fed on Wuchereria bancrofti Cases - Sangkhlaburi, 1974.

Species	No. Fed	No. Dissected	No. Infected	Percent Infected	No. Larvae
Aedes aegypti	271	207	0	0.0	0
Aedes albopictus	62	46	0	0.0	0
Aedes togoi	138	45	23	51.1	80
Armigeres annulitarsis	27	19	0	0.0	0
Culex quinquefasciatus	333	184	9	4.9	12

Table 3. Results of Comparison of Three Techniques for Detecting Microfilaremia - Kupadu and Nong Padong, 1974.

Technique	No. Patients	No. Positive	Percent Positive
Thick Film (20 c.mm. blood)	117	20	17
Counting Chamber (20 c.mm. blood)	117	22	19
Membrane filtration (1 ml. blood)	117	31	26

Table 4. Results of Examinations for Microfilaremia by Age, Using Membrane Filtration Technique - Kupadu and Nong Padong, 1974.

Age Group (Years)	Number Examined	Number Positive	Percent Positive
0-3	5	2	40
4-6	8	0	-
7-10	21	2	10
11-15	21	4	19
16-20	13	3	23
21-30	22	7	32
31-40	7	2	29
41-50	12	5	42
50+	8	7	88
Total	117	32	27

Table 5. Results of Examinations for Microfilaremia by Sex, Using Membrane Filtration Technique - Kupadu and Nong Padong, 1974.

Sex	No. Examined	No. Positive	Percent Positive
Male	73	17	23
Female	44	15	34
Total	117	32	27

Table 6. Twenty-four Hour W. bancrofti Microfilariae Counts in 10 Carriers, by Thick Film Technique.

Case No.	Microfilariae counts at hours/20 c.mm. blood											
	1200	1400	1600	1800	2000	2200	2400	0200	0400	0600	0800	1000
1	3	3	7	2	7	3	5	11	10	6	1	3
2	8	20	11	11	19	14	11	11	3	6	3	8
3	5	10	15	20	8	24	13	8	11	5	4	9
4	13	12	25	53	59	82	48	65	57	47	1	1
5	1	4	8	7	3	5	11	8	5	6	1	1
6	3	3	2	1	3	0	1	2	0	1	0	2
7	16	34	46	21	26	51	67	31	16	9	6	29
8	23	29	82	40	67	71	31	56	10	14	6	8
9	5	8	18	23	25	16	9	15	21	13	3	7
10	0	0	0	1	0	0	0	0	0	1	0	0

Table 7. Twenty-four Hour W. bancrofti Microfilariae Counts in 10 Carriers, Determined by Counting Chamber Technique.

Case No.	Microfilariae counts at hours/20 c.mm. blood											
	1200	1400	1600	1800	2000	2200	2400	0200	0400	0600	0800	1000
1	2	1	1	3	4	12	6	4	7	4	3	2
2	10	19	9	20	23	6	9	10	10	3	5	13
3	4	25	23	23	16	23	16	10	8	1	7	3
4	8	15	31	40	48	62	42	48	49	46	15	11
5	3	2	2	5	5	4	2	7	2	2	4	1
6	6	3	4	3	3	1	1	1	0	1	0	1
7	17	27	53	27	29	49	40	12	23	10	14	17
8	23	30	64	41	56	52	38	45	19	23	7	3
9	6	7	14	14	17	8	15	17	15	11	4	8
10	2	1	0	2	5	2	0	1	1	1	0	1

Table 8. Twenty-four Hour W. bancrofti Microfilariae Counts in 10 Carriers, Determined by Membrane Filtration Technique.

Case No.	Microfilariae counts at hours/1 ml. blood											
	1200	1400	1600	1800	2000	2200	2400	0200	0400	0600	0800	1000
1	109	96	194	245	334	271	433	318	270	200	128	64
2	444	924	560	1049	966	549	521	456	290	231	198	496
3	371	990	1171	1231	601	727	621	399	601	231	198	257
4	409	685	1560	2282	1789	3124	2653	2462	2841	2244	928	871
5	126	44	327	394	316	294	279	283	183	295	99	109
6	132	210	157	120	197	75	107	85	98	57	49	142
7	861	1824	2508	1686	1231	2102	2093	1917	616	769	756	1310
8	925	1672	3239	2358	3681	2891	2306	2950	877	945	273	334
9	439	564	749	1101	1070	1212	675	1036	473	1033	620	294
10	8	10	22	16	23	43	15	22	2	14	13	5

The nocturnally subperiodic character of the Sangkhlaburi strain of W. bancrofti is obvious, for at no time was the microfilaremia level less than 20 per cent of peak count. It appears from the above data, that the direct chamber count is at least as sensitive as the standard thick film, with the advantage of providing an immediate result without the necessity of drying and staining slides. The disadvantage is the need for microscopy at the site of specimen collection. Membrane filtration, although venipuncture is required, is still convenient enough for routine field use, and is much more sensitive than either the thick film or the counting chamber. The value of this technique in low density infections is illustrated by the case of periodicity patient No. 10 (Table 8).

Seventy-five individuals in the village of Kupadu submitted to full physical examinations during the course of those surveys. Only five of these yielded positive findings as summarized in Table 9.

Table 9. Summary of Clinical Findings in Kupadu Villagers Suggestive of Filariasis.

Case No.	Age	Sex	Finding	Microfilariae Present
1	52	M	Large hydrocoele	Yes
53	25	M	Large inguinal nodes, no other apparent cause	No
55	25	M	Large inguinal nodes, no other apparent cause	No
58	21	M	Thickened spermatic cord	No
62	52	M	Thickened spermatic cord	Yes

The pathology normally associated with infections of W. bancrofti is apparently at a very low level in this particular endemic area and seems to be limited to minor genital abnormalities in males. No cases of elephatiasis have been observed in the study villages.

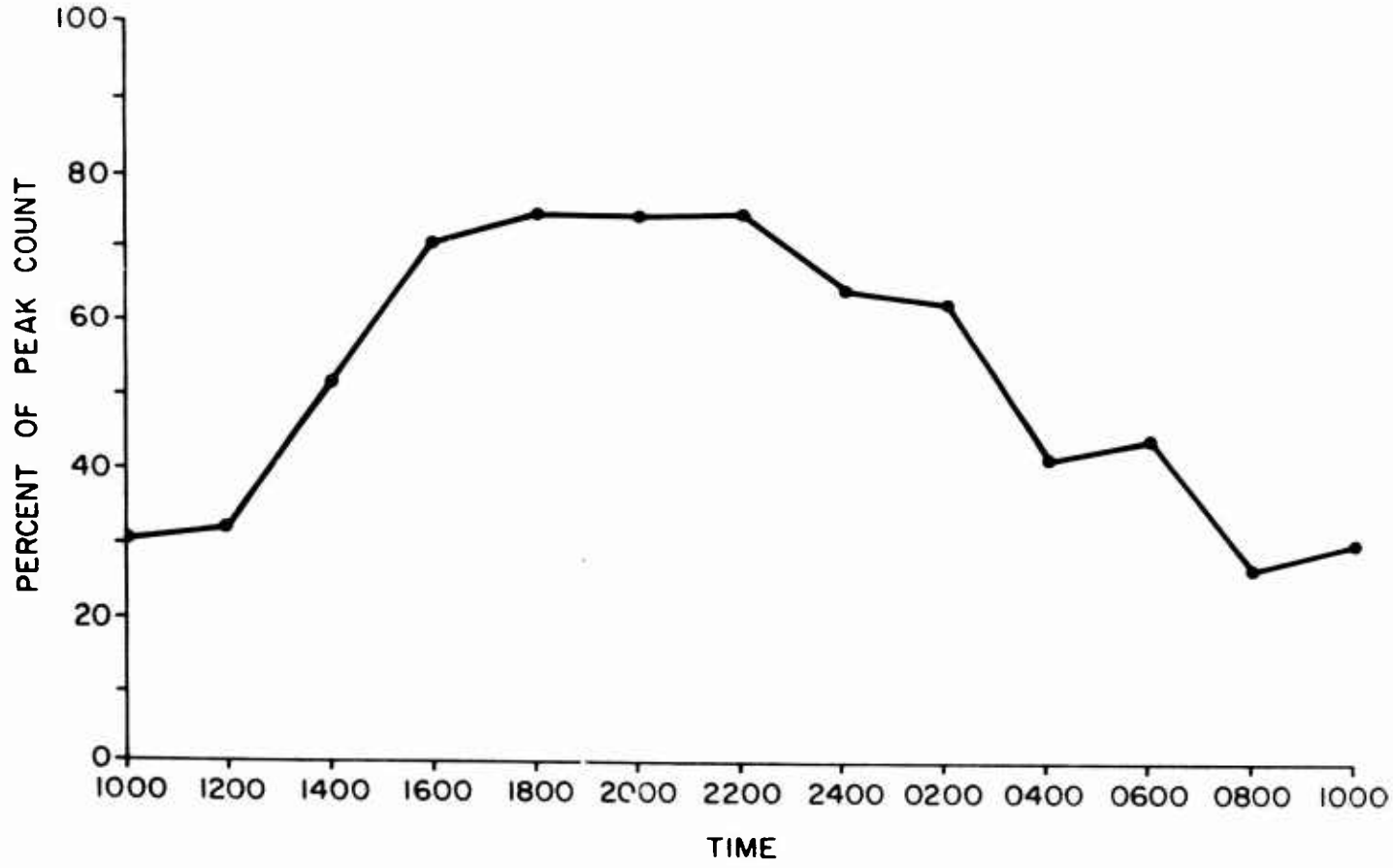

FIGURE 1. MEAN VALUES OF MICROFILARIA DENSITY FOR 10 PATIENTS, DETERMINED AT HOURLY INTERVALS BY 1 ML. MEMBRANE FILTRATION.

B. GNATHOSTOMIASIS

1. Studies of New Experimental Intermediate and Paratenic Hosts and Modes of Transmission of Gnathostoma spinigerum

OBJECTIVE: To attempt to identify new experimental host animals susceptible to Gnathostoma spinigerum as reported in the SEATO Medical Laboratory Annual Report April 1972 to March 1973.

BACKGROUND: Some species of crustacean, namely fresh water crabs, shrimps and prawns, are occasionally eaten raw or insufficiently cooked by man. Experimentally it has been shown that fresh water crabs (Paratelphusa sexpunctatum and Potamon smithanus) could be infected with G. spinigerum advanced third stage larvae; therefore, they may be considered as a potential source of natural infection to man (41). After experimentally feeding shrimps and prawns (Macrobrachium rosembergi De Mann, and M. mirabile Kemp) with gnathostome larvae it appeared that they could not be infected; however, only a few shrimps and prawns were utilized in the study (82). This study was expanded to include all larval stages of G. spinigerum and larger numbers of shrimps and prawns.

DESCRIPTION: In addition to the prawns obtained from sources appearing in the above report, six more living adult prawns (M. rosembergi) were obtained from a restaurant in Pathum Thani near Bangkok. These prawns were caught from the Chao Phya River a few kilometers north of Bangkok. Before initiating this study the prawns were maintained in fresh-water aquaria for four to five weeks for acclimation to the laboratory environment. From a total of 28 adult prawns obtained from the experimental farm of the Department of Fisheries and those from the Pathum Thani restaurant, 12 were autopsied for the presence of gnathostomes. The remaining 16 were experimentally fed with varying numbers of larvae fully developed in cyclops, and also advanced third-stage larvae from infected mice.

Two methods were used for feeding gnathostome larvae to prawns. Natural feeding was accomplished by presenting the prawns with both cyclops and minced mouse tissue containing known numbers of infective larvae. After feeding, the prawns were observed visually every one to two hours until all cyclops and mouse tissue were consumed. This usually occurred in less than six hours. Artificial feeding was performed by use of a polyethylene tube attached to a needle and a 1.0 ml. syringe containing a known number of larvae in a few drops of fresh water. The tube was easily inserted into the mouth of the prawns and the larvae were injected. Autopsies were performed from 4 to 57 days after feeding.

Table 1. Results of Feeding Gnathostoma spinigerum larvae to Fresh-water Shrimp and Prawn.

Method of Feeding	Recipients		No. Larvae Used			Remarks
	Shrimp	Prawn	Newly Hatched or First Stage	Fully Developed in Cyclops	Advanced Third-stage from Mice	
Natural	100	-	20000	-	-	Autopsies negative after 1-35 days
Natural	100	-	-	1000	-	Autopsies negative after 1-81 days
Artificia	-	3	-	250	-	Autopsies negative after 31 and 57 days
Natural	-	8	-	-	215	Autopsies negative after 4-42 days
Artificia	-	5	-	-	48	Autopsies negative after 10-30 days
None	100	12	-	-	-	Control autopsies negative

Shrimp proved to be too small for successful artificial feeding; therefore, only natural feeding was used.

PROGRESS: A review of the experimental feeding of G. spinigerum larvae to fresh water shrimps and prawns is presented in Table 1.

SUMMARY: Fresh water shrimps and prawns (Macrobrachium rosembergi, De Mann) were not infected by feeding on larvae of G. spinigerum. The evidence does not indicate that these crustaceans can act as intermediate or paratenic hosts for G. spinigerum.

2. Chemotherapy of Gnathostomiasis

OBJECTIVE: To continue to search for chemicals with chemotherapeutic activity against advanced third-stage larvae of Gnathostoma spinigerum.

BACKGROUND: These studies are a continuation of the work reported in previous years. Many antihelminthic drugs have been evaluated for possible chemotherapeutic activity against experimental G. spinigerum infections of mice with advanced third stage or migrating larvae. All drugs have been ineffective so far.

DESCRIPTION: Mice of the ICR strain were infected by oral administration of five advanced third-stage larvae of G. spinigerum. After infection for some days, the test drug or combination of drugs dissolved in distilled water was administered orally or parenterally in a predetermined regimen. Infected control mice were given distilled water orally. After completion of the treatment regimen, the mice were sacrificed at intervals and necropsied. Parasites were counted in the liver and/or body muscles and the results recorded.

The drugs tested during this reporting period were: Astiban (sodium antimony dimercapto-succinate), Lucanthone (Miracil D. or Nilodin) (1-methyl-4-diethylamino-ethylaminothioxanthone hydrochloride), Hycanthone (Etrenol) an active metabolite of Lucanthone, and Iodine in Lugol's solution.

PROGRESS: Drug screening tests on mice infected with G. spinigerum advanced third stage larvae gave the following results.

Astiban: This drug was administered last year in five daily oral doses of 640 mg/kg or with a single oral dose of 1920 mg/kg to gnathostome-infected mice without effect. This year an oral dose of 1920 mg/kg daily for two days was also found to be ineffective in significantly reducing the numbers of the larvae in treated mice. Therefore the drug appears to have no therapeutic effect on infected mice (Table 1).

Table 1. Treatment of Gnathostoma spinigerum Infected Mice with Astiban Oral Administration

Astiban Drug Dose (mg/kg/day	No. of Infected Mice Treated	No. of Third-stage Larvae Found	Time of Necropsy (Days)*
1920**	30	90	20
Control	30	93	19

* Days after administration of last drug dose

** For two days

Table 2. Treatment of Gnathostoma spinigerum Infected Mice with Lucanthone Oral Administration*

Lucanthone Drug Dose (mg/kg/day)	No. of Infected Mice Treated	No. of Third-stage Larvae Found	Time of Necropsy (Days)**
150	10	31	24
Control	5	16	24

* Two doses per day for five days

** Days after administration of last drug dose

Table 3. Treatment of Gnathostoma spinigerum Infected Mice with Hycanthone Oral Administration*

Hycanthone Drug Dose (mg/kg/day)	No. of Infected Mice Treated	No. of Third-stage Larvae Found	Time of Necropsy (Days)**
100	15	45	4-27
Control	15	46	14-28
200	13	42	10-27
Control	15	46	14-28
300	15	45	4-40
Control	15	44	33-40
400	14	40	1-40
Control	15	44	33-40

* One dose per day for five days

** Days after last dose of drug

Table 4. Treatment of Gnathostoma spinigerum Infected Mice by Oral Administration of Iodine in Lugol's Solution*

Iodine Drug Dose (mg/kg/day)	No. of Infected Mice Treated	No. of Third-stage Larvae Found	Time of Necropsy (Days)**
40	5	19	7
Control	5	20	3-7
200	5	16	17
Control	5	17	17
400	5	17	All mice died six hrs. after last dose

* Two doses per day for five days

** Days after last dose of drug

Table 5. Treatment of Gnathostoma spinigerum Infected Mice by Subcutaneous Injection of Iodine in Lugol's Solution*

Iodine Drug Dose (mg/kg/day)	No. of Infected Mice Treated	No. of Third-stage Larvae Found	Time of Necropsy (Days)**
20	10	30	17
Control	5	16	17

* One dose daily for five days

** Days after the last dose

Lucanthone: Gnathostome-infected mice were treated with two doses of 150 mg/kg/day for five days (ten doses). The results are shown in Table 2. This drug is judged to have no therapeutic value in the treatment of G. spinigerum infection.

Hycanthone: This drug was administered orally over a five day course using doses of 100, 200, 300, and 400 mg/kg. The results are shown in Table 3. There was no significant reduction in the number of gnathostome larvae in the treated mice. Therefore Hycanthone is considered to have no therapeutic effect on the infection.

Lugol's solution: The prescription of the solution was Iodine, two grams; Potassium iodide, four grams; and purified distilled water, 100 ml. An in vitro experiment of various dilutions of the solution (1:1000 to 1:20,000 or iodine solution equivalent of 1:500 to 1:10,000) on living G. spinigerum advanced third-stage larvae obtained from the experimentally infected mice caused the death of the larvae in ten minutes to four days compared with the control in distilled water where the larvae lived for eight days.

The screening tests on infected mice (average body weight of 25 grams per mouse) were done by oral administration of various doses of the solution containing iodine 40, 200, and 400 mg/kg (or iodine solutions in mice of 1:625, 1:125, 1:60) twice daily for five days. The results showed no therapeutic value on the infection (Table 4), and all five infected mice who received 400 mg/kg died of toxicity about six hours after the administration of the last dose.

The drug was also given by subcutaneous injection of Lugol's solution using a dose of 20 mg. iodine/kg body weight of the infected mouse or equivalent to about 1:1250 solution of iodine in the mouse body. The result is shown in Table 5. This dose of the drug by subcutaneous administration is judged ineffective.

SUMMARY: Oral administration of Astiban, Lucanthone, Hycanthone and Iodine in Lugol's solution and subcutaneous injection of iodine in Lugol's solution were ineffective in the chemotherapy of Gnathostoma spinigerum in experimentally infected mice. Further investigation on iodine in Lugol's solution given by subcutaneous injection is in progress and the combined therapy with Astiban and Ambiltar has shown a modest chemotherapeutic effect and will be investigated further (1).

IV. MISCELLANEOUS

1. An Epizootic of Tropical Canine Pancytopenia in Thailand

OBJECTIVE: To study the epizootiology of Tropical Canine Pancytopenia in a population of military working dogs, and to evaluate the efficacy of currently recommended prophylactic and therapeutic measures in a natural outbreak.

BACKGROUND: Tropical Canine Pancytopenia (TCP) is a tick-transmitted infectious disease of dogs caused by the rickettsia-like organism Ehrlichia canis. Infected dogs may be almost asymptomatic, or they may develop a frequently fatal syndrome characterized by fever, anemia, leukopenia, thrombocytopenia, and hemorrhage. The fatal syndrome has been observed most frequently in German Shepherds. Outbreaks of TCP have occurred in many tropical and sub-tropical countries, but until now the disease has not been recognized in Thailand. The present report describes an epizootic of TCP among military working dogs at the War Dog Training Center, Pakchong, Korat Province, Thailand, 185 km northeast of Bangkok.

DESCRIPTION: The War Dog Training Center is a modern facility established by the Thai Armed Forces to breed, train, and issue working dogs to all military services. The population of dogs at the Center averaged about 175 during the period of the study. The Center also supports an additional 125-150 dogs at remote stations. The composition of the dog population at the Center is constantly changing as dogs are issued to and return from remote stations. Most working dogs return to the Center annually for retraining; dogs also return for treatment of serious disease problems, since veterinary care is not generally available except at the Center.

Dogs at the Center are maintained in individual pens in several groups of screened or unscreened buildings which are separated by distances as great as several hundred meters. These groups of buildings are designated as the breeding area, the young adult area, the training area, the working dog area, and the hospital area. There is limited direct daily contact between groups, although dogs are periodically moved from one functional area to another as needs of training and utilization dictate. Contact is also possible in the hospital area which services dogs from all areas and from outside the Center. In addition, indirect contact is possible in common exercise, training and working areas. The common brown dog tick (Rhipicephalus sanguineus) has been collected from dogs and kennels in all areas. No other species of tick has been found.

TCP was first suspected in March, 1974, among a group of seven German Shepherds working at a military base in Lopburi, 133 km north of Bangkok. Within a two month period, three of these dogs died after episodes of epistaxis. The four surviving dogs, which were in poor condition, were transported to Pakchong and placed in the hospital for observation and treatment. It is believed that these dogs may have initiated the epizootic at Pakchong, although a retrospective analysis of clinical records suggests an increase in the prevalence of febrile episodes among dogs at Pakchong as early as January, 1974. No dogs were imported to the Center from outside Thailand during this time nor during the year prior to the recognition of TCP at Pakchong.

The progress of the epizootic of TCP has been followed by clinical, pathological, and laboratory studies. A clinical record is maintained on each dog, which contains an indication of clinical symptoms observed and treatments administered whenever a dog is brought to the hospital. Complete physical examinations are performed at regular intervals, including surveillance and treatment for heartworm and intestinal parasites. All dogs are regularly vaccinated for canine distemper, canine hepatitis, leptospirosis, and rabies.

A program to control the epizootic was developed based upon the experiences in Vietnam (83, 84) and upon laboratory studies conducted at the Walter Reed Army Institute of Research in subsequent years (85, 86). Serologic identification of infected dogs was the conerstone of the control effort (87). The basic elements of the control program were as follows:

1. Identification of infected dogs by serologic testing, clinical signs, and laboratory studies.

2. Treatment of infected or suspect dogs with tetracycline hydrochloride orally 30 mg/lb/day for 14 days. Supportive therapy was utilized as appropriate in severe clinical cases.

3. Prevention of infection or re-infection by continuous daily oral administration of approximately 3 mg/lb/day of tetracycline hydrochloride. (A single 250 mg capsule was opened, and the powder lightly mixed with the pre-weighed food in each dog feeding pan).

4. Elimination of ticks by regular spraying of kennels and dipping of dogs with insecticide.

5. Isolation and treatment of newly introduced dogs.

The laboratory procedures utilized to identify TCP-infected dogs for treatment included serology, hematocrit, total and differential leukocyte counts, and serum protein electrophoresis. The serological method utilized was the indirect immunofluorescent test (87). At each bleeding 10 ml of venous blood was collected. Two to three milliliters were placed in tubes with EDTA for hematologic studies; the remainder was allowed to clot and serum collected for serology and serum protein determinations. For serologic screening, a 1:10 serum dilution was used, and results were reported as "positive" or "negative."

Blood samples were collected from all dogs at Pakchong (except puppies less than six months of age) at three month intervals. During the intervals between quarterly bleedings some additional dogs were bled, including new arrivals missed at the previous bleeding. On occasion, sera were collected at other military bases, but the bleeding of dogs at remote stations could not be comprehensive. Quarterly bleedings were performed on 4 June, 4 September and 18 December, 1974, and on 4 March 1975. In addition, all dogs at Pakchong were bled on 25 July 1974, just before initiation of the tetracycline treatment program. A total of 316 dogs were studied between June 1974 and March 1975. This included 287 German Shepherds, 10 Doberman Pinschers, 9 Labrador Retrievers and 10 Labrador-Shepherd cross-breeds.

Individual dogs were given a complete 14 day therapeutic course of tetracycline for any of the following reasons:

1. Suspicious clinical signs
    a) Unexplained fever
    b) Anemia (hematocrit less than 39)
    c) Leukopenia (WBC less than 6000)
    d) Bleeding (epistaxis, hematuria)
    e) High serum gamma globulin ($>$ 2.5 gm%)
2. Serologically positive for TCP
3. Known or suspected exposure to TCP
4. Admitted to the Center with unknown prior history

In addition, on 26 July, all dogs at the Center older than six months of age (172 dogs) were placed on a 14 day therapeutic course of tetracycline. This was done with the knowledge that 49 per cent of the dogs had been serologically positive on 4 June, and with the suspicion than numerous additional dogs were incubating the disease (31 dogs developed fevers between 4 June and 25 July). At the completion of the course of treatment, all dogs were continued on prophylactic levels of tetracycline daily until 9 September. After a 60 day interruption, tetracycline prophylaxis was again reinstituted on 9 November, and has been maintained until the present time. If at any time a previously treated dog converted serologically, or showed any of the suspicious clinical signs listed above, it was retreated with tetracycline therapeutically.

PROGRESS: The existence of TCP in Thailand has been demonstrated conclusively for the first time. The evidence for the disease includes the observation of characteristic clinical, hematological and pathological signs among a group of Thai Military Working Dogs, and the confirmation of the disease serologically and by direct observation of morulae of Ehrlichia canis in tissue macrophages.

In April, 1974, Krisda, a one year old, male German Shepherd, was transported from Pakchong to SEATO Laboratory for observation and diagnostic studies. This dog had a history of recurrent fever (102°F - 104°F), inappetance, and progressive weight loss. On admission, the hematocrit was 35; leukocyte count 5100 per cmm; fecal specimen negative for ova and parasites. The serum was serologically positive for TCP. During a month-long period of observation, rectal temperature was consistently elevated (103°F-105.8°F), the appetite was poor, and the dog became progressively more debilitated, lost weight, and developed pressure ulcers of the skin of the abdomen and over bony prominences. The hematocrit fell to 17; leukocyte counts ranged from 6,600 to 30,000 per cmm. No chemotherapy was given. The dog was ultimately euthanized. At necropsy a moderate, multifocal bronchopneumonia was observed. Multifocal plasma cell infiltration was prominent in lymph nodes, in portal areas and around central veins of the liver, and interstitially in the kidneys. Lymphocytic and plasmacytic vasculitis, occasionally with minimal perivascular hemorrhage, was observed in the brain. Typical inclusions or "morulae" of Ehrlichia canis were observed in macrophages in giemsa-stained impression smears obtained from cut surfaces of lung at necropsy (86).

Between early April and 25 July, many dogs exhibited clinical or hematological abnormalities suggesting TCP. The number of dogs treated with tetracycline because of fever, anemia or epistaxis

during these months is indicated in Table 1. During this period of rapid disease transmission, 47 dogs were treated with tetracycline on the basis of clinical signs without knowledge of their serological status (results of 4 June serology were not known until after 25 July). Only two of these dogs were later found to be serologically negative, and thus 97% of these treatments were appropriate. On the other hand, of the 86 dogs serologically positive on 4 June, only 45 (52%) were selected for treatment on the basis of clinical signs or hematology.

After the mass treatment of dogs on 25 July, a dramatic improvement in the general condition of dogs was noted. Thin dogs gained weight, work performance improved, breeding performance improved, and the number of dogs hospitalized was reduced. We continued to initiate tetracycline therapy in dogs developing fever, anemia or epistaxis, as indicated in Table 2. Thirty-eight dogs were treated for symptoms between 26 July 1974 and 31 March 1975. Nineteen of these courses of treatment (68%) were apparently inappropriate, since they were administered to dogs which were serologically negative both before and after treatment.

Compared to the experience in Vietnam, the severity of the clinical disease at Pakchong was mild. Between April 1974, and March 1975, only 19 dogs died at Pakchong. Six of these deaths were from causes other than TCP (accident, heartworm-2, drug toxicity, cystic calculi, heat exhaustion). Three dogs died of unknown causes. In ten dogs the history, clinical findings, laboratory studies, and gross necropsy findings suggested that TCP was the primary or contributing cause of death. Histopathologic confirmation of TCP was possible in only two cases. Only two of the TCP deaths occurred after 26 July. Both of these dogs were returned to Pakchong from remote sites in moribund condition.

Tetracycline therapy had been instituted in only one of the ten dogs which died of TCP. In this case, the dog was exhibiting epistaxis, the hematocrit was 14, and the dog died three days after initiation of therapy. In all other severe cases tetracycline therapy caused remission of symptoms. Nine dogs with epistaxis and 13 dogs with hematocrits of 15-25 have been successfully treated.

Severe symptoms of TCP were observed only in German Shepherds, although 7 of 9 Labradors, 5 of 10 Dobermans, and 1 of 10 Shepherd-Labrador cross-breeds were serologically positive. Two of the serologically positive Labradors had febrile episodes, and two had low hematocrits (28 and 31); they were otherwise asymptomatic. Three Dobermans were febrile, but exhibited no

Table 1. Major Clinical Signs of TCP Observed at Pakchong During June - July 1974

Clinical Signs	Number of Dogs Exhibiting Signs	
	Serologically Positive	Serologically Negative
Fever (a)	31	2
Anemia (b)	8	0
Epistaxis (c)	6	0
Total	45	2

(a) Rectal temperature above 103°F. Depression and inappetance were frequently associated with the fever, but diarrhea or vomiting were seldom observed. None of these dogs exhibited anemia, leukopenia or epistaxis.

(b) Hematocrit less than 39. None of these dogs exhibited epistaxis; some were leukopenic, and most were febrile intermittently.

(c) These dogs were also anemic, leukopenic and febrile.

Table 2. Major Clinical Signs of TCP Observed at Pakchong Between August 1974 and March 1975

Clinical Signs (a)	Number of Dogs Exhibiting Signs	
	Serologically Positive	Serologically Negative
Fever	2	0
Anemia	7	19
Epistaxis	0	0
Total	9	19

(a) See footnote for Table 1.

Table 3. Results of Serologic Studies at Pakchong - June 1974 to March 1975

Date	Cumulative Number of Dogs Studied(a)	Serologically Positive Dogs			Serologically Negative Dogs			Cumulative Known Positive Dogs	Cumulative Known Negative Dogs	Percent Positive
		Converted To Positive	Added To Study	Total New Positive	Converted To Negative	Added To Study	Total New Negative			
4 Jun 74	176	-	86	86	-	90	90	86	90	49%
25 Jul 74	200	30	17	47	8	13	21	120	80	60%
Sep-Oct 74	242	13	12	25	6	33	39	136	106	56%
Dec 74-Jan 75	294	3	2	5	45	53	98	93	201	32%
Mar 75	301	0	1	1	23	9	32	69	232	23%

(a) Dead Dogs Excluded

Some, but not all, infected dogs at Pakchong have exhibited increased gamma globulins. In most cases this was also reflected in a reduced A/G ratio. Comparison of the mean values for serologically positive and serologically negative dogs revealed no significant differences; however, individual dogs among the infected groups did have gamma globulin values which differed significantly from values observed in serologically negative dogs. Among serologically positive dogs prior to treatment, 30 of 85 (35%) had significantly elevated gamma globulins on 4 June, and 13 of 38 (34%) had elevations on 25 July. Abnormal gamma globulin levels returned to normal after drug treatment more rapidly than the dogs converted to negative serologically. By 25 July, and 6 of 24 treated dogs (25%) had elevated gamma globulins, and by 4 September only 4 of 80 (5%) still had abnormal values.

The ability to identify infected dogs serologically has been indispensible to the control effort. The treatment of dogs using clinical or hematologic criteria has proven unsatisfactory. Many dogs have been treated unnecessarily using symptomatic criteria, and, more importantly, many asymptomatic carriers would have been overlooked.

An important limitation of the serologic screening method is its inability to identify re-infections or failed treatments. Dogs are susceptible to re-infection even though they have serologically demonstrable antibody to Ehrlichia canis (85). After treatment, for as long as the dog remains serologically positive, only clinical and hematological observations serve to identify re-appearance of active infection. As of March 1975, 69 treated dogs remained serologically positive. It is entirely possible that some of these dogs are harboring the organism. Hopefully, control of ticks and the continuation of prophylactic tetracycline will prevent transmission of the organism until such time as any carriers among this group can be identified.

Efforts to control the epizootic in Thai military working dogs are continuing. Tetracycline is still being administered prophylactically, and dogs which exhibit suspicious clinical or hemotologic signs, or which convert serologically, are being treated with tetracycline. While many dogs have become serologically negative, the efficacy of the combined therapeutic and prophylactic administration of tetracycline cannot be evaluated until the infective status of the remaining 69 serologically positive dogs has been resolved. Efforts to control ticks are being maintained.

SUMMARY: An epizootic of Tropical Canine Pancytopenia (TCP) has been studied in a population of 316 Thai Military Working Dogs. To date, 161 cases have been identified serologically, of which 54 were clinically or hematologically apparent. The prevalence of severe clinical symptomatology was low. Epistaxis was observed in only 9 dogs (2.8%), and only 10 dogs died (3.2%).

A control program including tick control, serologic identification and treatment of carriers, and tetracycline prophylaxis has been instituted. Administration of tetracycline 30 mg/kg/day for 14 days has produced clinical remission of symptoms in all but one of the severely ill dogs. Serologic remission has been observed in all but 69 dogs to date. Early intervention with tetracycline is probably largely responsible for the mildness of the epizootic, but the possibility that the organism was less virulent, or the dogs more resistant than in the Vietnam epizootic, cannot be ruled out.

Clinical, hematological, and serological surveillance is continuing.

2. Prediction of Illicit Drug Use by United States Servicemen

OBJECTIVE: For a description of the objectives of this study see the SE Annual Progress Report, March 1972.

PROGRES[illegible] views, at four-month intervals, of 425 soldiers in Thail[illegible]e been completed. We were able to obtain complete information on 345 of these men. The remaining individuals were interviewed only one or two times. Twenty-five additional individuals left Thailand within four months after arriving and were therefore not interviewed at all.

Data derived from these interviews have been collated and coded for automatic data processing. Analyses of these data are currently being conducted. These will be used to determine: a) the predictive validity of the questionnaire instrument, b) variables which relate to development of risk for drug use and c) variables which interfere with the ability of the instrument to predict drug use.

In addition to behavioral information concerning drug abuse, we have drawn bloods from these individuals and obtained medical histories. Review of medical and laboratory records was also conducted. This will provide unique data on development of inapparent disease in this population during a tour in Thailand.

SUMMARY: Interviews have been completed on 425 soldiers during their first year in Thailand. Drug use classifications based on the clinical interview and the questionnaire instrument will be compared to evaluate the predictive validity of the questionnaire. The analyses will focus on variables contributing to risk for drug use. Information concerning development of inapparent infection will provide unique epidemiological data on this population.

3. A Behavioral Survey of Thai Prostitutes

OBJECTIVE: a) To collect behavioral information from a prostitute population concerning attitudes and knowledge about venereal disease (VD), b) to correlate these with certain concurrently collected laboratory data and c) to determine what difference exists between these variables for women coliciting American and those women who do not have contact with Americans.

BACKGROUND; The prevalence of venereal disease among American troops seems to many observers to be too high. Considerable man-hours and money are spent on treatment of VD in American troops and on programs for the control of VD among prostitute populations. An evaluation of the variables under study will provide information on the effectiveness of US military sponsored venereal disease control programs directed at these prostitute populations.

DESCRIPTION: Laboratory and interview data were collected from 520 women. Four hundred and forty-four of these solicit US military personnel and 76 solicit only from the local national (Thai) population. Most women were selected from those attending VD clinics, but 20% were selected from other sources in order to gain information most representative of the entire population.

PROGRESS: Data are currently being analyzed. It is anticipated that analysis and write-up will be completed by July 1975.

SUMMARY: Laboratory and interview data were collected from a total of 520 Thai prostitutes. This will provide unique behavioral and laboratory information on VD in this population and effectiveness of VD control programs. Data analysis and write-up are currently being conducted.

4. Mosquito Fauna of Thailand

OBJECTIVE: To collect, identify, catalogue and redescribe the mosquito species of Thailand. Information is also being gathered on

the distribution, larval habitats and other aspects of the bionomics of various species. The eventual goal is the production of monographs on the mosquitoes of the area, together with keys, handbooks and other identification aids, for use of workers in public health and associated fields.

DESCRIPTION: Mosquitoes are collected from many areas of Thailand in connection with various studies on malaria and other arthropod-borne diseases. Additional collections of a specialized nature are made to obtain a correlated series of larvae, pupae and adults for illustration and taxonomic studies. The majority of this material is shipped to the Smithsonian Institution for study by specialists in the Southeast Asia Mosquito Project (SEAMP).

PROGRESS: During this year 887 collections of mosquitoes were made in Lampang, Chiangmai and Kanchanaburi provinces. These collections resulted in 7,300 pinned adults, 6,190 slide mounts of larvae, larval and pupal skins and 20 slide mounts of terminalia. Progeny rearings of nine Aedes (Finlaya) niveus group mosquitoes from filariasis study sites in Kanchanaburi province yielded a total of 190 pinned adults, 182 slide mounts of larvae, larval and pupal skins.

Culex: An attempt to collect all species of the Culex (Culex) vishnui subgroup of Thailand is nearing completion. Culex barraudi and Culex whitei, rare species of this group, were collected from seepage pools, ground pools, wells and paddy fields in Chiangmai and Lampang provinces. Collections of all stages of Culex alienus, C. annulus, C. perplexus, C. pseudovishnui and C. tritaeniorhynchus were obtained during the previous period.

Aedes: Work on the important niveus complex of species in Aedes (Finlaya) has been concentrated mainly at Sangkhlaburi in Kanchanaburi province. Aedes niveoides seemed to be one of the most abundant species in this area. Another three unidentified species were obtained from bamboo cup collections.

Ten species of Aedes (Stegomyia) were collected during filariasis studies in Sangkhlaburi district. The immature stages of A. albopictus have been collected in association with those of A. pseudalbopictus.

Heizmannia: Approximately nine species of this genus were collected at Sangkhlaburi. H. reidi, H. mattinglyi, H. covelli

females and an unidentified male were collected and reared from immature stages. Heizmannia mattinglyi which is known only from the adult female was collected in association with H. covelli. Associated larval and pupal skins of both these species are indistinguishable, but the male terminalia are typical of H. covelli.

5. Pathogens of Medically Important Mosquitoes of Thailand

OBJECTIVE: To determine the kinds of insect pathogens present in medically important species of mosquitoes in Thailand and to elucidate the biology of selected pathogens sufficiently to assess their potential as biological mosquito control agents.

BACKGROUND: Successful and economically feasible biological control of certain important agricultural and forest insect pests with pathogens, used alone or in combination with other control agents, has been thoroughly documented. Several pathogens for use against agricultural pests have been approved by the Food and Drug Administration and the Environmental Protection Agency and are currently being produced commercially and used in the United States. Interest in the potential of pathogens for the control of medically important arthropods is not new, but the successful use of pathogens in agriculture and forestry, combined with widespread environmental interest, has provided impetus in recent years for more vigorous investigation of their potential value to medical entomology. The SEATO Medical Research Laboratory is an ideal location for such investigations, for in Thailand there are more than four times as many species of mosquitoes (400+) as in the United States. Among these are several species of primary international importance as disease vectors.

DESCRIPTION: During the first six months of this project the slide-mounted mosquito larvae in the taxonomic collection of the Medical Entomology Department was screened for microscopically detectable pathogens. Also, a field survey for mosquito pathogens was initiated. The survey has concentrated on Culex pipiens quinquefasciatus in the Bangkok-Thon Buri area. Large numbers of larvae collected at various locations were transported to the laboratory. These were visually examined for gross signs suggesting presence of pathogens, such as loss of pigmentation, presence of abnormal body proportions.

Larvae displaying gross signs of pathology were segregated. Some of these abnormal larvae were examined microscopically in wet-mounts and/or as Giemsa-or hematoxylin-stained squash-smears,

while the balance were prepared for paraffin sectioning and hematoxylin-eosin staining. When large collections of larvae showing grossly abnormal signs were made, a portion of the collections were reared in the laboratory and the mortality rate was recorded. Smears were made of larvae and pupae that died. Surviving pupae were allowed to develop to adults and the progeny of these examined for evidence of transovarial (vertical) transmission. Attempts to transmit pathogens by per os exposure of uniinfected laboratory-reared larvae were also made. Survivors of these tests were reared and their progeny examined for evidence of vertical transmission.

PROGRESS: Five distinct species of fungus of genus Coelomomyces were found in the slide-mounted larval collection. Culex tritaeniorhynchus and C. fuscocephala were infected with apparently the same species, three different species were found in Anopheles vagus and one in A. nivipes. One nematode infection was found in C. tritaeniorhynchus.

Seventy-eight collections of C. pipiens quinquefasciatus were made at 54 locations in the Bangkok-Thon Buri area. Stained smears of larvae from 67 of the 78 collections were examined. Examination of all material collected is not complete, but the following observations have been made. Of 2073 larvae displaying grossly abnormal signs, microbial agents were found in 1971 (95%). Microbial agents were often seen within tissues or hemolymph of larvae examined in wet-mounts prior to squash-smearing. The presence of numerous microbial contaminants on the integuments and within the alimentary canals of larvae possibly obscured infections that will become apparent with the examination of sectioned material.

Two microbial agents were present in almost all specimens showing gross signs of disease. One was a dark-staining cytoplasmic inclusion, about four microns in length, that appeared to replicate by transverse fission. This agent could be seen within cells in larvae examined in wet-mounts. It has been transmitted to uninfected larvae in the laboratory. The other common agent was of minute bacilliform structure at the limit of resolution of the light microscope, somewhat less than one micron in length. This agent could be detected within the hemolymph of larvae examined in wet-mounts by its intense Brownian motion and in smears stained with Giemsa's stain at pH 7.4. Larvae containing this agent died almost without exception. The agent has been transmitted to uninfected C. quinquefasciatus larvae in the laboratory. Microsporidia were found in 27 of 67 (40%) collections from 15 of 54 (28%) locations surveyed. Preliminary transmission attempts have not been successful,but definitive attempts are planned.

One fungal agent has been found which presents by turning mosquito larvae orange. All transmission attempts with this agent have been unsuccessful. More material is being sought in the field.

An additional agent in the size range and with the staining characteristics of a polyhedral virus was collected from 11 of 54 (20%) locations. This agent was transmitted in the laboratory, but transmission rates were lower than those expected with a polyhedral virus and the identity of the agent remains in doubt. Further studies are underway.

DISCUSSION: Distortion of Coelomomyces sporangia resulting from the mounting techniques used for mosquito larval taxonomic specimens made species determination impossible. Adult forms are required for identification of insect nematode parasites, so the larval nematode found could not be identified.

Studies are underway to determine the identity and to define the biological characteristics of agents found in surveys of Culex pipiens quinquefasciatus. Efforts in the following areas will be required: (1) culturing of bacteria and fungi, (2) electronmicroscopy of suspected virus, rickettsiae, and microsporidia, (3) transmission experiments to determine optimum conditions and methods for transmission and propagation, and (4) experiments to establish host ranges. The survey for mosquito pathogens will be extended both geographically and to include other species of medical importance.

Access to the scientific literature in invertebrate pathology is essential for the identification of mosquito pathogens, and the literature is a time-saving source of methods in the propagation and study of pathogens. Therefore, a high priority will be placed on accumulating a bibliography of pertinent literature and a collection of reprints of previous reports of pathogens in mosquitoes.

6. Evaluation of Systemic Insecticides for Control of Trombiculid Mites

OBJECTIVE: To determine if rodent baits treated with organophosphate insecticides are effective against larval trombiculid mites (chiggers) on wild rodents in areas of Thailand where these mites are known to carry scrub typhus.

BACKGROUND: In World War II more than 7,000 casualties were caused by scrub typhus in U.S. Forces stationed in the Western Pacific and Burma. Control measures used at that time to

prevent scrub typhus included the burning of grass and the stripping of soil with bulldozers in encampments; DDT was effective for control of mosquitoes and flies, but ineffective against the scrub typhus mites. Impregnation of clothing with repellents such as dimethyl pthalate was the most effective method of personal protection against chiggers, but it did not enjoy a high degree of troop acceptance. Scrub typhus continued to be a problem during the Korean Conflict; control and prevention measures used during that period were essentially the same as used during WWII. some of the later chlorinated hydrocarbons, such as chlordane, dieldrin and lindane, were found to be effective for area control of trombiculid mites. However, large quantities of these insecticides were required (up to 5 lbs/acre) for effective control, and because of their non-specificity these compounds destroyed innocuous and beneficial organisms. More recently, organophosphates, such as ronnel and Ruelene, have been employed as systemic insecticides to control fleas on rats and dogs and ticks on cattle with a significant degree of success. Another of these compounds, Phoxim, has been used in New Mexico to control fleas and several types of mites on wild rodents which fed on phoxim-treated grain (88).

The principal vector of scrub typhus in Thailand is Leptotrombidium (L.) deliense (Walch) which parasitizes rodents, chiefly Rattus rattus, living in scrub forest. This mite is widely distributed throughout Thailand. During the reporting period preliminary tests of Phoxim were carried out on wild-caught Rattus rattus trapped near Sakaerat in Nakornratchasima province. This area is located near the site of an outbreak of scrub typhus which occurred in a unit of Thai Army personnel in 1965 (89).

DESCRIPTION: In each series of tests, wild-caught Rattus rattus trapped in the vicinity of Sakaerat, were inspected for the presence of larval trombiculid mites in their ears. Previous experience trapping rats in that area indicated that a high proportion of the trombiculid mites infesting R. rattus were T. deliense. Infested rats were separated into two equal groups; one was given a diet of phoxim-treated corn and the other untreated corn. During these tests each rat was confined to a cage suspended over pans of water to catch all mites that detached from their hosts. The water was removed daily from these pans, after a visual inspection for mites on the surface,and filtered through cotton muslin which was examined under a stereoscopic microscope for chiggers. Mites found were removed and placed in vials containing a moistened plaster of Paris-charcoal base and examined daily until it was established whether death had occurred or if metamorphosis had taken place. Dead mites were placed in

Table 1. Effects of 0.24% Phoxim-treated Bait on Trombiculid Mites Infecting Rattus rattus.

Day	TEST ANIMALS (12)		CONTROL ANIMALS (12)	
	Total No. Mites Recovered	Mite Mortality (Per cent)	Total No. Mites Recovered	Mite Mortality (Per cent)
1	285	15.1	172	23.8
2	341	27.5	323	31.8
3	71	39.4	92	15.2
4	11	27.3	25	32
5	4	100	14	50
6	10	70	46	26
7	2	50	28	0
8	1	100	5	0
9	0	-	0	-
10	2	100	0	-
11	2	50	0	-
12	2	0	0	-
13	1	0	1	100
14	667*	32.4	784*	15.6
Total	1399	28.6	1493	20.7

*Recovered after rats sacrificed

Table 2. Effect of 0.36% Phoxim-treated Bait on Trombiculid Mites Infecting Rattus rattus

Day	TEST ANIMALS (13)		CONTROL ANIMALS (13)	
	Total No. Mites Recovered	Mite Mortality (Per cent)	Total No. Mites Recovered	Mite Mortality (Per cent)
1	681	15	505	8
2	1519	10	953	9
3	292	20	311	9
4	107	24	159	11
5	25	56	28	53
6	3	33	20	10
7	1	100	8	62
8	12	17	2	100
9	2	50	5	80
10	2	100	3	33
11	0	-	0	-
12	0	-	5	80
13	0	-	7	86
14	359*	44	1844*	24
Total	3003	17	3850	17

*Recovered after rats sacrificed

Table 3. Effect of 0.36% Dimethoate-treated Bait on Trombiculid Mites Infecting Rattus rattus.

Day	TEST ANIMALS (10)		CONTROL ANIMALS (10)	
	Total No. Mites Recovered	Mite Mortality (Per cent)	Total No. Mites Recovered	Mite Mortality (Per cent)
1	2300	10.5	632	0.2
2	162	86	846	0.3
3	29	93	94	9.5
4	6	100	71	0.6
5	1	100	20	5
6	1	100	7	14
7	0	-	27	7
8	0	-	13	7
9	0	-	0	-
10	273*	91	1153*	2
Total	2772	24	2863	3

*Recovered after rats sacrificed

70% alcohol to be later mounted and identified. Larval trombiculid mites which underwent metamorphosis were discarded and were considered to have survived. Upon conclusion of the tests all rats were sacrificed, and any chiggers still attached were removed and processed in the same manner as those recovered during the tests.

PROGRESS: Uncracked, dried corn, treated with two concentrations of phoxim (0.24% and 0.36%), was fed to wild-caught Rattus rattus infected with trombiculid mites. No significant increase in mortality of mites on the rats fed treated grain was observed in either test (Tables 1, 2). In the two phoxim trials the proportions of T. deliense in the mites recovered from the R. rattus were 53 and 70 per cent for the 0.24% and 0.36% trials, respectively. In a single test with 0.36% dimethoate-treated corn, the overall mortality in mites on the animals fed treated grain was greater (24%) than among mites on the control animals (3%), but not significantly higher than was observed for either groups in the phoxim tests (Table 3). However, an unusually high proportion of the mites on the rats fed dimethoate detached on the first day of the test; 83% of the mites recovered from these animals detached on day 1, while at the same time only 22% of the mites on the control animals dropped off. If the data for the first day are excluded, the toxic effects of dimethoate appear more impressive: 89% of the mites recovered between days 2 and 10 from rats fed dimethoate treated grain were dead, while only 3% of the mites recovered during the same period from control animals died. It is probable that the dimethoate-treated grain (which had a powerful odor) had a fumigant effect on the mites, causing large numbers to detach from their host on the first day of the test before the test rats had a chance to ingest much of the treated grain. Seventy-two per cent of the mites recovered from the animals in these tests were T. deliense.

7. Studies on the Growth, Development, and Reproduction of Gibbons in Captivity

OBJECTIVE: To collect information on the growth, development, and reproduction of gibbons in captivity, and to collect normal biological data which may be useful in biomedical research.

BACKGROUND: A colony of 42 gibbons (Hylobates lar) is maintained at SEATO Medical Research Laboratory for use in essential medical research projects of the laboratory. An active breeding program has been conducted for the past several years, and 27 young have been born in the colony. Physiological, biochemical and hematological observations are made on a regularly scheduled basis.

Table 1. Newborn Gibbons 1974 - 1975

Baby Number	Date of Birth	Parents	
		Female	Male
Pc 24	13 Sep 74	B-7	P-16
Pc 25	30 Oct 74	B-6	B-12
Pc 26	6 Jan 75	B-4	B-8
Pc 27	20 Feb 75	B-11	B-64
Pc 28	17 Mar 75	B-59	B-12

Table 2. Tooth Eruption in Colony-born Gibbons

Tooth	Decidual		Permanent	
	Observations	Age*	Observations	Age
1st incisor	2	1.0	6	14.6±1.5
2nd incisor	2	1.5	5	20.4±2.2
Canine	7	3.4±1.3	1	48.0
1st premolar	9	3.4±0.5	1	31.0
2nd premolar	10	5.9±1.3	1	41.0
1st molar	8	15.1±1.6	1	41.0
2nd molar	2	18.0	1	41.0

* Mean ± one standard deviation

Table 3. Summary of Hematologic Findings in Mature Captive Gibbons - Mean Values ± 1 S.D.

Parameter		Non-splenectomized		Splenectomized	
		Male	Female	Male	Female
	RBC $X10^6$	7.70 ± 1.36 (385)*	8.02 ± 1.4 (260)	7.60 ± 1.29 (368)	7.12 ± 1.28 (384)
	WBC $X10^3$	11.32 ± 4.16 (489)	11.35 ± 5.76 (477)	12.46 ± 4.05 (426)	13.95 ± 4.92 (443)
	PCV (%)	48.92 ± 5.93 (496)	48.13 ± 5.72 (339)	48.65 ± 4.93 (388)	46.77 ± 4.90 (507)
	Hb (gm/100 ml)	14.74 ± 1.97 (439)	14.17 ± 1.78 (354)	14.53 ± 1.82 (321)	14.23 ± 1.70 (358)
Differential in percentage	Lymphocytes	53.19 ± 12.28 (493)	48.01 ± 16.52 (428)	51.21 ± 23.01 (410)	57.70 ± 33.31 (437)
	Neutrophils	43.47 ± 17.56 (491)	47.03 ± 19.78 (457)	39.92 ± 17.58 (404)	38.20 ± 15.10 (434)
	Basophils	1.05 ± 1.06 (491)	1.20 ± 1.18 (457)	1.18 ± 0.99 (404)	1.34 ± 1.33 (434)
	Eosinophils	2.29 ± 2.38 (491)	3.05 ± 3.06 (457)	2.48 ± 2.38 (404)	2.21 ± 2.17 (434)
	Monocytes	3.61 ± 2.29 (491)	4.00 ± 2.83 (457)	3.65 ± 2.60 (404)	3.41 ± 2.35 (434)
	Bands	1.18 ± 1.07 (491)	1.15 ± 0.70 (457)	1.21 ± 0.68 (404)	1.14 ± 0.60 (434)

* Numbers in parentheses represent total number of determinations.

Table 4. Summary of Hematologic Findings in Immature Captive Gibbons (< 4 years old) - Mean ± 1 S.D.*

Parameter		Value
	RBC $X10^6$	7.18 ± 1.07 (49) **
	WBC $X10^3$	9.87 ± 1.58 (119)
	PCV (%)	44.75 ± 4.48 (124)
	Hb (gm/100 ml)	14.20 ± 1.57 (43)
Differential in percentage	Lymphocytes	64.33 ± 13.39 (105)
	Neutrophils	31.19 ± 12.65 (105)
	Basophils	1.02 ± 0.78 (105)
	Eosinophils	2.44 ± 3.14 (105)
	Monocytes	3.41 ± 2.13 (105)
	Bands	0.60 ± 0.32 (105)

* Values from both sexes included.

** Numbers in parentheses represent total examinations performed.

PROGRESS: Vaginal swabs were taken daily from nine adult gibbons and the time of menstruation was recorded. Fifty-two menstruations were observed. The duration of menstruation was 1-4 days, with 58 per cent of these lasting only one day.The interval between menses was 17-120 days with the most common interval being 17-25 days. This information is similar to that recorded in previous annual reports. Information concerning copulation behavior and fetal development is being collected and will be reported when sufficient data are collected to allow interpretation. Since the last reporting period five gibbons were born in the colony. Birth data are listed in Table 1.

Colony-born gibbons have been examined monthly from birth to adulthood, and the sequence of tooth eruption has been recorded. Table 2 summarizes these observations to date.

Blood samples have been collected from all gibbons within the SEATO Medical Research Laboratory colony quarterly as a screening procedure for early detection of granulocytic leukemia and other diseases. This procedure has resulted in a large accumulation of hematologic data which have been compiled and are presented in Tables 3 and 4. Although many of the older gibbons in the colony were inoculated with various experimental agents during the late 1960's, the values presented represent samples taken from animals that were free of clinical evidence of experimental or spontaneous disease. The immature gibbons have not been used experimentally.

SUMMARY: Five gibbons were born in the breeding colony of eight pairs during the year. Observations on normal growth and development of colony born gibbons are presented, and normal hematologic values in young and in mature gibbons are reported. These studies are continuing.

8. Laboratory Animal Disease in Thailand: Its Occurrence and Importance to Comparative Medicine

OBJECTIVE: The objective of this study is to detect and investigate spontaneous diseases of laboratory animals. This information will aid in defining and improving the health of laboratory animals models for the study of human diseases.

DESCRIPTION: In order to accomplish the objective, a program of continuous surveillance of the health status of the animal colony has been developed. Four areas are emphasized in this program:
1. the disease screening program conducted in the laboratory

animal breeding colony, 2. the recurring clinical and laboratory examination of animals housed in the colony including those procedures performed during the quarantine of newly purchased animals, 3. the post mortem examination of animals that die in the colony, and 4. the development of standards for operation and quality control. When indicated by the findings, experimental studies are initiated to explore in detail the problems that occur

PROGRESS: The prevalence of spontaneous infectious diseases in the rodent breeding colony remained at a low level during the year. This observation is consistent with findings during the previous reporting period. Annual production of mice, rats, hamsters and guinea pigs was reduced slightly due to a decrease in demand.

Disease screening was conducted quarterly utilizing retired breeders from the mouse, hamster and guinea pig production units. Results of histopathologic findings are summarized in Table 1. All lesions observed were mild and focal in nature and are not uncommon in old animals maintained under conventional conditions. Lesions observed were considered degenerative in nature, except the pulmonary lesions in mice which were consistent with chronic murine pneumonia, and the nematodes in the intestinal tract of mice which were identified as pinworms (Aspicularis sp.).

Table 1. Frequency of Histopathologic Findings in the Rodent Breeding Colony - 1974

Species	Number Examined	Pulmonary No. (%)	Gastro-Intestinal No. (%)	Genito-Urinary No. (%)	Hepatic No. (%)
Mouse	50	14(28)*	5(10)*	12(24)	11(22)
Hamster	30	0	2(6)	2(6)	4(13)
Guinea Pig	35	14(40)	0	3(8.5)	6(17)

* Nematodiasis

Table 2. Prevalence of Virus HI Antibody in 50 Mice

Virus Antibody	Percent Positive
GD VII	69
Sendai	60
Reovirus 3	60
Minute Virus of Mice	57
Pneumonia Virus of Mice	0
K	0
Polyoma	0

Table 3. Rhesus Monkey Losses During Quarantine
April 1974 - March 1975

Month	Number Received	Number Deaths	Intestinal Disease	Pulmonary Disease	Un-determined
Apr 74	85	5(5.9%)	3	2	0
Jul 74	85	3(3.5%)	2	0	1
Feb 75	170	4(2.3%)	1	3	0
Total	340	12(3.5%)	6	5	1

Bacteriologic examinations identified organisms similar in type and prevalence to those published in previous annual reports. Virologic screening studies were performed using sera from 50 retired breeder mice. The hemagglutination-inhibition test (HI) was utilized for detecting antibodies to GD VII, Sendai, Reovirus 3, Minute Virus of Mice, Pneumonia Virus of Mice, K, and Polyoma viruses. Hepatitis, Lymphocytic Choriomeningitis, and Mouse Adenovirus antibodies were detected utilizing the Complement-fixation test (CF). Results of the HI tests are shown in Table 2. Results of the CF tests were not available at the time of publication.

During the period 1 April 1974 through 31 March 1975, 340 rhesus monkeys were imported directly from India. Twelve (3.5%) monkeys died during the quarantine period, primarily from gastrointestinal and respiratory diseases (See Table 3). Intestinal parasitism and measles were prevalent in newly arrived monkeys. One monkey that was sacrificed in February, 1975 after exhibiting clinical signs of a CNS disturbance had a lobar pneumonia; a Pneumococcus sp. was isolated from the lungs.

Three gibbons in the SEATO Medical Research Laboratory colony died during the year. One adult male (S-58) died of pneumonia due to migrating Strongyloides sp. larvae. Two immature colony-born gibbons (PC 22, PC 23) died this year. Gibbon PC 22 died of a bacterial pneumonia; no lesions were seen at necropsy examination which would account for the death of PC 23.

Between October and December 1974, ten colony rabbits developed a moderate to severe necrotic dermatitis of the limbs. The infection generally began on the paw and progressed proximally. Clinically, the disease was characterized by swelling, redness, pain and loss of hair over the affected areas. Later the skin became necrotic and sloughed. In some rabbits the entire limb was involved, while in others only the distal portion of the limb was affected. Six animals responded to 14 days of Kanamycin therapy (15 mg/kg/day), while four animals did not respond and had to be sacrificed. Histologically, the disease was bacterial colonies visible in the necrotic debris. Bacteriologic examination revealed coagulase positive Staphylococcus aureus. Affected tissues were collected at necropsy, ground in a sterile glass Tenbrook grinder and inoculated into the dermis and subcutaneous tissue of the hind-paw of three clinically normal rabbits. One rabbit developed a subcutaneous abscess 12 days after inoculation which persisted until the animal was sacrificed at four weeks. S. aureus was recovered in pure culture. The portal of entry has not been

clearly established, but may have been through the nail bed following trauma produced during toenail clipping operations.

Mice in the breeding colony were found to be infested with mites in October 1974. Alopecia, pruritis and inflammation were the predominant clinical signs. The mite was identified as Myobia musculi. All rooms were contaminated and all ages were affected. The condition was brought under control by whole body immersion of mice in a 2% malathion solution. Pregnant animals were not dipped until after their young were weaned. Baby mice were not dipped until the time of weaning. All bedding and cages were autoclaved before and after use for six weeks. Mortality from the dipping process was less than 0.4%. No evidence of mite infestation has been observed during the last three months.

9. Vertebrate Reservoirs of Disease

OBJECTIVE: To provide prompt identification of specimens and to advise concerning the ecology of vertebrates involved in the transmission of human disease.

BACKGROUND: Our large vertebrate collection was moved to the Applied Scientific Research Corporation of Thailand, where it became the nucleus of the Thai National Reference Collections under the curatorship of the late Mr. Kitti Thonglongya. A Definitive collection of Asian rats and mice is still housed at SEATO Medical Research Laboratory.

Vertebrate studies starting with birds in the ecology of Japanese encephalitis virus later shifted to mammals. Surprising discoveries in Thai mammals upset previous taxonomies, necessitating revisions throughout the entire Asian range of some genera. (Travel outside of Thailand was at personal expense).

DESCRIPTION: All possible evidence for species-limits has been assembled including scientific study-skins and skulls, tape recordings of vocalizations, observations of behavior and ecology in the natural state, host-specific ectoparasites, karyograms, and breeding experiments. Native mouse colonies have been distributed to laboratories at Yale, Roswell Park Memorial Institute, NIH, Houston, Woods Hole, Bonn, Cambridge, Lausanne, and New Zealand. At the moment we have requests for shipment of mice to Buffalo, NIH, Houston, and Hannover. The above laboratories are studying viruses, cancer, cytogenetics, chromosome banding patterns, and satellite DNA. Our colony of the Asian house mouse, Mus musculus castaneus, has been expecially valuable for

Table 1. Identification of Infected Animals

Study Project	Animals Identified	Remarks
Medico-ecology and Economic study, Nepal	Bandicota bengalensis	Large mounds in fields, burrows to 60 feet long, also in houses; first records for Kathmandu Valley high altitude villages; also fields at Kathmandu
	Mus musculus homourus	
	Mus musculus castaneus	Houses at Kathmandu and Hetaura
	Rattus turkestanicus	High altitudes, in the house
	Rattus nitidus	Kathmandu, buildings
	Rattus brunneus	Buildings and fields, Kathmandu. Giant form of, or related to, Rattus rattus. Karyotype unlike R. rattus fide A. Gropp.
Rabies (positive, diagnosed by CPT Dill)	Rattus rattus mindanensis	Young male, no. 4-499; on 16 Sept 74 entered base bowling alley and bit sailor; Subic Bay, Philippines
	Rattus exulans	Host of fleas; indoors at Paktongchai and Bangkok
	Rattus rattus	Host of chiggers; forest at Sakaerat Station, fields at Paktongchai, houses and trees at Bangkok
Kaeng Khoi virus	Rattus hinpoon sp. nov.	Limestone cliffs, mouth of cave
	Rattus rattus	Cave mouth, also far inside
	Rattus neilli sp. nov. (Table 3)	Limestone cliffs, including mouth of cave
	Falco severus	A falcon which takes bats during their exit flight at dusk
	Taphozous theobaldi	A larger bat in the cave
	Tadarida plicata	The lesser, more numerous bat, and the one subject to fatal liver disease from the virus
Tick virus survey	Small carnivores, rodents	Hosts of ticks
Gnathostoma spinigerum	Mustelidae	Coordination of scientific names used by Russian authors
Rickettsiae; systemic poisons against ectoparasites	Bandicota indica	Hosts of ticks; fields at Paktongchai and Bangkok
	Bandicota savilei	Host of ticks; fields at Paktongchai
	Rattus surifer	Host of fleas; forest at Sakaerat Station
	Rattus norvegicus	Host of fleas; indoors at Paktongchai (Black phase) and Bangkok
	Rattus losea sakeratensis	No known ectoparasites; fields at Paktongchai

cytologic and cancer research because of its wealth of novel alleles unknown in the laboratory house mouse.

PROGRESS: Identification of Infected Animals.-- Some identifications for various studies are listed in Table 1.

Taxonomic Research:--Table 2 is an addition to the Taxonomic checklist previously reported (1). Determination of the number of species of gibbons (Table 3) grew out of interest in rearing these apes at SEATO Medical Research Laboratory, where vocal distinctions were first noticed, and where karyotypes of three species were prepared. With E. Marshall, tape recordings were made of every species (in the wild except for Hylobates concolor (Figure 1). We discovered previously unknown areas of Hylobates agilis in Southern Thailand and Central Kalimantan (Figure 2). We are the first to possess data permitting a definitive enumeration of the species (Table 3).

Table 2. Changes in Checklist of Thai Rats and Mice (1)

Former Name	New Name
Subgenus Leggadilla	Subgenus Pyromys
Rajah Rats	Subgenus Maxomys
Niviventer Group	Subgenus Niviventer
Rattus niviventer	Rattus confucianus
Rattus fulvescens	Rattus bukit
Rattus fulvescens fulvescens	Rattus bukit gracilis
Rattus nielli is added, as number 37, in the Subgenus Leopoldamys.	
"Rattus sakeratensis"	Rattus losea sakeratensis

Table 3. Taxonomy of Gibbons (Lesser Apes), Genus Hylobates

Scientific Name	Common Name	Characteristic
A. Subgenus Symphalangus		50 chromosomes
1. Hylobates syndactylus	Siamang	
B. Subgenus Nomascus		52 chromosomes
2. Hylobates concolor	Hainan gibbon	
C. Subgenus Hylobates		44 chromosomes
I. Peripheral, isolated species		
3. Hylobates moloch	Javan gibbon	
4. Hylobates hoolock	Hoolock	
5. Hylobates klossi	Mentawai gibbon	
II. The Lar complex		
a. Superspecies of pileatus/muelleri		Bubbling great-call
6. Hylobates pileatus	Pileated gibbon	
7. Hylobates muelleri	Bornean gibbon	
b. Superspecies of agilis/lar		"Soaring" great-call
8. Hylobates agilis	Agile gibbon	
9. Hylobates lar	White-handed gibbon	

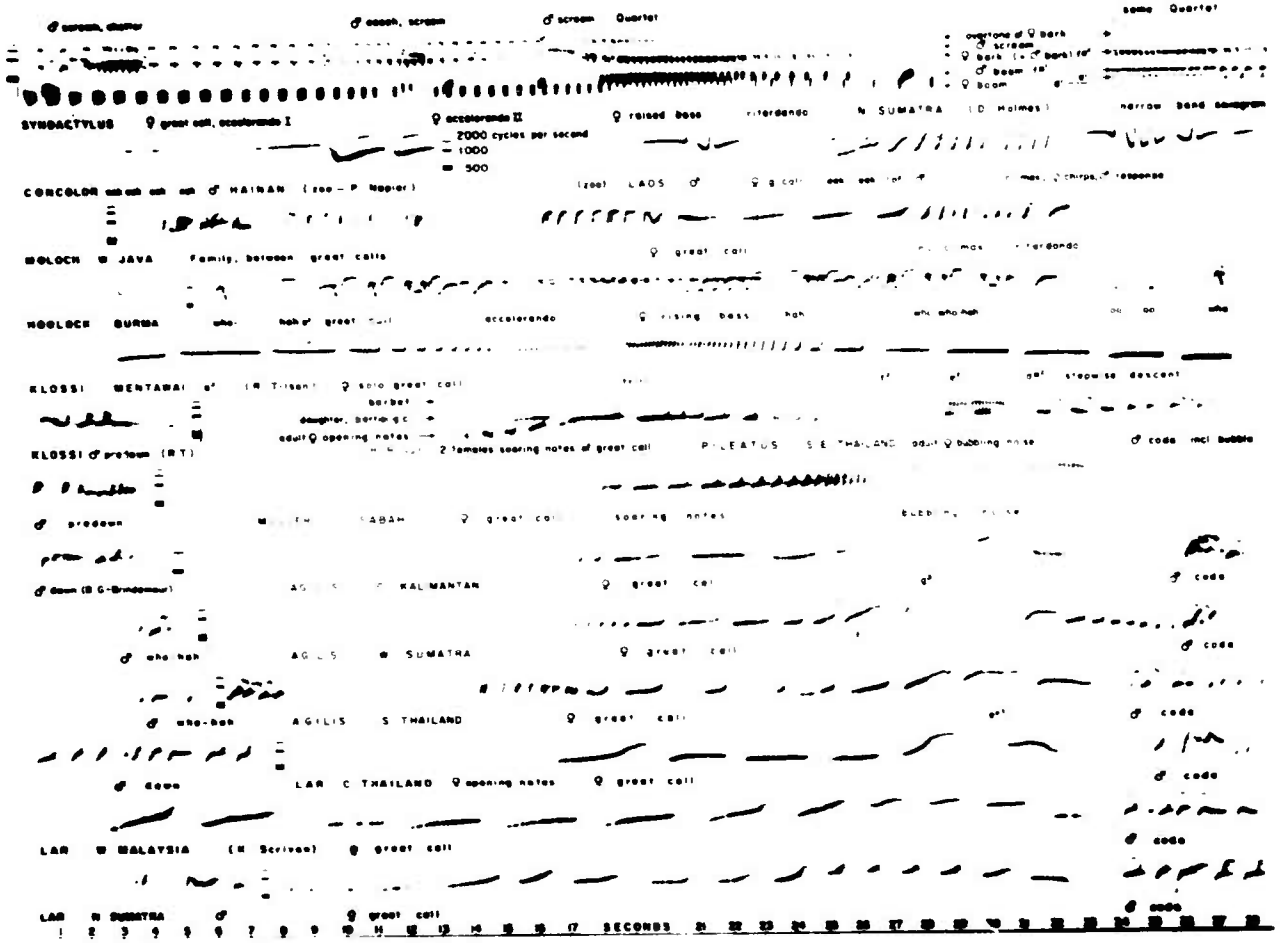

Figure 1 Sound spectrograms of territorial songs of gibbons. There are nine types of songs divided among the 14 isolated populations shown in Figure 2.

Figure 2. Distribution of species of gibbons. Solid circles indicate the places where the author tape-recorded wild gibbons; open circles are places whence recordings were supplied by colleagues.

10. Migratory Animal Pathological Survey

OBJECTIVE: The objectives of the MAPS program as set out in the original proposal of 1963 was to learn the migration routes of birds of East Asia, the ectoparasites that were harboured on the birds and the haematozoa which infected birds as correlative data for use by epidemiologists interested in the dispersal and periodic occurrence of human infections, especially the arthropod borne viruses and rickettsias.

BACKGROUND: In October 1973 the Migratory Animal Pathological Survey activity was closed and all of the files and library moved from the Applied Scientific Research Corporation of Thailand (ASRCT) to the SEATO Medical Laboratory. A final report on the study of the bird migration in Eastern Asia has been completed and published in book form (90). The first quarter of the year was devoted to proof reading and composition of the book which came off the press in June. More than 1,000 copies were mailed to ornithologists and libraries throughout the world and there has been a steady demand for copies ever since.

This report summarized the results from the banding of more than 1,200,000 birds of 1,218 species in ten countries of Eastern Asia; India, Thailand, Malaysia, Indonesia, Philippines, Taiwan, Hong Kong, Japan, and Korea. There were more than seven thousand recoveries from these birds and their movements demonstrated that there were four major flyways across Asia, the studies being made in the East Asian and Indo-Asian flyways. The other two flyways are the Eastern European and Western European. Within these four flyways are numerous migration routes related to species and populations. Most species or populations remained in a given flyway without much overlap to adjacent ones. A given species with a wide distribution may have populations restricted to certain flyways and migration routes. Other information arising from these studies demonstrated that there was migration within the temperate zone and limited to it, migration within the tropics and limited to it, nomadism, altitudinal migration, and local dispersion as well.

Survival among the birds followed patterns already demonstrated in Europe and North America. Juvenile mortality is 60% or more until they gain experience. Following the first year survival rises until 75% or more live through each succeeding year. Data from Malaysia where there was a long term survival study indicated that many species were long lived, even tiny sunbirds which were still alive and active at 12 years.

PROGRESS: The work of MAPS was divided into three phases; bird migration, ectoparasites, and avian haematozoa. Summary of the ectoparasite studies appeared in book form in 1973 (91). During the remainder of 1974 much time was devoted to the preparation of a report on the haemotozoa studies. These had involved the examination of more than 50,000 blood films from 1,147 species which had been completed before the project was closed at ASRCT. Summary, analysis, and revision are still underway and it is expected that the volume will be completed in 1975 or 1976 to make up the third report from this extensive study in Asia.

In the meantime recovery records continue to come in from hunters and bird students in Eastern Asia gradually increasing our knowledge of the survival of birds. Many species have now passed 10 years.

Project 3A762759A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 074 Tropical and subtropical diseases in military medicine

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6442	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762759A831	00	075
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with security classification code)

(U) Rickettsial Diseases of Military Personnel

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
55 08	CONT	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

16. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	3	200
FISCAL YEAR CURRENT 76	4	214

18. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security classification code)

(U) Rickettsial infections; (U) Laboratory diagnosis; (U) Vaccines; (U) Epidemiology; (U) Ecology; (U) Vectors and reservoirs

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23. (U) 1. Detection of rickettsial diseases. 2. Improvement of methods for clinical serologic diagnosis and retrospective seroepidemiology. 3. Immunological studies on host response, both humoral and cellular, to infection with wild type and attenuated rickettsial agents, which represent a serious hazard to troops operating in the field.

24. (U) 1. Development of a hemagglutination assay for use in field studies. 2. Assay of protective effects afforded by passive transfer of humoral antibodies and cellular components in mouse model. 3. Survey of physical and chemical methods for rickettsial inactivation which preserve immunogenicity.

25. (U) 74 07-75 06 1. An indirect hemagglutination test for human antibody to typhus and spotted fever group rickettsiae has been developed. The test employs glutaraldehyde fixed sheep erythrocytes sensitized with rickettsial ESS. The reagents are stable for six months at 4C and 3 months at room temperature. 2. Host defenses in experimental scrub typhus have been characterized in a murine model. Using the inbred BALB-c mouse line production of complement fixing antibody has been assayed; relative protection offered by prior infection with a less virulent strain was assessed and the relative contributions of cellular and humoral immunity determined by assessing the protection effected by passive transfer of immune cells and sera. 3. Studies of physical and chemical methods for rickettsial inactivation have led to vaccine studies in outbred mice using formalinized and gamma-irradiation-inactivated scrub typhus organisms. Comparison of results with these two methods of inactivation shows that gamma-irradiation inactivated rickettsiae are more immunogenic than formalinized rickettsiae and preliminary studies suggest that complete protection against homologous challenge can be obtained with reasonable quantities of irradiated organisms. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 075 Rickettsial Diseases of Military Personnel

Investigators.
Principal: Joseph V. Osterman, Ph.D.; Akira Shirai, Ph.D.; MAJ George H. G. Eisenberg, MSC
Associate: Janis Campbell; SP4 Robert Davis

Description.

To develop new techniques for diagnosis of rickettsial disease and to characterize both the humoral and cell-mediated immunological responses to rickettsial infection.

Progress.

I. Development of new diagnostic techniques.

A. Indirect hemagglutination test for human antibody to typhus and spotted fever group rickettsiae.

The indirect hemagglutination technique has been utilized in the study of rickettsial infections by sensitizing erythrocytes with extracts of Proteus OX strain bacteria or rickettsial organisms. The Proteus OX strains are easily grown in large quantities and provide a convenient source of bacterial erythrocyte sensitizing substance (ESS), but certain rickettsial infections, such as Brill-Zinsser disease and rickettsialpox do not elicit the production of Weil-Felix agglutinins. Treatment of erythrocytes with rickettsial ESS broadens the scope of the test to include both these diseases, but erythrocyte fragility necessitates frequent sensitization of fresh cells and restricts employment of the technique in field situations. This work describes an indirect hemagglutination test for rickettsial antibodies which employs rickettsial ESS bound to glutaraldehyde stabilized erythrocytes. The test retains the sensitivity and simplicity of the original technique, but broadens its application by removing the need for frequent sensitization and standardization of erythrocytes and by extending its practical utilization to field studies.

The indirect hemagglutination (IHA) test was adapted to the microtiter technique to conserve reagents. Serum samples, initially diluted 1:40, subsequently underwent serial two-fold dilutions in the

wells of U-type microtiter plates containing 0.025 ml of PBS - 0.4% BSA - 0.1% NaN_3, followed by the addition of 0.025 ml of sensitized erythrocytes to each well. Non-sensitized erythrocytes were incubated with the lowest dilution of each serum employed in a test, as a control for non-specific agglutination. Plates were sealed with plastic tape, gently agitated and maintained at room temperature. After 2 hr and 18 hr incubation, the plates were inspected for agglutination and results were recorded as 1^+ (positive; smooth mat of cells covering bottom of cup, edges occasionally folded); + - (borderline; small mat of cells surrounded by compact circle of sedimented cells); 0 (negative; button of cells forming small open circle or compact button of sedimented cells in center of well). The endpoint was defined as the maximum dilution exhibiting 1^+ agglutination.

Stablilized erythrocytes were sensitized with various dilutions of rickettsial ESS and tested against serial dilutions of positive control serum. A 1:5 dilution was found optimal for both maximum titer and sharpness of endpoint. This dilution of typhus ESS contained 59 µg/ml of protein and 3.5 µg/ml of carbohydrate; spotted fever ESS contained 54 µg/ml of protein and 6.5 µg/ml of carbohydrate.

The undiluted typhus ESS antigen and the 10% suspension of fixed erythrocytes have been stored at 4C for periods of 9 and 15 months, respectively, with no signs of deterioration and upon sensitization, the antibody titer recorded with a positive control serum remained identical to the initial test. The assembled IHA reagent, composed of stabilized, sensitized erythrocytes has been stored for over 9 months at 4C and 3 months at room temperature. Neither preparation has shown any decrease or fluctuation in titer when tested weekly against a positive control serum. Fig. 1 includes a comparison of the agglutination pattern achieved with freshly sensitized cells and those stored as complete reagent for 3 months at room temperature.

Fig. 1 shows a representative IHA test employing antisera from patients with various rickettsial diseases. Only those antisera from Brill-Zinsser disease and epidemic or murine typhus infections agglutinated erythrocytes sensitized with ESS from R. typhi. The titer of the murine typhus antiserum in (A) and (H) is 1:10,240; the epidemic typhus antiserum in (B) is 1:640 and the Brill-Zinsser serum in (C) is 1:2560. Fig. 2 shows a similar IHA test employing erythrocytes sensitized with ESS from R. rickettsi. Only the antisera of patients with Rocky Mountain spotted fever caused erythrocyte agglutination. The titer of the spotted fever serum in (A) is 1:10,240.

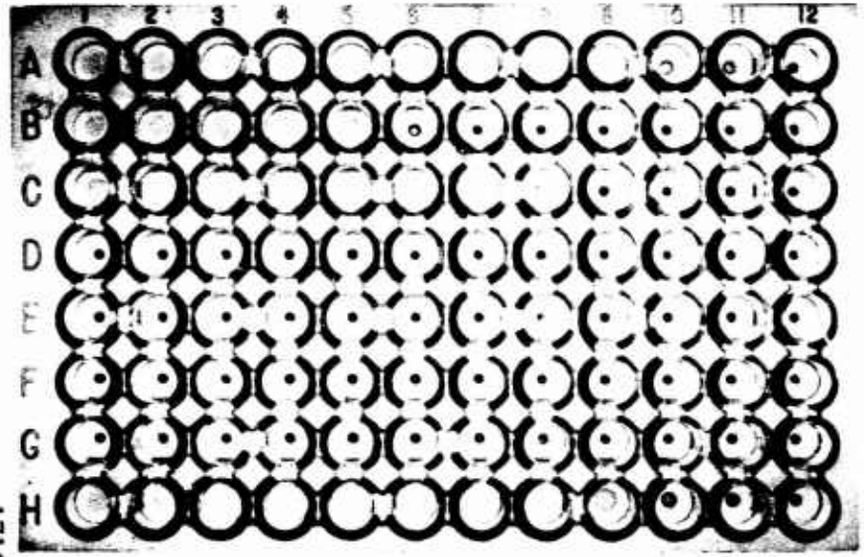

Fig. 1 Indirect hemagglutination test using glutaraldehyde stabilized erythrocytes sensitized with ESS from R. typhi. (A) Murine typhus antiserum; (B) Epidemic typhus antiserum; (C) Brill-Zinsser disease antiserum; (D) Rocky Mountain spotted fever antiserum; (E) Q fever antiserum; (F) Scrub typhus antiserum; (G) Normal serum; (H) Glutaraldehyde stabilized sensitized erythrocytes stored at room temperature 3 months and tested against same murine typhus serum as in (A). Column 12 is a control for non-specific agglutination; each well contains a 1:40 dilution of serum combined with non-sensitized stabilized erythrocytes.

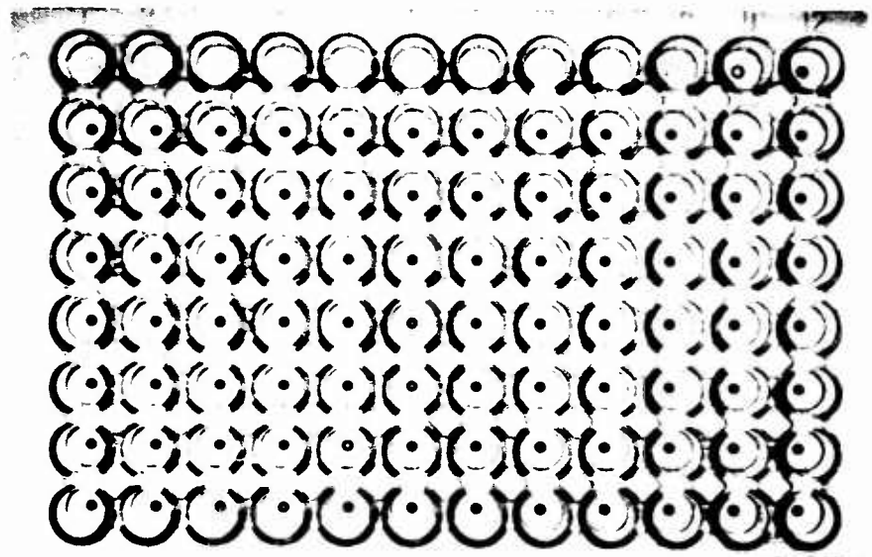

Fig. 2 Indirect hemagglutination test using glutaraldehyde stabilized erythrocytes sensitized with ESS from R. rickettsi. (A) Rocky Mountain spotted fever antiserum; (B) Murine typhus antiserum; (C) Epidemic typhus antiserum; (D) Brill-Zinsser disease antiserum; (E) Q fever antiserum; (F) Scrub typhus antiserum; (G) Normal serum; (H) Diluent only. Column 12 is a control for non-specific agglutination; each well except 12H contains a 1:40 dilution of serum combined with non-sensitized stabilized erythrocytes.

Table 1 summarizes the results obtained when R. typhi or R. rickettsi antigen was used in either the IHA or IFA test against antisera from known, but different, rickettsial infections. Acute or early convalescent epidemic and murine typhus antisera tested by the IHA procedure showed 86% positive reactors compared to 88% detected with the IFA test. Similar sera from spotted fever patients tested with the IHA reagent indicated 74% positive reactors, while 86% were identified by the IFA test. The typhus ESS sensitized erythrocytes and the spotted fever ESS sensitized cells were not agglutinated by Q fever or scrub typhus antisera and no typhus-spotted fever cross reactions were observed. Table 2 indicates the IFA test was more sensitive than the IHA when examining murine and epidemic typhus antisera, with a 2-4 fold difference in titer. Rocky Mountain spotted fever antisera did not show this constant relationship and approximately 50% of the sera tested showed higher titers in the IHA than IFA test. In most instances, a rise in titer demonstrated in paired sera by the IFA was paralleled by similar results in the IHA test. Only 3 paired sera from the Rocky Mountain spotted fever group (C.H., H.D., T.M.) showed an increase in titer by the IFA test with total absence of reactivity in the IHA test.

When erythrocytes sensitized with murine typhus ESS were incubated with rat serum having a murine typhus complement fixing titer of 1:64, no agglutination was detectable. Attempts to enhance agglutination by rodent sera included: a) addition of 1% normal rabbit serum as a stabilizing agent; b) extraction of immune serum with acetone; c) adjustment of the pH of the reaction mixture from 6.0 to 8.0 in 0.2 pH increments. None of these techniques was successful in obtaining reproducible agglutination of sensitized erythrocytes by rodent sera. Similar results were obtained with immune sera from guinea pigs and rabbits, although occasionally an individual serum did produce interpretable agglutination patterns using the standard technique.

In summary, our results indicate that when sera was used at a screening dilution of 1:40, the IHA test was less sensitive than the IFA in identifying positive reactors, particularly in spotted fever infections. However, we feel the practical advantages of this IHA test compensate for its reduced sensitivity, particularly for epidemiological studies conducted in remote areas without access to the sophisticated equipment necessary for the IFA test. The IHA procedure is simple to perform, requiring little equipment and only rudimentary technical training. The development of a reagent easily and economically prepared, with a long shelf life and minimal refrigeration requirements will allow immediate on-site serological testing by field teams with the possibility of subsequent bleedings from individuals found to be of interest through this IHA screening test.

Table 1. Detection of rickettsial antibodies in human sera by the indirect hemagglutination (IHA) test and indirect fluorescent antibody (IFA) test.[a]

Sera	IHA		IFA	
	Typhus ESS	Spotted Fever ESS	R. typhi	R. rickettsi
Murine typhus	29/31[b]	0/31	31/31	0/31
Epidemic typhus	15/20	0/20	14/20	0/20
Brill-Zinsser disease	3/3	0/3	3/3	0/3
Rocky Mt. spotted fever	0/42	31/42	0/42	36/42
Scrub typhus	0/102	0/102	0/102	0/102
Q fever	0/16	0/16	0/16	0/16
Normal	0/14	0/14	0/14	0/14

[a] The initial dilution for all sera was 1:40.

[b] Denominator indicates number of sera tested; numerator indicates number of positive reactors.

Table 2. Comparison of antibody titers in human sera determined by indirect hemagglutination (IHA) and indirect fluorescent antibody (IFA) tests. [a]

Sera	Titer		Sera	Titer		Sera	Titer		Sera	Titer	
	IHA	IFA		IHA	IFA		IHA	IFA		IHA	IFA
Murine typhus			Epidemic typhus			Rocky Mountain Spotted Fever					
6-7-9(A)[b]	160	640							74-021615	1280	640
7-7-9(B)	640	1280	154(A)	40	160	W.S.(A)	< 40	160	74-021616	1280	320
34-7-9(A)	< 40	80	154(B)	1280	1280	W.S.(B)	80	640	74-021619	2560	1280
35-7-9(B)	640	2560	176(A)	< 40	< 40	C.H.(A)	< 40	< 40	74-021620	640	2560
49-4-9(A)	2560	640	176(B)	1280	1280	C.H.(B)	< 40	160	74-021621	2560	640
50-4-9(B)	10240	2560	179(A)	80	160	W.H.(A)	< 40	< 40	74-091741	2560	2560
105-4-9(A)	2560	2560	179(B)	320	2560	W.H.(B)	640	640	74-091963	2560	2560
106-4-9(B)	1280	2560	191(A)	< 40	< 40	H.D.(A)	< 40	< 40	75-008042	2560	160
200-7-9(A)	< 40	80	191(B)	640	1280	H.D.(B)	< 40	80	75-008730	< 40	320
201-7-9(B)	320	640	203(A)	< 40	< 40	T.J.(A)	640	640	75-009966	160	640
518-6-9(A)	640	1280	203(B)	1280	1280	T.J.(B)	320	320	75-010517	640	320
519-6-9(B)	1280	1280	210(A)	40	< 40	L.H.(A)	40	40	75-021781	640	320
204-5-9(A)	40	80	210(B)	5120	2560	L.H.(B)	640	320			
205-5-9(B)	640	640	228(A)	< 40	< 40	C.E.(A)	640	40			
169-4-9	80	160	228(B)	160	320	C.E.(B)	1280	160			
507-7-9	320	640	230(A)	< 40	< 40	S.C.	< 40	1280			
780-7-9	1280	2560	230(B)	160	2560	J.A.	1280	320			
349-7-9	160	320	233(A)	160	320	M.T.	2560	160			
670-7-9	5120	2560	233(B)	1280	640	J.D.	40	40			
358-5-9	320	640	236(A)	40	40	A.F.	2560	1280			
209-7-9	320	1280	236(B)	640	1280	G.G.(A)	40	160			
164-2-0	80	640				G.G.(B)	80	40			
302-2-0	320	2560	Brill-Zinsser disease			B.H.(A)	< 40	< 40			
38280(A)	5120	5120				B.H.(B)	80	160			
38507(B)	2560	10240				T.L.(A)	40	< 40			
37576(A)	2560	10240	Nes	40	80	T.L.(B)	160	80			
38492(B)	320	2560	Kap	5120	5120	T.M.(A)	< 40	< 40			
38347(A)	160	160	Cap	320	2560	T.M.(B)	< 40	160			
38488(B)	160	640				74-015913(A)	10240	10240			
37416	1280	5120				74-023780(B)	2560	640			
38558	2560	5120				74-013331	320	320			

a The initial dilution for all sera was 1:40.

b The letters (A) and (B) indicate sequential bleedings from the same individual, but not necessarily taken during acute and convalescent stages of infection.

II. Characterization of humoral and cell-mediated immunity to rickettsial infection.

A. Host defenses in experimental scrub typhus - Characterization of a murine model.

Host defense mechanisms operative in primary rickettsial infections are incompletely understood, but experimental and clinical data suggested that both humoral and cellular immunity may participate in protection. Immune serum prepared in other species confers protection upon mice in neutralization tests and when passively transferred. A role for cellular immunity was suggested by the appearance of dermal hypersensitivity in both humans and animals recovering from infection. It was also shown that lymphocytes from such individuals undergo blast transformation and elaborate migration inhibitory factor upon specific in vitro antigenic stimulation. It has been known for sometime that infection of mice with R. tsutsugamushi conferred on the survivors protection against subsequent challenge not only with the homologous strain but also heterologous strains in this group. Also, there exists a hierarachy of virulence of scrub typhus in mice. Realizing this, Kuwata (1952) was able to protect mice against lethal challenge with a virulent strain of scrub typhus by preinoculation with a less virulent strain. This situation was equivalent to possessing an "attenuated" strain and made possible the study of immunity following active immunization. In this study, we have modified the Kuwata system by choosing a different virulence pair; the less virulent Gilliam strain for immunization and the lethal Karp strain for challenge. The fact that this hierarchy of virulence exists in mice also permitted the use of an inbred (BALB/c) murine model with all the advantages accruing to a system that allows exchange of tissue without subsequent immunological rejection. In this report, we present the model; assay for the production of complement fixing antibody; assess the relative protection offered by prior infection with the less virulent Gilliam strain and finally determine the relative contributions of cellular and humoral immunity by assessing the protection effected by the passive transfer of immune cells or sera.

The kinetics of morbidity and mortality following infection are summarized in Fig. 3. BALB/c mice receiving 100 MID_{50} of the Gilliam strain of R. tsutsugamushi intraperitoneally (IP) exhibited no signs of illness, but 1,000 MLD_{50} of the Karp strain IP inevitably resulted in morbidity and mortality. Morbidity, characterized by inactivity and distinctive ruffling of mouse fur, usually preceded mortality by one or two days. Most animals were sick on or before day 9 and died by day 11 post inoculation.

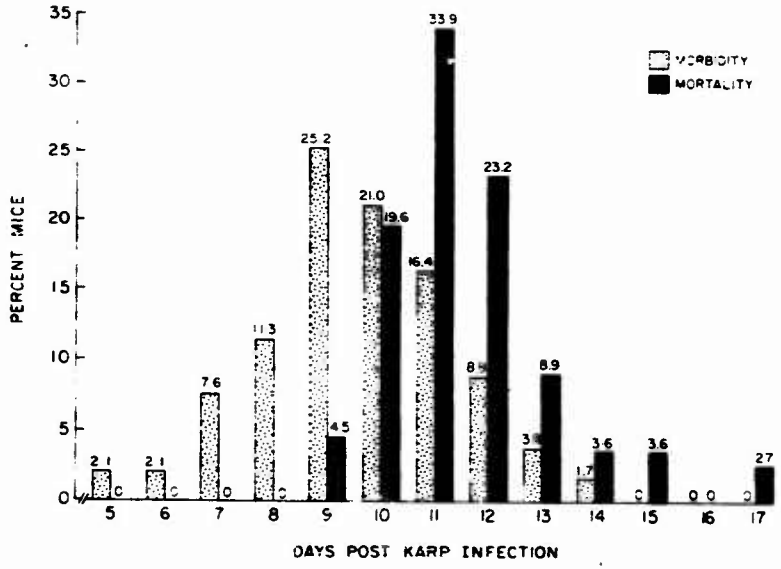

Fig. 3 Morbidity and mortality of BALB/c mice following intraperitoneal inoculation of 1,000 MLD_{50} of R. tsutsugamushi, strain Karp. The number above each bar indicates the non-cumulative percentage of mice evidencing first symptoms of illness or mortality on each day post inoculation. Total sample size was 250 mice.

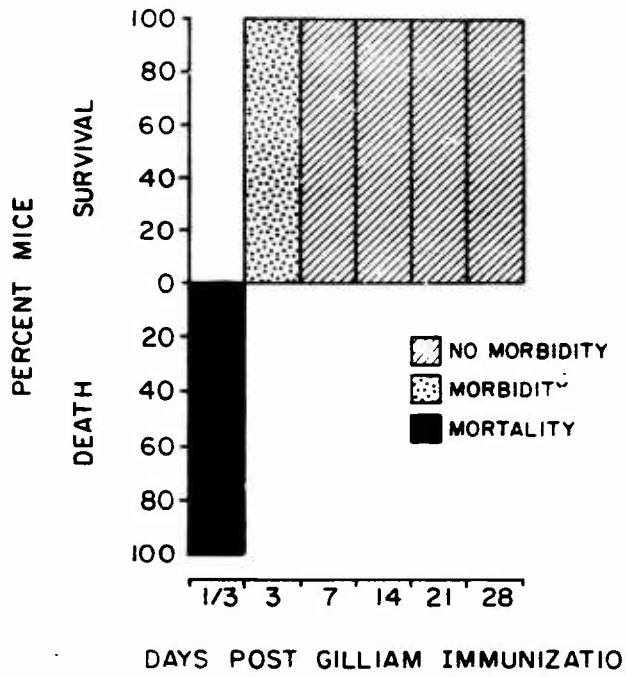

Fig. 4 Survival of BALB/c mice after immunizing infection with R. tsutsugamushi, strain Gilliam (100 MID_{50}) and challenge infection with strain Karp (1,000 MLD_{50}). All mice were immunized with Gilliam, then challenged with Karp on the days indicated. Sample size for each challenge infection was 5 mice. Protection first appears three days after immunization but all survivors exhibited signs of illness. Full protection against mortality and morbidity occurred seven days after immunization.

The protective effect of Gilliam infection was evidenced by the fact that mice inoculated with the less virulent Gilliam strain were able to resist lethal challenge with the Karp strain. The kinetics of development of this resistance are summarized in Fig. 4. At 8 hrs, 3 days, 7, 14, 21 and 28 days after infection with 100 MID_{50} of the Gilliam strain, groups of 5 animals were challenged with 1,000 MLD_{50} of the virulent Karp strain. Protection against mortality appeared after 3 days; however, all surviving animals at that time exhibited morbidity. Complete protection against both mortality and morbidity was detected at 7 days and continued through 28 days when these experiments were terminated.

In previous studies by Salvin and Bell (1955), using outbred mice, it was shown that sublethal doses of H. capsulatum afforded protection against lethal challenge of k. typhi and R. tsutsugamushi. In our laboratory, using inbred BALB/c mice, animals infected IP with 1×10^6 yeast phase organisms of H. capsulatum were challenged 14 days later with 1,000 MLD_{50} of Karp. Non-specific protection was not afforded against this severe challenge and all mice died.

The kinetics of antibody production are shown in Fig. 5, indicating that primary infection with 100 MID_{50} of the Gilliam strain results in the production of CF antibody specific for the Gilliam strain and showing no detectable cross reaction with the Karp strain. A booster injection with 100 MID_{50} of the Gilliam strain, 35 days after the primary injection, caused no anamnestic response. The first detectable sign of antibody occurs at day 14, long after the Gilliam-protected animals are resistant to lethal Karp challenge. Maximum titers were reached between 21 and 28 days. Antibody levels declined 4-8 fold during the following 6-7 weeks. Immune sera with mixed reactivity to both Karp and Gilliam were obtained from Gilliam protected mice which had survived a lethal Karp challenge 21-28 days previously. This serum had a 1:40 anti-Karp CF titer and a 1:80 anti-Gilliam CF titer.

Protection with immune sera was attempted by passive transfer of anti-Gilliam sera, either from primary infected or boosted animals, but it failed to establish protection against subsequent Karp challenge. The respective homologous (anti-Gilliam) CF titer of the sera employed was 1:80, 1:40 and 1:40. Three groups of 5 mice each received 0.7 ml (approximately one mouse equivalent) IP of the respective sera, and all were challenged 8 hrs later with 1,000 MLD_{50} of the Karp strain. No animals survived the lethal challenge. To test whether this lack of protection was merely a quantitative phenomenon, 2 groups of 5 mice each were given 1.4 ml of anti-Gilliam serum (1:320) IP and challenged with only 100 or 500 MLD_{50} of the Karp strain. Again, no

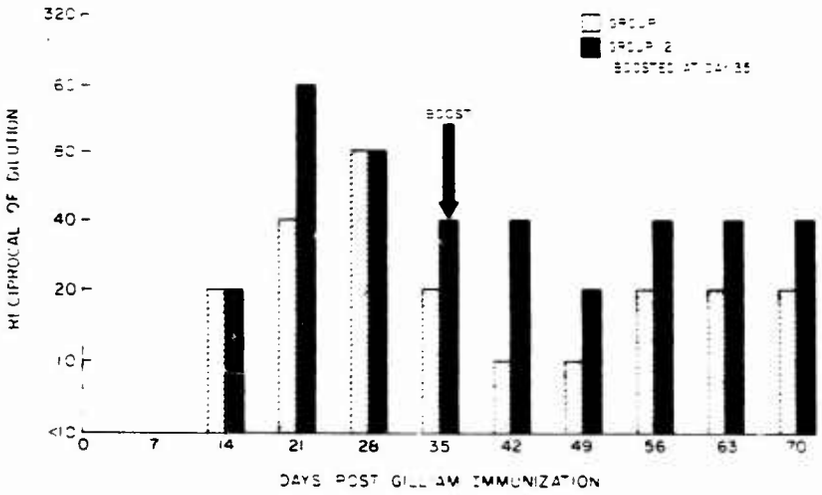

Fig. 5 Comparison of complement fixing antibody levels following immunization with R. tsutsugamushi, strain Gilliam. Two groups of mice were immunized by intraperitoneal injection of Gilliam (100 MID_{50}) at day 0. One group received a booster inoculation of Gilliam (100 MID_{50}) IP 35 days after initial immunization. Although the group receiving a booster injection exhibited a consistently higher CF titer, an anamnestic response was not observed.

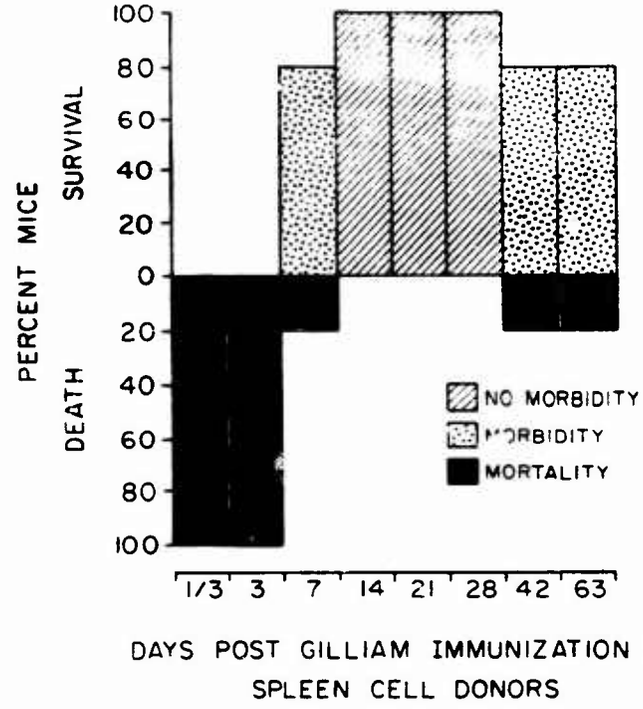

Fig. 6 Survival of BALB/c mice after receipt of immune spleen cells followed by infection with R. tsutsugamushi, strain Karp (1,000 MLD_{50}). All mice serving as spleen cell donors were immunized with Gilliam (100 MID_{50}). On the days indicated, one spleen equivalent was transferred to each recipient mouse which was challenged 8 hrs later with Karp. Sample size for each challenge infection was 5 mice.

animals thus "protected" survived the challenge. However, when the sera mixedly reactive against both Karp (1:40) and Gilliam (1:80) was passively transferred IP (0.7 ml), 60% of the animals subsequently challenged with 1,000 MLD_{50} of Karp survived.

The cellular contribution to host protection was evaluated by a series of cell transfer experiments. Mice were immunized with 100 MID_{50} of the Gilliam strain and at various days after this inoculation, a group of 5 mice were sacrificed, their spleens aseptically removed, and a suspension of single cells prepared. One spleen equivalent or approximately 1 X 10^8 leucocytes in 0.2 ml were given to each recipient. These animals were subsequently challenged 8 hrs later with 1,000 MLD_{50} of the Karp strain. The results, summarized in Fig. 6, indicate that spleen cells from animals immunized 3 days previously with Gilliam conferred no protection against the Karp challenge. At 7 days post-immunization, 80% of the challenged mice became ill, but survived, and complete protection against illness as well as death was observed from day 14 to 28. This total protective capacity of immune spleen cells declined 20% on days 42 and 63. Experiments were not extended beyond this point.

Transferred whole spleens consisted of a heterogeneous collection of cells including macrophages, and it was of interest to determine if protection could be conferred by lymphocyte-rich preparations. The latter were prepared by exposing spleen cell suspensions to a plastic surface and harvesting the non-adherent and adherent cells. Non-adherent cells were greater than 95% lymphocytes by morphologic criteria. Removal of adherent cells did not affect the ability of spleen cells to transfer protection. Experiments indicated that 30 X 10^6 non-adherent spleen cells (i.e., lymphocytes) were sufficient to transfer protection at any time from 7 to 63 days after infection with Gilliam. In contrast, 30 X 10^6 adherent cells (i.e., macrophages) from Gilliam infected animals did not effect transfer of protection against lethal Karp challenge. Finally, it was also of interest to determine the number of lymphoid cells necessary to achieve protection. Spleens were harvested from mice infected 21 days previously with Gilliam and 5 X 10^6 to 30 X 10^6 immune lymphoid cells (non-adherent) were transferred. Animals were challenged with 1,000 MLD_{50} of Karp and Fig. 7 illustrates that complete protection was achieved at concentrations between 15 X 10^6 and 30 X 10^6 cells.

The identity of the lymphocyte subpopulation responsible for protection was obtained using 30 X 10^6 spleen lymphocytes from 21 day immune animals. They were treated with anti-theta or anti-light chain serum and complement, or were untreated. Table 3 shows that anti-theta serum abrogates the protective effect of immune splenic lymphocytes and the ability of such cells to respond to Concanavalin A, but not lipopolysaccharide. Anti-light chain serum spares the protective ability of immune spleen lymphocytes and their ability to respond to Concanavalin A, but sharply reduces lipopolysaccharide directed proliferation.

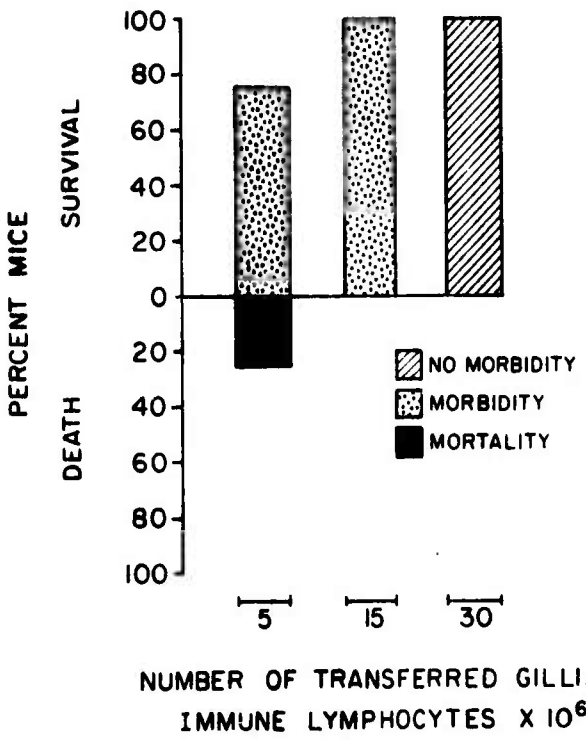

Fig. 7 Survival of BALB/c mice after receipt of various numbers of immune lymphocytes followed by infection with R. tsutsugamushi, strain Karp (1,000 MLD_{50}). All mice serving as lymphocyte donors were immunized with Gilliam (100 MID_{50}); at 21 days post-infection, spleens were harvested, cells separated by glass adherence properties and various numbers of non-adhering cells (lymphocytes) were transferred to recipient mice. Eight hours after transfer, recipient mice were challenged with Karp (1,000 MLD_{50}). Sample size for each challenge infection was 5 mice.

Table 3. The effect of specific cytotoxic anti-lymphocyte serum on mouse survival and cellular proliferation.

Treatment of spleen cells	Mouse survival Sample size/% survivors	Concanavalin A stimulation CPM X 10^3/% control	Lipopolysaccharide stimulation CPM X 10^3/% control
Control [a]	5/100	78/100	19/100
Untreated [b]	10/70	57/73	12/63
Anti Θ + C	10/20	8/10	8/42
Anti Ig + C	10/100	82/105	4/20

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[a] Spleen cells transferred immediately after harvest.

[b] Spleen cells untreated with either anti-serum but incubated under similar temperature conditions and transferred in parallel with treated cells.

The specificity of cellular protection is illustrated in Table 4 which summarizes the results of 2 separate control studies. The protective capabilities of the Gilliam-sensitized cells against other intracellular organisms were investigated in the first study. Spleen cells from Gilliam-infected mice were harvested and inoculated into 4 groups of mice. Eight hrs later, one group was challenged with the Karp strain; 2 other groups were challenged with representative typhus and spotted fever group rickettsiae; one group was challenged with a non-rickettsial intracellular parasite, Plasmodium berghei. The dosages of the inoculum selected were such that the infected mice would be killed within 10 days. In all cases, the Gilliam-sensitized cells were incapable of protecting against non-specific challenge infections. The ability of a non-infectious stimulation of the immune system to resist challenge with the Karp strain was also investigated. Spleen cells were harvested from 2 groups of mice injected with complete Freund's adjuvant or 50% sheep red blood cells 21 days previously. After transfer of cells, recipient mice were challenged at 8 hrs with Karp. In both situations, the challenge inoculum was uniformly lethal, indicating that non-specific stimulation of the immune system had not provided protection against scrub typhus infection.

III. Development of an inactivated scrub typhus immunogen.

A. Formalin inactivation of R. tsutsugamushi.

In our initial experiments an immunogen was made from a suspension of the Karp strain containing approximately 10^8 rickettsia per ml. The Karp strain was chosen because it provides a definite endpoint in mouse titrations; a single infecting organism consistently causes death. The procedures used to make the immunogen and immunize mice were the same as those used by Smadel et al (1946). However, while Smadel's group always used freshly harvested rickettsia to make their immunogens, it was hoped that the large stock of previously titrated, frozen suspensions stored in this department could be used for our studies. To test this possibility, a 20% (w/v) suspension of Karp strain was prepared from freshly harvested infected yolk sacs. One aliquot of this material was immediately titrated to determine the LD_{50} in mice. A second aliquot was treated with formalin and merthiolate to produce Karp immunogen F. The remaining material was rapidly frozen at -80C as is routinely done in this laboratory to prepare suspensions of rickettsia for storage. Following our standard procedures for recovering frozen suspensions, part of the frozen material was rapidly thawed at 37C. A small aliquot was titrated in mice. The remaining suspension was immediately treated as before to produce Karp immunogen FT. The two immunogens were stored at 4C for 21 days, the time required for the mouse titrations to be completed. Both suspensions contained

Table 4. Specificity of immune spleen cells in the protection of mice challenged with rickettsiae and plasmodia.

Immunization of donor animals[a]	Organism used in challenge infection of recipient animal[b]	% survival[c]
R. tsutsugamushi (Gilliam) ($100MID_{50}$)	R. tsutsugamushi (Karp) ($1000MLD_{50}$)	100
R. tsutsugamushi (Gilliam) ($100MID_{50}$)	R. akari (Hartford) ($20MLD_{50}$)	0
R. tsutsugamushi (Gilliam) ($100MID_{50}$)	R. typhi (Wilmington) ($68MLD_{50}$)	0
R. tsutsugamushi (Gilliam) ($100MID_{50}$)	P. berghei (2×10^7 organisms)	0
Complete Freund's Adjuvant (0.4 ml)	R. tsutsugamushi (Karp) ($1000MLD_{50}$)	0
50% Sheep Red Blood Cells (0.1 ml)	R. tsutsugamushi (Karp) ($1000MLD_{50}$)	0

[a] Mice serving as spleen cell donors were immunized with Gilliam or non-replicating immunogens; at 21 days post immunization, one spleen equivalent was transferred to each recipient mouse.

[b] Challenge infections were performed 8 hours after spleen cell transfer.

[c] Sample size for each challenge infection was 5 mice.

approximately 10^8 rickettsia per ml, which is greater than the minimum concentration required for a formalin-inactivated immunogen to be effective (Smadel et al, 1946). Therefore, the formalin-inactivated suspensions were used to immunize weanling ICR mice. Each mouse received three injections of 0.5 ml of immunogen IP over a 10 day period. Two weeks after the final injection had been given, groups of immunized mice were challenged with from 1 to 10,000 LD_{50} of fully virulent Karp. Control mice that had not been immunized were challenged with from 0.1 to 10,000 LD_{50} Karp. The data and results are shown in Table 5 (challenge dose recorded is based on the death pattern in the control mice).

Several conclusions may be drawn from the results: (1) immunogens must be made from freshly harvested rickettsia, the freeze-thaw process, while having little ultimate effect on viability, does decrease immunogenicity by about 90%; (2) a freshly harvested and formalin-inactivated immunogen containing 5×10^7 rickettsia per ml is effective, there is significant protection against challenge with 1,000 LD_{50} of the homologous strain; and, (3) absolute protection against a 1,000 LD_{50} challenge, or even against a 75 LD_{50} challenge, is not achieved. The latter two observations confirm those reported by Smadel et al (1946). Since a reasonable criterion for vaccine acceptability would be absolute protection against a challenge of 1,000 LD_{50} and since immunization with the formalin-inactivated immunogen does not provide this level of protection, even when the challenge is with the homologous strain, other techniques for inactivation and for immunization are being pursued in an effort to enhance protection. The results of the formalin-inactivation studies will be used for comparison in the evaluation of these other techniques.

B. Irradiation inactivation of R. tsutsugamushi.

The experiments completed to date have been designed to determine both the dose of γ radiation that is required for complete inactivation of a suspension and the effect of this dose on the immunogenicity of the suspension when it is used to immunize mice. The same suspension of Karp used in the formalin-inactivation studies was used in these experiments. Due to the lability of suspensions of scrub typhus rickettsia, all irradiation was done on vials immersed in a thick slurry of dry ice and ethanol. The γ source was ^{60}Co. The irradiated specimens were thawed rapidly, diluted as required, and injected into weanling ICR mice with each mouse receiving a single injection IP of 0.2 ml. At 24-25 days, surviving mice were challenged by IP injection of approximately 1,000 LD_{50} of Karp. The results are depicted in Fig. 8. The PI50, or 50% Protective Index, is used as a measure of the ability of the immunogen to protect a mouse against a subsequent

Table 5. Resistance of immunized mice to challenge with the homologous strain of scrub typhus.

Immunogen	Challenge Dose - LD_{50}						No. LD_{50} for 50% Death
	7500	750	75	7.5	0.75	0.075	
None	5/5	5/5	5/5	5/5	1/5	2/5	1
Karp F	1/10	3/10	3/9	0/9	0/8		1000
Karp FT	8/9	4/9	4/8	2/8	2/8		130

Numerator: Number Dead
Denominator: Number Challenged

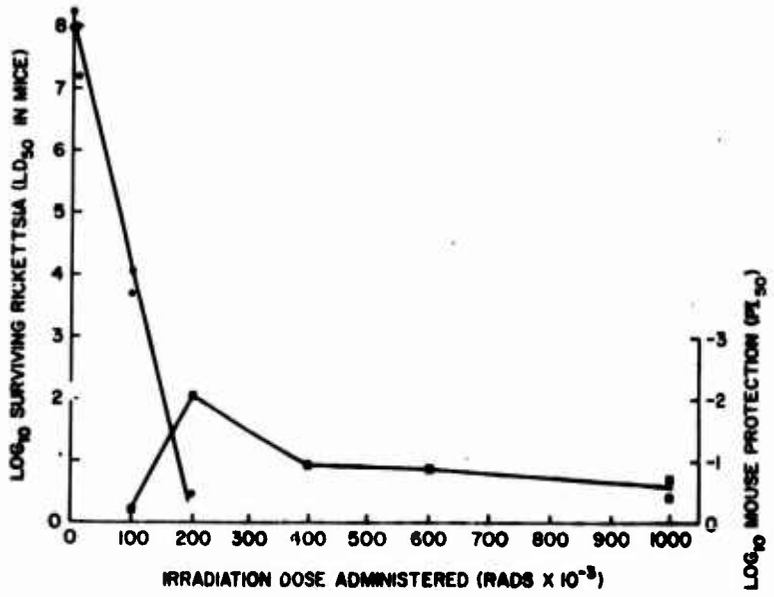

Fig. 8 Effect of irradiation on survival and immunogenicity of Karp scrub typhus.

Symbols: o LD_{50} ■ PI50

1,000 LD_{50} challenge. At the PI50, 50% of immunized mice will survive a 1,000 LD_{50} challenge. In Fig. 8, the PI50 is expressed as the dilution (w/v) of infected yolk sac that must be made in addition to the dilution required to remove all surviving virulent rickettsia in order to protect 50% of vaccinated mice against a 1,000 LD_{50} challenge. As can be seen, up to a radiation dose level of about 100,000 rads, the antigenic mass remaining after the dilution required to remove viable rickettsia is insufficient to yield a measurable PI50, at least when the mice are given only one injection of immunogen. The PI50 is greatest at approximately 200,000 rads, the minimum dose level giving complete inactivation. This is because irradiation reduces immunogenicity at a slower rate than it reduces viability and because the available antigenic mass containing no viable organisms increases as radiation dose level increases until the 100% lethal radiation dose is reached.

Of course, at higher radiation dose levels the only visible effect is the reduction in immunogenicity. Further inspection of the PI50 plot reveals that at the 200,000 rad dose level one injection of 10^6 inactivated rickettsia per ml provides significant protection for mice. Not shown in the plot is the fact that all mice immunized with a 20% yolk sac suspension that had received 200,000 rads survived immunization without morbidity and, though there was evidence of slight distress, survived the subsequent 1,000 LD_{50} challenge; 100% protection was achieved. Comparison of these results with those of the formalin-inactivated immunogen indicates that complete protection was not achieved with formalin-inactivated immunogens and, further, three injections of 5×10^7 rickettsia per ml were required to achieve a PI50. Therefore, the irradiation-inactivated immunogen is considerably more immunogenic than the corresponding formalin-inactivated preparation. This conclusion is further strengthened by the fact that Smadel's group found that formalin-inactivated immunogens containing 10^7 or fewer rickettsia per ml were ineffective and the level of protection conferred on immunized mice was less than 10 LD_{50}.

Work with irradiation-inactivated immunogens is continuing using a dose of 300,000 rads to provide a safety factor that will insure complete inactivation of rickettsia. These experiments are designed to test protection when mice are immunized by administration of one to three injections of either a 2% or a 20% (w/v) yolk sac suspension and given homologous challenges of up to 10,000 LD_{50}.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 075 Rickettsial Diseases of Military Personnel

Literature Cited.

References:

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Publications:

1. Shirai, Akira, and Wisseman, Charles, Jr. Serologic classification of scrub typhus isolates from Pakistan. Amer. J. Trop. Med. Hyg. 24: 145-153, 1975.

Presentations:

1. Shirai, Akira. The role of cell mediated immunity (CMI) in scrub typhus infection. By Shirai, Akira, Catanzaro, P.J., and Osterman, J.V. Annual meeting of American Society for Tropical Medicine and Hygiene, 1-8 November, 1974, Honolulu, Hawaii.

2. Osterman, J.V. General rickettsiology. University of Maryland, College Park, Maryland, 16 April 1975.

PROJECT 3A762758A833
BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00
Biomedical Factors in Drug Abuse

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6491	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. AGGRADING	8A. DISPN INSTRN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	101
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Include with security classification code)

(U) Assay Methodology for Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR 75 (CURRENT)	8	280
FISCAL YEAR 76 (BUDGET)	6	238

19. RESPONSIBLE DOD ORGANIZATION

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20. GENERAL USE

Foreign intelligence not considered

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21. KEYWORDS (Provide KEY ONE phrase classification code) (U) Mass Spectrometry; (U) Drugs of Abuse; (U) Toxicology; (U) ESR; (U) Chromatography; (U) Immunoassay

22. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by numbers, precede text of each with security classification code.)

23. (U) The technical objective of this work unit is to develop and evaluate analytical procedures for the assay of drugs and their metabolites and to assess the applicability of these methods to drug abuse detection and treatment programs in the armed forces.

24. (U) Efforts will be concentrated on the development and evaluation of simple, rapid and accurate systems of drug detection for the operational laboratory, on the establishment of appropriate standardization techniques for the research laboratory as well as the drug screening laboratory, and on the design of sophisticated, highly sensitive and specific analytical methods for investigating the pharmacokinetics of drugs of abuse.

25. (U) 74 07 - 75 06 Efforts continued in the development and evaluation of methods and instrumentation for assaying drugs of abuse and their principal metabolites in body fluids. A study of methaqualone metabolism in humans continues. Initial data indicate that patterns of urinary metabolites may be useful in establishing time of ingestion. Extensive use made of glass capillary columns prepared in-house in gas chromatography and mass spectroscopy studies. Columns 50 meters in length with separation efficiencies of 1100-1500 theoretical plates per meter can be readily prepared. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

* Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Assay methodology for drugs of abuse

Investigators.

Principal: LTC (P) Douglas J. Beach, MSC
Associate: SP4 Kenneth P. Arnold, B.S.; Billy G. Bass, MS; Ann R. Berman, B.S.; Betty J. Boone, Ph.D.; CPT James A. Cella, MSC; LTC Gale E. Demaree, MSC; James E. Doolittle, A.A.; Laurence R. Hilpert, B.S.; Leo Kazyak, B.S.; CPT James A. Kelley, MSC; SSG Frank E. Johnson, B.S.; SP4 James P. McGrath, B.S.; SP5 John T. McLennon.

The technical objectives of this work unit are to develop and evaluate analytical methods for the detection, identification and quantification of drugs of abuse, pharmaceutical compounds and their metabolites in biological fluids and to exploit these techniques for application to mass screening, rehabilitation and chemotherapy management. Efforts were concentrated in these areas:

1. Evaluation of drug screening kits.

   a. Latex flocculation test for morphine.

   b. Radioimmunoassay for methaqualone.

2. Methaqualone metabolism.

3. Characterization of cannabinoids in human urine after smoking marihuana.

1. Evaluation of latex flocculation test for morphine.

A latex flocculation kit marketed by Roche Diagnostics, Nutley, N.J. was evaluated for its efficacy in detecting morphine in urine. The basis for the test is the flocculation of a latex morphine complex in conjunction with normal urine - the reaction is inhibited by urine containing morphine. The degree of inhibition depends on the amount of morphine in the urine specimen.

a. Latex flocculation test for morphine.

All of the test reagents and materials were supplied in the commercial kit, including disposable pipettes and droppers. Urine, which required no preliminary treatment, was added to the tube

containing antimorphine serum. Latex solution was then added and the tubes were incubated for 2 hours in a heat block at 37°C. Normal and positive urine control samples were also included in the kit and were run in duplicate with each group of tests. The flocculation reaction was observed at the end of the incubation period.

A total of 113 urine samples were run in duplicate. Eighteen of these were samples obtained from the Communicable Disease Center, Atlanta, GA, for use as quality controls in various drug tests. Another 18 urine specimens, known to be free of drugs of abuse, were spiked with various amounts of morphine or other drugs. The remaining 77 urine specimens were obtained from laboratory volunteers and hospital patients, including a group of known morphine addicts.

There were 9 positive and 9 negative results in the 18 quality control samples. The positive reaction samples all contained varying amounts of morphine as well as combinations of other drugs of abuse including amphetamines, methadone and barbiturates. The samples with a negative flocculation reaction contained combinations of these same drugs with the exclusion of morphine.

The 18 urine specimens which were spiked with morphine and other drugs of abuse had 9 positive and 9 negative reactions to the test. All of the positive reactive urine specimens contained morphine in concentrations of 2.5 to 10 µg/ml. Other morphine dilutions of 0.01 to 1 µg/ml failed to elicit a positive response.

The group of 77 urine specimens from laboratory volunteers and hospital patients resulted in 59 negative and 18 positive reactions. The positive samples were from patients who were known to have taken morphine or codeine. The 59 negative samples were from volunteers who had received no drugs of abuse as well as from hospital patients receiving many drugs such as demerol, valium, antibiotics, diuretics, vitamins, etc.

A group of 5 urine specimens were repeated to evaluate reproducibility of results. There was no change in the final results of 3 negative and 2 positive reactions in the repeated tests.

Total Tests		Positive	Negative
Unknown Urines:	77	18	59
Quality Control (CDC):	18	9	9
Spiked Urines:	18	9	9
Total:	113	36	77

The conclusion of the evaluation was that the latex flocculation test for morphine detection in urine appears to be a simple valid test and does not require costly equipment to process. Our evaluation showed no cross-reactions with other drugs except for other similar structured drugs (codeine) which occur in immunological morphine tests. Reproducibility of results is excellent, with no false positive or negative reactions. The cut-off level of morphine in this test, as evidenced by our evaluation, is 2.5 µg/ml. Agreement between duplicate samples was very good.

b. Radioimmunoassay for methaqualone.

The ^{125}I radioimmunoassay kit for methaqualone (Roche Diagnostics) was supplied for experimental testing, containing enough reagents for 2500 tests. The technical requirements for the test were uncomplicated and did not require any pretreatment of the urine specimens. All the reagents could be stored under refrigeration, and then brought to room temperature for use.

Several variations of the experimental protocol provided by Roche Diagnostics were tested before analyzing urine specimens.

The protocol recommended 60 minutes for incubation, 10 minutes for precipitation and 10 minutes for centrifugation. Incubation times of 10, 30, 60 and 90 minutes as well as 10 and 20 minute precipitation and centrifugation times were tested. No systematic differences were seen in the results of this experiment.

Standard methaqualone concentrations of 1, 10, 20, 50 and 100 µg/ml were prepared from the positive control urine (100 µg/ml) supplied in the kit, using the normal control urine as a diluent. Another set of methaqualone concentrations of 1, 10, 20, 50, 100, 200, 500 and 1,000 µg/ml were prepared from crystalline methaqualone, using pooled normal urine collected from laboratory volunteers. There was excellent agreement between the two standard curves up to 100 µg/ml of methaqualone, the maximum concentration available with the standards provided in the kit.

Using the laboratory standards proviously prepared (1 to 1,000 µg/ml), five different reagent mixing times were evaluated: (1) the reagents were mixed immediately before sample testing; (2), (3), (4) reagents were premixed 1 hour, 2 hours, and 17 hours before sample testing; and (5) reagents were pipetted separately into samples. There were no important differences among the five test situations.

Four unconjugated monohydroxy metabolites of MTQ were synthesized and the reactivities of each were compared with MTQ standards of the same concentration. The reactivity of the 4'-OH metabolite was

similar to that of MTQ, with the 3'-OH being slightly less reactive. The α-OH appeared to have about 80% of the reactivity of MTQ, while the 6-OH reacted very weakly. Concentrations of the 4 combined metabolites plus MTQ in equal amounts reacted similar to the standrad curve, showing an additive effect.

Spiked samples of other drugs of abuse were diluted in pooled negative urine or distilled water and were studied for cross-reactivity with the MTQ antibody. These drugs included LSD, morphine, barbiturates, methadone and amphetamine in varying concentrations. There was no cross-reactivity noted in any of the tested drugs.

Standard curves were prepared, using 3 different volumes (10, 50, and 100 µl) of each MTQ concentration to study the effect on sensitivity due to sample volume. The volume of antiserum and ^{125}I-MTQ was kept constant. Decreased reactivity was shown in the lower volumes. However, it is speculated that the linearity of curve could be extended by decreasing the sample volume.

Many urine samples containing MTQ were too concentrated to accurately quantify directly. Quantification was accomplished by diluting the urine specimens in distilled water (1:10, 1:50, 1:100, 1:500, 1:000). The agreement of values from sequential dilutions was very good.

A total of 250 urine specimens were selected at random from samples obtained from United States Air Force Europe/Drug Abuse Detection Laboratory to be assayed for comparison of results obtained by TLC/GLC. There was excellent agreement between the two methods. The comparison is summarized in the table below.

Results by TLC/GLC	Positive - 196	Negative - 54
Results by RIA	Positive - 196	Positive - 38
	Negative - 0	Negative - 16

Of the 54 specimens found negative by TLC/GLC, 38 gave a positive reactivity with RIA which has a 100 µl/ml cut-off level. GLC does not have the sensitivity to detect MTQ or its metabolites at this level and therefore these samples would be considered negative.

Comparison of quantitation results showed reasonably good correlation between GLC and RIA. The GLC data also emphasized that the materials in greatest concentration associated with the positive responses by RIA are the metabolites.

A total of 322 urine specimens were assayed in duplicate. The agreement between duplicates was good. Ninety-four of these samples were again assayed in duplicates. All samples gave the same results on the second assay except for 2 high count negatives which were considered positive on the re-run.

Urine specimens were obtained from the recovery ward of Walter Reed Army Medical Center. The patient had received one or more drugs within 24 hours preceding the specimen collections. Other urine specimens were collected from laboratory volunteers, some of whom were on medication while others did not admit to drug use of any type. Many of the drugs taken by patients and staff members included antibiotics, cold remedies, etc. None of these specimens showed any reactivity with the RIA for MTQ.

The conclusion drawn from the evaluation was that the radioimmunoassay for methaqualone appears to be technically feasible for use in the screening for the presence of the drug or its metabolites in urine. No interfering substances were observed. Two of the unconjugated metabolites (3'-OH and 4'-OH) react almost as strongly with the assay as the parent drug itself. Confirmation by GLC/TLC of the positive RIA findings is good, except where the level of drug and metabolite in the urine is low. This is due to the fact that RIA is a much more sensitive test. Reproducibility of results is also very good.

2. Methaqualone metabolism.

During early investigations into the detection and quantification of methaqualone the observation was made that certain urinary metabolite patterns might be indicative of time of ingestion. A study was designed to investigate this possibility through the use of human volunteers who received single therapeutic doses of methaqualone and the timed collection of urine post ingestion. Two of the volunteers have taken the drug to date, and indications are that both methaqualone and at least four of its principal urinary metabolites follow a dose response pattern. Interestingly, the metabolite 2-methyl-hydroxy-3-o-tolyl-4 (3H) quinazolinone consistently decreases within the first ten hour period post ingestion to a concentration below the present measurable limit. This would tend to infer that the absence of 2-methyl-hydroxy-3-o-tolyl-4 (3H) quinazolinone in a random urine specimen that contained the other four metabolites would set the ingestion of the methaqualone beyond the initial period. The concentration ratio of methaqualone to 2-methyl-hydroxy-3-o-tolyl-4 (3H) quinazolinone within the ten hour period may provide a reliable estimate of the actual time of ingestion.

A gas chromatographic method has been developed for methaqualone analysis. Based on the data from the limited number of volunteers studied and from urine specimens of methaqualone users obtained from a drug screening laboratory (United States Air Force, Europe) DADL (Drug Abuse Detection Laboratory) the procedure appears to be reliable and promises to be a useful method for establishing the presence of methaqualone in urine from the characteristic metabolite excretion pattern. The procedure appears especially useful for very low urinary concentrations of the drug.

3. Characterization of cannabinoids in human urine after smoking of marihuana.

Early success in detecting a small concentration of 11-hydroxy-Δ9-tetrahydrocannabinol in the urine of a casual marihuana smoker prompted further efforts to develop effective methodology for the detection of cannabinoids in body fluids. Other urine specimens of suspected marihuana smokers have been analyzed during the past year and a carboxylated analogue of tetrahydrocannabinol (THC) has been found in greater concentrations than the 11-hydroxy-THC metabolite. This carboxylated metabolite has been further characterized, and confirmation of its existence has come from other sources (e.g. National Institute of Drug Abuse). An arrangement has been made with Dr. J.H. Mendelson (M.I.T.) to obtain urine specimens from human volunteers who took part in a controlled DOD funded study. These urines will be analyzed to obtain new data on levels of 11-hydroxy-Δ9-THC and other cannabinoids that might be detected in the urines.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OB 6492	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	102
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Military Performance and Drug Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology 012600 Pharmacology 012900 Physiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:

b. NUMBER:

c. TYPE: — d. AMOUNT:

e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 75	4	240
FISCAL YEAR — CURRENT 76	2	258

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Drug Abuse (U) Military Performance (U) Drug Tolerance (U) Psychology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Specific objective of studies managed within the work unit is to specify the probable impact of drug abuse on miltary performance using laboratory models and performance measures logically equivalent to critical aspects of military performance. In addition, data are obtained from studies managed within related work units. These data are used to establish the significance of changes in endocrine function, physiology and social environment brought about by the abuse of drugs in terms of their implications for the performance of military personnel.

24 (U) The techniques of experimental psychology and behavioral pharmacology are applied to the assessment of performance decrements associated with the abuse of drugs. Important data are derived from related studies employing epidemiological, neuroendocrinological and psychophysiological methods for use in constructing an integrated concept of how social, pharmacological, and physiological consequences of drug abuse may interact to alter the performance of military personnel.

25 (U) 74 07 - 75 06 Tolerance to behavioral effects of delta-9-THC could be controlled by manipulating the contingencies supporting on-going behavior, thus, whether tolerance does, or does not, occur to the behavioral effects of this drug depends upon behavioral rather than physiological variables. Studies of heroin self-administration by baboons indicate sustained intake at doses as low as .7 micrograms per kg and general insensitivity to unit dose once the organism begins using the drug. Studies of withdrawal indicate marked suppression of testosterone output. Heroin use disrupts the normal circadian rhythmicity of several behavioral measures. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75 - 30 June 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 102 Military performance and drug abuse

Investigators
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Description.

The purpose of this work unit is to evaluate the consequences of drug abuse for the performance of military personnel. Research managed within this work is an extension of research conducted on the analysis and management of behavior (Work Unit 025) and is related to, and coordinated with, research being conducted under Work Units 103, 110 and 111, as well as certain MRDC contracts. Specific objectives of studies managed within the work unit are to specify the probable impact of drug abuse on military performance using laboratory models and performance measures logically equivalent to critical aspects of military performance. In addition, data are obtained from studies managed within the related work units cited above. These data are used to establish the significance of changes in endocrine function, physiology and social environment brought about by the abuse of drugs in terms of their implications for the performance of military personnel. Data from such studies are used as a guide for the development of appropriate laboratory models and constitute a critical input to this work unit.

Progress.

The research program is being conducted in several phases, each being addressed in turn. The principal drug being utilized as a model for drugs of abuse is heroin. The animal model being employed is the baboon. The initial phase of the research program is nearing completion. This phase of the research program was designed to

examine the conditions under which acquisition of drug self-administration would occur. Simultaneously, data were acquired which would permit characterization of some of the major features of the natural history of heroin abuse under conditions of relatively free-access. The same data were also applicable to the documentation of validity of the baboon as a model for human heroin abuse, from a behavioral, physiological and endocrinological perspective. At the same time, the model served as a source of plasma samples for the development of the analytic technology needed to begin to assess the pharmacodynamics of heroin and its metabolites in a biological system in which the drug is voluntarily self-administered.

The second phase of the program described in the original protocol was initiated during the reporting period. This phase involves an examination of the variables that support, or maintain, established drug self-administration behavior.

The final phase of the program is to be directed at the prevention and elimination of drug self-administration behavior in an environment in which a drug, i.e. heroin, is available. Although, the research completed and in progress has provided data from which inferences may be drawn as to profitable approaches to these final issues, formal experiments have not yet begun.

All of the research to be described below has employed male baboons (Papio anubis) which have been prepared with chronic venous catheters to permit the animal to infuse drug solutions. Infusions occur whenever the animal operates a pushbutton located on a panel within its individual experimental cubicle. Operation of other pushbuttons yield food and water. The performance criteria which must be met in order to obtain food, water or drug vary with the particular experimental design being used. Furthermore, the times at which food, water or drug are available to the animal may also vary with the particular design being employed.

Acquisition of heroin self-administration behavior.

Nine baboons have been exposed to an opportunity to infuse heroin under conditions of relatively free-access. Five of these animals had unlimited heroin available for 22 hrs of each day for periods ranging up to 125 days. Food, water or an infusion could be obtained

by a single press of the appropriate pushbutton. Two of the animals received .02 mg/kg of heroin with each infusion and two received .1 mg/kg/infusion. The remaining animal received .5 mg/kg/infusion. Two additional animals also received .5 mg/kg/infusion; however, because of concern about the possibility of overdose, a contingency was added to limit the number of infusions. This constraint was systematically relaxed as acquisition progressed. One of these latter animals was again run through the acquisition procedure, at the same dose, but without any constraints on usage after 14 drug-free months in order to at least partially replicate observations made in the single high dose animal run through the procedure without overdose protection. The two remaining animals (.1 mg/kg/infusion) acquired heroin self-administration under different conditions. For these animals, an attempt was made to provide additional support for heroin usage by requiring the animals to use heroin at least six times a day in order to initiate six periods of time when food would be available for operation of the food pushbutton. Thus, in order to get an opportunity to earn food pellets, some heroin usage was necessary. The major results from these animals can be summarized as follows:

1. No baboon failed to administer heroin when afforded an opportunity to do so. This observation can be extended to several additional baboons which have been exposed to heroin under other conditions. The unit doses used were .02, .10, and .50 mg/kg/infusion. There is every reason to believe that unit doses considerably lower than those already employed would also be effective in establishing heroin self-administration. Thus far we have seen no evidence to suggest that any baboon or monkey would fail to use heroin under the conditions described. However, we have experienced several failures to obtain acquisition under what are apparently more complex conditions of behavioral control.

2. Each animal exposed to the drug showed a pattern of acquisition that consisted of a gradual approach to a stable daily rate of usage. Conventionally, psychologists interpret gradually changing response rates which reach some stable asymptotic level as evidence of that behavior is gradually coming under control of the contingencies supporting behavior. However, in the case of

drug self-administration the interpretation is less straightforward. Increasing rates of drug usage could also be construed as reflecting the time course of some physiological change, e.g. tolerance. The data obtained thus far may favor the latter interpretation. The elements which favor the hypothesis include the fact that the baboon is an extremely intelligent animal and the performance required to obtain heroin was extremely simple. It is difficult to conceptualize the animal as needing 30 to 50 days to achieve stable performance with respect to the controlling contingencies. In fact, although all animals increased their usage of heroin across days, generally 10 to 30 days of varying amounts of usage of heroin preceeded the increasing usage. In several cases, initial exposure to heroin actually depressed the rate of responding on the drug pushbutton below the baseline rate established when a response produced only saline. This initial depression in rate indicates rather clearly that the animals were encountering the consequences of their behavior and adjusting their response rates accordingly, probably as early as their first day of exposure to the drug. The fact that several animals immediately adjusted their baseline rates of response downward when heroin combined with the fact that it is inconceivable that an organism like the baboon could require 30 to 50 days to learn to push a button seem to favor the idea that the negatively accelerating functions obtained for heroin administration across days reflect a parallel physiological change rather than learning. If this premise is correct, then the stable levels of intake achieved following this period of "acquisition" may indicate a point at which some steady state is achieved. It is not likely that stable intake simply indicates the absolute limit of tolerance because the intake levels at which individual animals stabilized their daily intake differed depending upon both the unit dose to which they were exposed and the contingencies controlling acquisition.

3. The stable intake levels obtained appear to be functionally related to unit dose as well as the contingencies controlling the acquisition of drug self-administration behavior. Two animals were run at a unit dose of .02 mg/kg under conditions of free access, i.e., each response produced an infusion of heroin. Heroin was available 22 hrs each day from 1000 to 0800 hrs the next

morning. No limit was placed on the number of infusions which could be taken each day. The animals were run under identical conditions, however, one year intervened between the running of the two animals. For reasons to be described below, stable intake was determined by selecting median intake of the 28th through 43 daily sessions occurring following the first day on which intake levels rose above initial basal levels. Accordingly, one animal stablized at 2.92 mg/kg/day and the second at 2.86 mg/kg/day. In order to achieve this, both animals had to administer approximately 140 infusions/day. Two other animals were run under identical conditions except for unit dose which was .1 mg/kg. These animals stablized (defined as above) at 4.4 mg/kg/day and 2.6 mg/kg/day, intake levels requiring only 30 to 40 infusions per day at this unit dose. The four animals described above achieved virtually identical stable heroin intake levels despite the fact that their unit doses differed by a factor of 5 and the number of infusions required also differed by a factor of five.

Two additional animals were run at a dose of .1 mg/kg under acquisition conditions designed to provide further support for drug infusion behavior by linking the availability of food sessions to use of about 6 infusions/day. This had the effect of driving up the baseline rate of response on the drug pushbutton. Thus, when drug was introduced the animals would encounter more drug sooner than was the case of the other two baboons run at this dose. Using this procedure both animals achieved stablity (defined) as above at 9 and 10 mg/kg/day, respectively. Thus, the procedure effectively increased the level at which both animals stablized their intakes when compared with the "free access" condition. Furthermore, the procedural change appeared to have an identical effect in both animals. These data exemplify the fact that the conditions under which heroin self-administration behavior is established are powerful determinants not only of initial rate of heroin usage, but also the level at which intake will stablize under conditions of free access.

The remaining animals were exposed to heroin at a unit dose of .5 mg/kg. The first two animals in this group were run as were the previous "free access" animals. However, because we were concerned about the possibility of overdose, a contingency was added such that 2 infusions within 10 min precluded further drug administration for 30 minutes. Within several sessions following the onset of acquisition it became clear that

these animals were contacting the overdose contingency and that a ceiling was being placed on intake. Therefore, the contingency was relaxed, first from 2 to 4 infusions and subsequently to 10 infusions in any 10 min period to preclude further infusions for 30 minutes. Under this latter condition, drug intake was so high that after only six days, we decided to again protect against overdose by reducing the contingency, this time to 5 responses. This decision was made solely because the conditions in effect during these sessions (38 through 43) most nearly approximated those in effect for the "free access" animals. It is for this reason that it was necessary to use the same sessions in calculating the stable intake levels of the remaining animals. With respect to the animals exposed to a dose of .5 mg/kg, intakes peaked at 105 and 107 mg/kg/day. These values, although similar to each other, were vastly different from those obtained at the lower doses. One possible reason for this result could have been the presence of the overdose contingency, therefore, an additional animal was run at this same dose, .5 mg/kg, but without constraint on the number of drug infusions. Stability (sessions 38 through 43) was obtained for this animal at 115 mg/kg/day confirming the data obtained from the previous two animals. As a further confirmation, one of the animals which had been run on the procedure with an overdose contingency was recatheterized after 14 months without heroin and allowed to reacquire heroin self-administration behavior without overdose protection. Following reacquisition, behavior stabilized at 105 mg/kg/day, or within 2 mg/kg/day of the level obtained during original acquisition. Reacquisition was more rapid than original acquisition, however, original acquisition had been constrained by the overdose contingency. Thus, this latter difference may be more apparent than real. In fact, the slope of reacquisition is very similar to that of the animal that originally acquired heroin administration without overdose protection.

In summary, all animals showed gradual acquisition of heroin self-administration behavior. They also achieved and maintained some stable level of daily usage which was very similar for unit doses of .02 and .1 mg/kg and vastly higher for .5 mg/kg. Acquisition with added support for responding provided by an opportunity to work for food re-

sulted in a higher stable intake at .1 mg/kg indicating a role for behavioral variables as well as unit dose in determining terminal level of usage. However, the rate at which terminal usage levels are achieved appears to be independent of both unit doses and conditions of acquisition explored thus far.

4. Withdrawal. Several animals have undergone withdrawal. Usually, this has occurred because of the development of some unforeseen problem, so the available data are somewhat unsystematic. Behaviorally, withdrawal appears as a very pronounced increase in the rate of response on the drug key which develops two or three days after heroin has been replaced by saline. This high rate of responding may persist for three to ten days and then fall gradually to baseline. It is not at all clear why there is a delay of two or more days following replacement of heroin with saline before increased responding is observed. One possible reason may be related to conditioned reinforcing properties of stimuli associated with heroin administration. With the paradigms employed, each operation of the drug key produces an infusion, a change in the color of a stimulus light and auditory stimulation produced by the operation of the pump. All of these stimuli are present during withdrawal. The only difference is that saline rather than heroin solution is infused. It is conceivable that these stimuli may predominate in the control of behavior early in withdrawal and that the absence of heroin is of secondary importance. Some support for this hypothesis comes from an observation made on a number of different occasions. We have, in the course of several years of work, had occasions on which a drug catheter has lost patency or the drug pump, although operating, has failed to infuse drug. Whenever, these conditions have arisen, there has never been an immediate change in the animal's behavior that would signal the onset of the problem. Therefore, it has been necessary to institute procedures for verifying patency and infusion each day. However, we have also had occasions when the drug pumps failed to operate at all, i.e., no auditory stimulus was produced by pump operation. Whenever this has occurred, there has been an immediate increase in the rate of response on the drug key, very similar to that eventually observed during withdrawal. Therefore, we have always seen an immediate change in behavior when an animal failed to receive both heroin

and the other stimuli associated with drug infusion and a delayed change in behavior when heroin was not delivered, but the stimuli associated with infusion were still present. This being the case, the delayed change in response rate in withdrawal is undoubtedly the result of a complex interaction of stimuli controlling behavior and the physiological events associated with withdrawal from heroin.

5. Effects on food and water intake. A primary effect of heroin was found to be the suppression of food intake. The effect was dose-related, with more suppression being seen in animals with higher drug intakes. All animals exposed to the 0.5 mg/kg unit dose showed an immediate and profound suppression upon commencement of heroin intake. In one of the three animals, food intake remained suppressed to below 10 pellets/day (7.5 gm of food) for the duration of exposure to heroin (60 days) when the animal died. In another, pellet consumption was reduced to about 25% of baseline levels, recovering to above 50% of baseline after about 72 days on the drug. The third high dose animal, showed an immediate suppression of food intake, but recovered to above 50% of baseline after about 15 days. This animal's intake remained between 50 and 75% of baseline for the remainder of his exposure to drug. All four animals on the medium dose (0.10 mg/kg/infusion) also showed immediate suppression of food intake to below 25% of baseline levels. The recovery of food intake in these animals was somewhat faster than the high dose animals, with the four animals requiring 15, 20, 50, and 62 sessions on the drug for intakes to climb above 50% of baseline. Three of the four animals eventually rose above 75% of baseline while still on this dose of heroin. Both low dose (0.02 mg/kg/infusion) animals showed the least suppression of eating with intakes generally remaining above 75% of baseline levels throughout their entire exposure to this unit dose.

When the unit dose was increased to a standard dose of 10 mg/kg/infusion in three of the animals (one low, one medium and one high dose animal), some change was seen in the frequency of self-administration behavior of all of these animals. A reduction in infusion rate occurred in the low and medium dose animals. However,

all three animals were infusing more heroin/day because of the change in unit dose. The effect of this increased intake of drug on daily food intake was different from the initial heroin-related suppression of food intake. The former low dose animal had been using about 3 mg/kg/day and was now infusing about 35 mg/kg/day, however, food intake was reduced only by about 30 percent. To produce a heroin intake level of 35 mg/kg/day, the animal had to infuse approximately 900 mg/day, an intake of heroin which would have totally suppressed intake of food and water in a naive animal. The medium dose animal doubled its intake of heroin. This new heroin intake level of 9 mg/kg/day was far lower, in both absolute and relative terms than that of the former low dose animal. However, the magnitude of the reduction of food intake subsequent to increased heroin intake was greater. This same animal also experienced a very large reduction in food intake upon initial exposure to heroin. As was the case with the former low dose animal, the duration of the effect was shorter than could be expected if the animal had been naive. The high dose animal experienced only a small increase in unit dose, 7.35 mg/infusion to 10 mg/infusion. No systematic change in the number of infusions/day was observed. Nonetheless, a decrease in food intake of about 20% did occur. This reduction was smaller and of shorter duration than would have been the case with a naive animal.

There was little relationship between suppression of food intake and any other variable we measured. Although both the recovery of food intake and the shortened duration of suppression in experienced animals receiving a higher unit dose suggest that tolerance to this effect of heroin occurs, the dose response relationships, time to recovery, and so on, were not so orderly that they may be easily interpreted. What is clear is that effect on food intake progresses independently of changes in the acquisition of self-administration behavior.

In addition to the reduction in food intake described above, it should also be noted that preliminary observations of food intake during withdrawal indicate that food intake is suppressed for a number of days after termination of heroin administration. The extent to which reduced food intake during withdrawal is functionally related to other physiological and endocrinological variables needs to be addressed.

All three high dose animals showed a suppression in water intake that persisted throughout the entire exposure to the high dose, with no evidence of any recovery in any of the animals. Terminal intakes on these animals ranged from between 25 and 50% of baseline levels. Thus, animals M372 and M370, both of which showed some recovery of food intake, remained suppressed in terms of water intake. Animal P102, whose food intake remained suppressed essentially to zero, continued to consume water, though at a rate much below his baseline.

6. Patterning of food and drug intake. Before exposure to heroin, baboons consume food in small quantities throughout the day portion of the day-night cycle maintained in their experimental chambers. Associated with the reduction in food intake secondary to exposure to heroin is a change in the pattern of food consumption which persists even after food intake recovers to baseline. Generally, the pattern consists of consuming one, or perhaps two, large "meals" during the day and little or no responding on the food key at other times. This is in sharp constrast to responding on the drug key which takes place fairly continuously at all hours of the day portion of the cycle with about 30% of the responses occurring at night. Originally it was believed that this pattern of relatively continuous responding on the drug key developed subsequent to exposure to heroin. However, further reassessment of baseline responding for saline now indicates that this same pattern of responding during both the day and night was present when the key produced merely a saline infusion. Behavior with respect to the drug was distributed over the 22 hr sessions in much the same way whether infusions were saline or heroin. The distribution of responding on the food key was altered by the drug.

ANALYSIS OF THE VARIABLES MAINTAINING HEROIN SELF-ADMINISTRATION IN THE BABOON.

Choice between food and heroin.

Baboons are periodically given an opportunity to choose between heroin infusion or food presentation. In the first experiment, three animals were allowed one choice every two minutes throughout the day. The drug parameters remained constant throughout the experiment, but the number of food pellets delivered for a food choice was varied between one and four. As the number of pellets

was increased, the number of heroin choices per day increased in all animals. In a second experiment, the interval between choice opportunities was varied from 2 to 12 minutes with the choice being between heroin and four pellets of food. The longer the inter-choice-interval, the less heroin was taken each day by two animals. These data are being replicated in additional animals. Present results suggest that the reinforcing effects of heroin depend strongly on the alternative choices available to an animal.

Effect of unit dose on maintenance of heroin self-administration.

Several animals are now being run under conditions of decreasing unit dosage. Although no definitive statement can be made as yet, it appears that exposure to successively decreasing unit doses of heroin leads to orderly increases in the number of infusions/day. For one animal, the range of unit doses explored so far has been .5 mg/kg down to .00075 mg/kg, a 700 fold reduction in unit dose. However, the number of daily infusions is only two to three times higher than it was at a unit dose of .5 mg/kg. The animal's daily intake of heroin has dropped from 105 mg/kg/day to about .25 mg/kg/day maintained by approximately 350 infusion responses each day. The animal's total daily intake of heroin is only about 5 mg, against an original level of 1,900 mg, yet responding is being maintained. Similar data are being generated by a second animal. It would appear that decreasing the unit dose of heroin, increases, rather than decreases, responding for the drug, but that the increase in responding is not inversely proportional to the decrease in dose. As was the case with increasing drug dosage, the previously established behavior of the animal, appears to be a more important determinant of future performance than does the dose of the drug.

The acquisition of heroin preference.

The first experiments conducted in house with baboons self-administering freely available heroin suggested that the rate of "drug" key pressing for heroin did not surpass the rate of "drug" key pressing for saline until after about 30 days of exposure to heroin. This could indicate that heroin does not immediately reinforce responding but requires a period of low level exposure before it becomes an effective

reinforcer. This observation seems to square well with informal accounts of heroin use with humans. However, there remained alternative explanations of the 30 day period of baseline responding for heroin. First, the responding on the "drug" key was usually above zero so that some drug was infused immediately after it became available. This was usually reflected in an immediate drop in food intake. It is plausible that heroin intake above this low level was not reinforcing but that the few infusions that were taken were reinforcers. Since every response produced an infusion, it was impossible for responding to increase (demonstrating reinforcement) above baseline without also approaching the subject's physiological limit for heroin. This ceiling could have "masked" the observation of reinforcement. Second, the baseline level of saline infusions may have been unusually high because of the sterility of the environment or superstitious association with simultaneously available food and water.

In order to observe heroin reinforcement without these potential confounding variables a procedure was developed that permited two measures of reinforcement that do not require the subject to infuse additional drug. The first measure was obtained by scheduling infusions for responses after a variable interval of time from the onset of a trial; only the one response after that unpredictable interval produced an infusion, earlier responses had no effect. This procedure allowed the rate of responding to increase during the interval without increasing the number of infusions which was fixed on a random time schedule. Experiments with monkeys working for food scheduled by this procedure show that as a reinforcer increases in value more responses occur for it during the intervals between scheduled deliveries of the reinforcer. This occurs even when the responses have no effect as far as increasing the frequency of reinforcers. The second measure of reinforcement was obtained by providing, simultaneously with the drug key, a second key that delivered saline on the same schedule. Pressing either the drug key or the saline key would occasionally produce an infusion and then provide the subject access to food. If the subject was indifferent between drug and saline it would press the two keys equally often in order to get the earliest

scheduled infusion and turn on the food condition. If heroin was preferred to saline, the subject would distribute proportionally more responses to the heroin key. This preference would indicate reinforcement even if the total number of responses did not exceed baseline.

One subject has completed eighty daily sessions on this procedure. Evidence for a small preference for heroin appeared within two weeks after exposure to the drug (the exact point within this period that heroin demonstrated reinforcement was obscured by a variable baseline preference). After thirty-two days of exposure to heroin, preference increased again from a stable 60% level to 70%. Analysis of absolute response rates for heroin and saline indicate that during the first month of heroin intake, responding for both saline and heroin tended to vary together eventhough more responses occurred for heroin. After thirty-two days, saline response rate went into a steady decline while heroin response rate remained stable. It appeared that during the early period of heroin intake the subject did not discriminate well between the heroin infusions and the placebo saline infusions. It was even conceivable that the saline infusions temporarily acted as conditioned reinforcers owing to their external similarity to heroin infusions. This poor discrimination may have acted to artificially dampen the observation of a strong heroin preference during the first thirty-two days of exposure.

This experiment is being replicated with other baboons with the provision that the alternative to heroin, while still functional in leading to food, does not provide any infusion or other external stimulation similar to an infusion. This should increase the likelihood that a clear discrimination between heroin infusions and the alternative will be quickly formed once heroin is made available. If this study confirms the observation of a two phase preference acquisition function with a break at about thirty days, then the preliminary observations made on the basis of free intake will be largely confirmed.

Conditioned reinforcers in the maintenance of heroin self-administration in baboons.

A number of observations of the heroin self-administration behavior of baboons suggest that stimuli associated with the infusion of heroin (conditioned reinforcers) become potent controllers of self-administration behavior. In fact, these stimulus may even predominate over heroin itself in maintaining stable patterning of responding. This is one inference that could be drawn from our observations of the persistence of established response patterns subsequent to changes in unit dose. Similarly, the delayed change in response rate which occurs in withdrawal as contrasted with the immediate increase in rate associated with pump failures lends additional support to this hypothesis.

The conditioned reinforcing properties of stimuli associated with heroin infusion are being studied in two baboons, one of which has completed the experiment. At this point the data analysis has been very superficial because the design of the study was complex to permit a number of questions to be addressed simultaneously. The basic paradigm permitted the animal to infuse either heroin or saline. Infusions of either instituted identical compound stimuli except for the heroin. In addition heroin infusions were limited to 10/day, whereas no limit was placed on the number of saline infusions. Initially the animal's choice was between two identical saline infusions which produced in a very low stable rate of response with choices equally distributed between the two alternatives. When heroin (limited to 10 infusions/day) replaced saline, the number of heroin infusions increased gradually to 10/day over a period of 12 days. As was the case with acquisition under conditions of free access several days of exposure were required for performance exceed basal levels. Responding also increased on the saline key but not until 25 days of exposure to the drug had elapsed. Increased responding on the saline key was correlated with a marked reduction in food intake. Unlike the animals receiving heroin under conditions of free-access, maximum suppression of food intake occurred after 30 days on the drug. It lasted for 16 days and then showed recovery. This animal was, by the time food intake dropped and saline responding increased, infusing all of the 30 mg of heroin available within 5-10 min of the start of each daily session at 1200 hrs. It may

be that the drop in food intake and sustained increase in saline infusions reflected the fact that the animal was experiencing something like withdrawal everyday on this procedure. What is clear, however, is that saline infusions developed unmistakable conditioned reinforcing properties. This animal also underwent withdrawal after 75 days on the drug. At that time, heroin was replaced by saline. Food intake dropped immediately and began returning to baseline after two days. Saline infusions increased immediately, to nearly 400 in a single day (200 ml infused) and gradually fell to basal levels over 10 days. Responding on the former drug key remained at 10/day for 10 days and above baseline for 40 days. Responding for saline increased abruptly 14, 19 and 26 days into withdrawal. Plasma samples are available for these periods and are being processed to determine levels of opiates present on these days.

PLASMA LEVELS OF OPIATES AND ENDOCRINOLOGICAL ASPECTS OF HEROIN SELF-ADMINISTRATION

For a report on plasma levels of opiates in the animals described in this report, as well as some preliminary observations on the relationships of withdrawal to plasma testosterone levels, see the description of Work Unit 111 in the report.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6493	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	103
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Drug Abuse Prevention in Military Personnel

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology 003500 Clinical Medicine 012900 Physiology 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	PRECEDING	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	75	6	230
	CURRENT 76	8	401

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Adjustment; (U) Psychiatric Illness; (U) Stress Performance; (U) Deviant Behavior

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This unit examines social, environmental, psychological, and organizational factors that influence the spread of drug abuse. The impact of drug abuse on unit health and the performance of soldiers has also been studied. This study has military relevance for the development of future prevention and treatment programs.

24. (U) The methods of clinical psychiatry, social psychology, experimental analysis of behavior, anthropology, epidemiology, physiology, and toxicology are used to identify and modify factors which contribute to drug abuse in the military.

25. (U) 74 07 - 75 06 The primary field phase of the study of the epidemiology of drug and alcohol abuse at a large Army post has been completed. This study is an attempt to determine the environmental and social factors which can be modified to decrease the likelihood of the initiation of drug abuse, disrupt its maintenance, or treat its consequences. The analysis and publication of the materials gathered through urine screening, individual and group interview questionnaires, studies of demography and population dynamics, participant observation and survey continues. Work continues to be coordinated with experimental psychology, observing primate behavior. The analysis of choice behavior in the presence of available heroin is being studied in primates in an environment characterized by qualitatively different reinforcers. The last prevalence survey made of the post demonstrated that heroin was used by 5% of a random sample of men E-5 and below (at risk personnel). Heroin use was intermittant and opportunistic. The largest choice of use was polydrug 22%, while 18% were assertedly exclusive marijuana users, and 7% exclusive users of drugs other than marijuana. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 74 - 30 June 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug Abuse Prevention in Military Personnel

Investigators.

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Description

The field studies phase of the epidemiology of drug and alcohol use at an army post has essentially been completed. The purpose of these studies was to determine individual, environmental, and social factors that contribute to the initation and maintenance of drug and alcohol use.

Progress

1. The first phase of these studies entailed analysis of 3200 individual urine specimens and non-anonymous questionnaires, depth interviews of 460 urine positive and negative individuals matched by race and rank, a post-wide sociometric survey, systematic interviews of officers and noncommissioned officers in each company at the post and systematic studies of the demography of the post.

2. The second phase was divided into four primary tasks. First; repeated interviews with both illicit drug users and non-users were used to trace the social networks and contacts critical to the transmission and maintenance of drug and alcohol use among individuals in social groups. Second; participant observors collected data concerning; the formal organization of company-sized units; the sets of informal relations within the companies; the interactions between the formal and informal systems; and the performance of both individuals and units in

mission-relevant tasks. Third; treatment, rehabilitation, and service systems at the post were examined by observers in both the alcohol and drug treatment facilities. Records from service organizations such as the Provost Marshal's Office, the hospital emergency room, and the Mental Hygiene Consultation Service were collected. A health diary study using a random sample of soldiers was completed to ascertain health problems and patterns of consultation and therapy that do not come to the attention of the Army medical system. Fourth; survey instruments were used on a random sample of 700 soldiers to map cognitive and organizational factors, attitudes, behavioral modalities, and patterns of drug and alcohol use.

3. A pilot analysis of the sociometry study is complete. There appear to be correlations between the sociometric structure of groups and the patterning of drug use and other behaviors. Our presently available technologies do not allow us to explore the broad scale topological and graphic relationships involved. A research plan; "Interactive Graphical Social Relations Representation", has been submitted and fine grained analysis awaits the approval of this plan.

4. The analysis of the demographic structure and population dynamics of Ft. Meade and their possible implications for drug and alcohol use and abuse continues. Draft sections covering such standard demographic variables as Marital Status, Race, Rank, GT Score, Age, Education, etc., have been completed as well as one section on population stability. The analyses of this data have demonstrated the non-ergodic implications of differential turnover rates of personnel. Stability parameters have been used to cluster units giving two-fold differences in drug abuse rates.

5. Biochemical test results, the non-anonymous questionnaire, the initial 460 depth interviews, and the sociometric results have been transferred to magnetic tape, merged into comprehensive individual files, and supplemented with demographic information contained on the master personnel roster. Additional coding operations are in progress preparatory to comprehensive analysis and write-up.

6. The structured portions of the network and social contact interviews has been coded, entered on magnetic tape, and reduced to preliminary descriptive summary tables. Tape recordings of the unstructured interviews are currently being reviewed, indexed, and abstracted, and working papers are in preparation.

7. The field notes taken by the participant observers along with their interviews have been reviewed and indexed. Abstracts and working papers are being prepared. An interpersonal knowledge questionnaire used in this study has been coded and transferred to magnetic tape for further statistical analysis.

8. The field notes from observers at the alcohol and drug treatment centers are being abstracted and incorporated into a series of working papers.

9. The health diary data has been coded, transferred to magnetic tape, and is currently being analyzed. Archival data from the service organizations is being coded, preparatory to analysis.

10. The questionnaire/survey data has been coded, transferred to magnetic tape, edited, and is being analyzed.

11. Certain observations from the data may be presented at this point.[1-6] Drug use, particularly poly-drug use appears to be a widespread, stable phenomenon at the post. If anything, the general prevalence of use of marijuana, amphetamines, and hallucinogens increased during the period covered by our studies. "Hard drug" use subsided during this period (1973-1974). Agents such as cocaine and the opiates were used in an intermittent "chipping" style rather than regularly or addictively. Rates of alcohol consumption have remained consistently high. Most of the soldiers we studied could be classified as efficient drug users. That is, individuals whose drug use is discrete and well managed and rarely interferes with effective performance or other behavior on duty. The use of drugs appears to have become normalized. That is, drug use no longer has the compelling metaphoric qualities that were observed about it during the Viet Nam era. It involves neither "anti-establishment" nor counter cultural political content or assertions. It is, today, simply something people do, recreationally and experimentally, like drinking alcohol. Drug users do, for the most part, tend to associate with fellow drug users, particularly when using drugs, but they also maintain viable social and interpersonal relationships with non-users. The group at greatest risk for drug use is, as we have noted before, that comprised of unmarried, barracks dwelling, lower ranking enlisted personnel. Patterns of use at the post tend to be organized in terms of barracks mates and members of the same military units, as do patterns of distribution. In a number of cases, changes in the level of individual drug use appear to be related to the levels of use established by the using social network. Frequency of polydrug use, then, appears to be less a function of psychophysiological levels established by individuals, but varies with both changing events and changes in the composition of the individual's social network. Patterns of use appear at present to have no direct overt relationship to unit organization and function and future analyses may help us to determine whether subtle relationships exist between the two.

12. When the analyses have been completed the results will be published in a series of articles in the professional literature, and incorporated into a project volume detailing the history, scope, methods, and results of the comprehensive project. Tentative chapter topics have been assigned with working outlines anticipated by 1 OCT 75 and first drafts completed by 1 JAN 76.

13. A major outgrowth of our drug field studies is the realization that little is known about the basic sociology of the military community, data on the single soldier who lives in the barracks at a single point in time, but we have limited information on the life of the married soldier and the impact of his unit and his family on his activities, values, beliefs and life style. There is reason to believe that non-barracks dwelling soldiers have quite different lives and problems. There is a clear need for cohort, cross-sectional, and retrospective studies that focus on the married soldier and encompass long life-cycles in the Army community. Future work will aim in this direction with a continuing focus on drug and alcohol use. Some preliminary steps have been taken during the course of this past fiscal year:

a. A comprehensive bibliography and resource file of alcohol and the military is being prepared from the literature.

b. A bibliography of diaries and historical studies that highlight life conditions in the army is being assembled to provide a socio-historical context in which to contrast contemporary observations.

c. A protocol has been written entitled "Alcohol Use in the Army: A Retrospective Study by Means of Social Psychological Biographies," that aims to describe the relationship of Army social structure to individual patterns of alcohol consumption within that institution. Respondents will be drawn from the Soldier's Home in Washington, D.C. and an attempt will be made to survey drinking patterns as they relate to life in the Army under various conditions during the period 1915 to 1975.

d. Cohort and rolling cohort studing of alcohol use are being planned with a view to describing the acquisition of drinking habits and the structure of drinking patterns in the Army. Ethnographics of the bars and services on the "strip" outside selected Army installations are also contemplated as is an intensive study of communal life at an on-post married soldiers' housing area.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug Abuse Prevention in Military Personnel

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6498	75 07 01	DD DRSE(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	105
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with security Classification Code)

(U) Cellular Aspects of the Metabolism of Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 012900 Physiology 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 10	CONT	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	2.0	175
	CURRENT 76	2.0	262

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Acetylcholine Toxicity, (U) Acetylcholinesterase (U) Cell Culture, (U) Cell Membrane, (U) Morphine Tolerance

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To investigate the alterations of cellular metabolism underlying the development of tolerance to and dependence on drugs of abuse, and important military problem.

24. (U) A cell culture system with well defined density dependent physiological responses is used in reproducing and analyzing selected actions of narcotic drugs.

25. (U) 74 07 - 75 06 Continuous culture of WRL-10A mouse cells in progressively increasing concentrations of morphine up to 1.0 mM resulted in the development of a cell population which 15 months later shows only a slightly depressed growth rate and minor cytopathology. Control cells cultured in the same concentration of the drug without pre-exposure die within 3 weeks. Tolerance to morphine extended also to the respiratory activity of the cells which upon addition of 1.0 mM morphine to control cells was depressed by 30 - 40% but remained unchanged in cells preexposed to 0.75 mM of the drug. As with neural tissues, morphine had no effect on basal respiratory rates such as exhibited by high density growth inhibited populations. In terms of survival, however, such populations exhibited considerably greater sensitivity than low density growing cultures. High density populations are characterized by the presence of acetylcholinesterase (AChE) in the external surface of the cell membrane and also released into the media. It was further found that addition of morphine inhibits AChE activity in the media while cellular synthesis of AChE is enhanced. This suggests a homeostatic mechanism whereby synthesis and release of AChE would be secondary to synthesis and release of acetylcholine (ACh) and would represent an adaptive process preventing the accummulation of toxic levels of ACh with increasing cell density. By inhibiting AChE activity morphine in high doses would lead to toxic ACh levels and cell death while in more moderate doses it would induce a compensatory increase of the rate of AChE synthesis. The validity of this concept is currently being tested. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 105 Cellular aspects of the metabolism of drugs of abuse

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: Edwin M. Bartos, Ph.D.; Richard C. Robinson, B.A.

Description

Attempts to reproduce the phenomena of drug tolerance and dependence in cultured cells thus paving the way for the eventual uncovering of the metabolic processes involved in drug abuse are not new. Beginning with the prewar heroic period of tissue culture[1] and up to the present time[2] a sizeable number of reports on the subject have accumulated with an approximately equal distribution of positive and negative results. Thus, it is characteristic that recently one group of investigators reported that levorphanol prevented the induction of acetylcholinesterase (AChE) in cultures of mouse neuroblastoma cells without development of either tolerance or dependence[2], a second group found that in a human neuroblastoma cell line acute exposure to morphine decreased acetylcholine (ACh) levels with no effects on choline acetylase (ChAc) or AChE while chronic exposure resulted in a significant decrease of ChAc and an increase in AChE[3,4], and a third obtained elevation of the activity of both ChAc and ChE by adding morphine to 7-day chick embryo neurons in culture but failed to do so if either the embryo or the cultures were pre-exposed to the drug; on the other hand, when the same experiment was performed with mouse neuroblastoma cells exactly the opposite effect was obtained, i.e., morphine had no effect on the enzymes of naive cells but did increase both ChAc and AChE activities when the cells were obtained from tumours born by mice treated with the drug[5,6,7]. The multitude of cell types, culture methods, treatment schedules and observational criteria used as well as the rather large variance inherent in long-term linear tissue culture experiments are undoubtedly responsible for these inconsistencies. It follows that to answer unequivocally the question as to whether it is possible to reproduce the phenomena of drug tolerance and dependence *in vitro* there is an urgent need to use a well characterized cell culture system in conjunction with a rigorously standardized methology. At this point, the system does not need to be neuronal as opiate tolerance and dependence have been reported in other cell types with no greater inconsistency than described above for neuronal cells[8,9]. Accordingly, we undertook a study of tolerance to morphine in cell culture using L-929 mouse fibroblasts.

TABLE I

EFFECT OF MORPHINE ON THE RESPIRATION OF LOW DENSITY
(4-8 X 10^5 cells/ml) EXPONENTIALLY GROWING CULTURES

NAIVE CELLS				CELLS PRE-EXPOSED TO .75 mM MORPHINE SULFATE			
A. Cells Suspended in Drug Free Media	Morphine Sulfate 1.0 mM Added	B. After Addition of the Drug	B/A	A. Cells Suspended in Drug Free Media	Morphine Sulfate 1.0 mM Added	B. After Addition of the Drug	B/A
3.30[1]	150[2]	2.29[1]	.69	4.15	150	4.38	1.03
3.52	200	2.33	.66	3.36	200	3.40	1.01
3.22	220	2.22	.69	4.03	220	3.98	.99
3.36	250	2.02	.60	3.36	250	3.28	.98

[1]Respiratory rate: fmoles O_2/cell/min
[2]Time of exposure: minutes

Progress and Results

The results of a longitudinal study demonstrating the development of tolerance to the toxic effects of morphine in a well defined suspension culture system using L-929, clone WRL-10A cells, has been reported previously (Walter Reed Army Institute of Research Annual Progress Report 01 07 73 - 30 06 74, II: 1511 - 1521). To date, tolerant cells have been in continuous culture for a period of 15 months in the presence of 1.0 mM morphine sulfate exhibiting only a slightly decreased growth rate and minimum cytopathology; naive cells exposed to the same concentration of the drug develop severe cytopathology and become nonviable within 2 - 3 weeks.

To investigate whether tolerance to the toxic effects of morphine extended to cellular respiration, as is known to occur in neural tissues[10], low density (4 - 8 X 10^5 cells/ml) cultures growing in the presence of .75 mM morphine were centrifuged and resuspended in drug free media. After counting the cells, a suitable aliquot was removed and intoduced into the chamber of a Yellow Springs respirometer employing an O_2 electrode in a closed system where the rate of oxygen depletion is a function of cellular respiration and the number of cells present. While the respiratory rate of the cells in drug free media was being determined in this fashion, a small volume of a stock solution of morphine sulfate calculated to yield a final concentration of 1.0 mM morphine was added to the culture. After a desired time interval the cells were counted again, a second aliquot removed and the determination of the cellular respiratory rate repeated. Naive cells obtained from low density control cultures growing in the absence of the drug were similarly treated. The results of eight such experiments are shown in Table I where it can be seen that while in the absence of morphine the respiratory rates of naive and drug pre-exposed cells were similar, when challenged with 1.0 mM morphine naive cells uniformly exhibited at 30 - 40% depression of their respiration rate while cells pre-exposed to 0.75 mM morphine showed no effect.

These results indicate that the development of tolerance to the toxic effects of morphine in our system extends to cellular respiration (1). Indeed, the magnitude, 30 - 40%, of the respiratory depression and its absence in pre-exposed cells parallels closely Takemori's findings on the effects of morphine on the *in vitro* respiration of cortical slices obtained from naive and morphine treated rats[10]. In these latter experiments morphine was shown to depress the K^+ stimulated respiratory activity of the cortical slices but not their basal respiratory rates. The question therefore, arose as to whether the same differential effects on stimulated vs basal respiratory activity did obtain in our cell system as well. While the respiratory activity of our cell line of fibroblastic origin does not vary with the concentration of extracellular K^+, it is reduced to a basal rate when the population density of the cultures reaches high levels and growth is inhibited[11].

TABLE II

EFFECT OF MORPHINE ON THE RESPIRATION OF HIGH DENSITY (10-14 X 10^6 cells/ml) NAIVE GROWTH INHIBITED CULTURES

A. Cells Suspended in Drug Free Media	Morphine Sulfate 1.0 mM Added	B. After Addition of the Drug	B/A
1.70[1]	160[2]	1.66[1]	.98
1.82	165	1.86	1.02
1.54	170	1.74	1.13
1.15	170	1.11	.97

[1]Respiratory rate: fmoles O_2/cell/min
[2]Time of exposure: minutes

In the four experiments summarized in Table II it can be seen that this basal rate is approximately 50% lower than in low density exponentially growing cells (Table I) and that morphine is without effect, precisely as in the case of the cortical slices investigated by Takemori.

The lack of a demonstrable inhibitory effect of morphine on the respiratory activity of high density growth inhibited cultures raised the question whether such populations might be inherently more resistant to the toxic effects of morphine. This, however, did not prove to be the case as all naive high density growth inhibited populations exposed to doses of morphine sulfate higher than 0.25 mM, which is nontoxic also for low density growing cultures, developed severe cytopathology and became nonviable within 3 weeks. Moreover, all attempts to develop high density growth inhibited populations from low density cultures tolerant to morphine conentrations higher than 0.25 mM also failed, thus demonstrating that in fact high density stable populations are more sensitive to the toxic effects of morphine than low density exponentially growing cultures.

As previously reported, an important metabolic difference between high and low density cultures is the presence of significant amounts of acetylcholinesterase in the former and its virtual absence in the latter[12]. The enzyme was found to be located on the external surface of the cell membrane (2) suggesting that it might also be released into

the cellular environment as in muscle cell cultures[13] and raising the question whether it could be involved in the higher sensitivity to morphine exhibited by the AChE rich high density populations. To investigate the first question, cellular protein and acetylcholinesterase activity in the cells and the media of a high density growth inhibited population of WRL-10A cells were determined over a 24-hour period representing the normal interval between media renewal in these cultures. The results obtained are shown in Fig. 1 where it can been seen that the enzyme is indeed released from the cells with an initial rate of 0.94 units/mg cell protein/hr during the first 8 hours followed by a lower rate of 0.28 units from 8 to 24 hours, the time weighted average rate being 0.5 units/mg cell protein/hr. These values

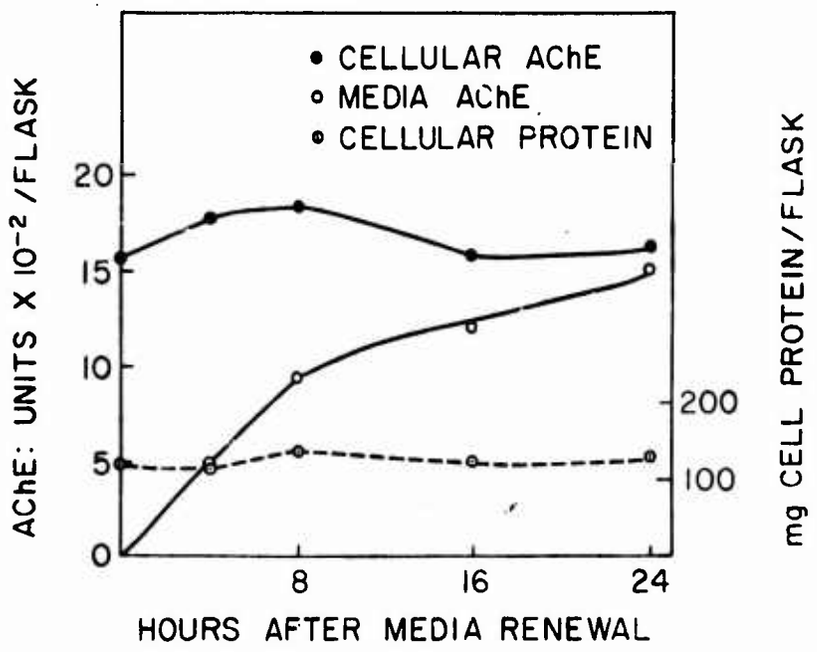

Figure 1. AChE activity in the cells and in the media, and cellular protein in a high density growth inhibited WRL-10A culture. At the indicated times after media renewal of a 50 ml culture maintained in a 125 ml flask, one ml samples were obtained, centrifuged, and the supernatant media was assayed for AChE; the cell pellet was washed, resuspended in buffer and lysed by sonication prior to assay for AChE and protein. The zero hour cellular protein and AChE was obtained just prior to media renewal since at least 2 hours are required for proper dispersion of the cells. Media AChE activity was corrected for the initial AChE activity of unused media.

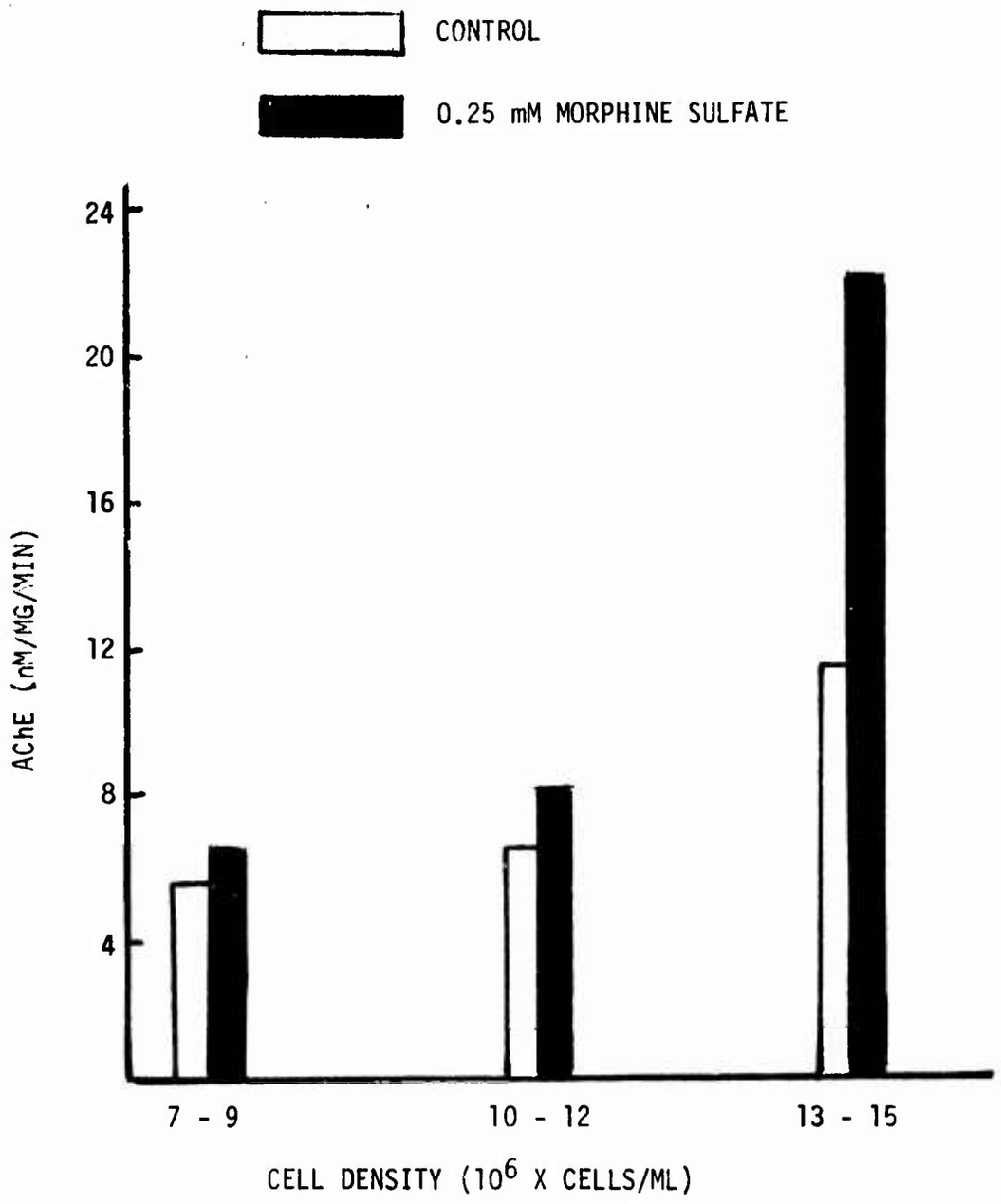

Figure 2. Effect of morphine on specific activity of acetylcholinesterase during development and maintenance of high density populations of WRL-10A cells. Morphine treated cells were obtained for a period of one year from the same culture by sampling for at least 3 days at desired population levels. Control cells were obtained from seven different cultures varying in duration from 2 weeks to one year and sampled at desired population levels. Bars represent means of at least 3 samples obtained as described.

represent minimal rates of enzyme synthesis since AChE degradation and turnover under these conditions are not known. The biphasic pattern of AChE release in these populations, with a higher rate during the first eight hours of the 24-hour media renewal cycle, is consistent with other previously observed heightened metabolic activities immediately following renewal of the media[14,15]. After 24 hours, the accumulated media AChE reached approximately 90% of the AChE activity associated with the cells. The cellular AChE during this time interval shows a slight increase in the first 8 hours followed by a return to the initial level by 16 hours, this level being maintained to the end of the 24-hour period. During this time, cellular protein remained at a nearly constant level reflecting the constancy of the cell density which was found to range from 15.5 - 15.8 X 10^6 cells/ml. In agreement with previous findings[14], this indicates that during the 24-hour media renewal cycle no significant cell breakdown occurs in these cultures.

Enzyme activity of cellular origin could not be detected in the media of high density cultures after addition of 1.0 mM morphine. This is in good agreement with the well known inhibitory effects of morphine on the hydrolysis of acetylcholine by AChE[16]. Inhibition of the activity of a neuroenzyme leading to increased rates of enzyme synthesis is one of the popular hypotheses proposed to explain the development of tolerance to opiates[17]. Accordingly, we examined the levels of cellular acetylcholinesterase in control and morphine treated high density cultures, the latter assayed after washing the cells free of the drug. The results are shown in Fig. 2 where it can be seen that the activity of the enzyme tended to be higher in the morphine treated cells and that the difference between the two types of cultures increased dramatically with increasing cell density.

To explain this finding as well as the previously noted greater sensitivity of high density populations to morphine it is proposed that besides acetylcholinesterase, WRL-10A cells contain also choline acetylase (ChAc) and consequently are capable of acetylcholine (ACh) synthesis and release. During the development of high density populations ACh accumlation in the media would tend to increase and this in turn would induce AChE synthesis and release as an adaptive process necessary for maintaining media ACh concentrations below toxic levels. Addition of morphine to the media of such cultures and the ensuing inhibition of AChE activity would have consequences depending on the amounts added: very high doses of the drug would result in toxic ACh concentrations and loss of viability of the cells while increase of ACh concentrations due to more moderate doses would induce a compensatory increase of AChE synthesis thus ensuring the survival of the cultures. These manifestations would be expected to be more pronounced with increasing cell density precisely as shown in Fig. 2 because of the relatively higher media ACh concentrations involved. This concept allows the meaningful integration of all our observations so far; additional work aiming to test its validity through direct measurements of acetylcholine synthesis and release in our system is in progress.

Summary and Conclusions

Continuous culture of WRL-10A mouse cells in progressively increasing concentrations of morphine resulted in the development of a cell population which after 15 months of exposure to 1.0 mM morphine remains fully viable, has a slightly depressed growth rate and exhibits only minor cytopathology. Control cells cultured in the same concentration of the drug without pre-exposure, die within 3 weeks. Tolerance to morphine extended also to the respiratory activity of the cells which upon addition of 1.0 mM morphine to the control cells was depressed by 30 - 40% but remained unchanged in cells pre-exposed to 0.75 mM of the drug. As with neural tissues, morphine had no effect on basal respiratory rates such as exhibited by high density growth inhibited populations. In terms of survival, however, such populations exhibited considerably greater sensitivity than low density growing cultures. High density populations are characterized by the presence of acetylcholinesterase (AChE) located on the external surface of the cell membrane and also released into the media. It was further found that addition of morphine inhibits AChE activity in the media while cellular synthesis of AChE is enhanced. These findings are interpreted in terms of a homeostatic mechanism whereby synthesis and release of AChE would be secondary to synthesis and release of acetylcholine (ACh) and would represent an adaptive process preventing the accummulation of toxic levels of ACh with increasing cell density. By inhibiting AChE activity, morphine in high doses would lead to toxic ACh levels and cell death while in more moderate doses it would induce a compensatory increase of the rate of AChE synthesis. The validity of this concept is currently being tested.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 105 Cellular aspects of the metabolism of drugs of abuse

Literature Cited.

References:

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1. Robinson, R.C., Bartos, E.M. and Glinos, A.D.: Tolerance to morphine in a fibroblastic cell line exhibiting growth related regulation of components of the cholinergic system. Fed. Proc. 34: 736, 1975.

2. Bartos, E.M. and Glinos, A.D.: Growth related regulation of components of the cholinergic system in a fibroblastic cell line. In Vitro 10: 365, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6409	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	106
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Procede with Security Classification Code)

(U) Clinical and Demographic Studies of Military Drug Abusers

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012600 Pharmacology 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	1.5	70
FISCAL YEAR CURRENT 76	.5	8

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide Each with Security Classification Code)

) Hepatitis B Antigen and Antibody; (U) Pulmonary Functions; (U) Immunog[illegible] (U) Demographic Variables

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. P[illegible] (Individual paragraphs identified by number. Proceed text of each with Security Classification Code.)

23. (U) Drug abuse has [illegible]y been a major problem in military populations. The objectives of these studies are to obtain information on medical problems related to drug abuse and to examine demographic variables and drug use histories of a military population.

24. (U) Clinical studies evaluated medical complications of drug abuse by extracting data from clinical records. A review of drug associated deaths in USARV was done by chart summary of all such autopsy protocols. Clinical laboratory studies done and/or results analyzed in heroin and non-heroin using military populations (USARV) include: biochemical studies by procedures according to Hycel; hepatitis B antigen and antibody determination by radioimmunoassay; determination of immunoglobulins by the immunodiffusion technique; pulmonary functions by means of an electric spirometer. Analysis of demographic variables and drug use histories of heroin and non-heroin using military (USARV) populations is in progress by frequency analysis and cross-tabulation.

25. (U) 74 07 - 75 06 A study of clinical aspects of acute abstinence in 320 heroin dependent USARV soldiers has been completed and reported. A review of drug associated deaths in USARV has been completed. Data from the following laboratory studies in heroin and non-heroin using soldiers (USARV) are being analyzed: hepatitis B antigen and antibody; immunoglobulins; and biochemical studies. Study of pulmonary functions in these populations has been submitted for review. Frequency analyses and cross tabulations have been completed on demographic variables and drug use histories in 3484 non-heroin using soldiers (USARV). For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 74 - 30 JUN 75.

*Available to contractors upon originator's approval

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Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task ØØ Biomedical Factors in Drug Abuse

Work Unit 1Ø6 Clinical and demographic studies of military drug abusers

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Description

The objectives of this work unit are to complete clinical and demographic studies initiated in Vietnam related to heroin use in the military, to carry out further studies on biological specimens and data derived from these studies and to conduct other clinical studies related to drug abuse in military populations.

Progress

1. Prevalence of Hepatitis B Antigen and Antibody Among Heroin Users in Vietnam

In our preceding progress report (1) results of a study of the prevalence of hepatitis B antigen (HBAg) and antibody (HBAb) in sera of heroin and non-heroin using populations (USARV), as determined by solid phase radioimmunoassay, were reported. In addition, the prevalence of HBAg and HBAb in subgroups of the heroin using sample according to mode of self-administration of heroin were reported. Further work is being done with regard to the possible effect of three variables (age, race and time in Vietnam) on the prevalence of HBAg and HBAb in the non-heroin using sample of this study. During this reporting period analysis of data consisting of liver function tests performed on subsamples of the heroin and non heroin using samples of this study has been completed. Liver function studies were done to check for possible correlation between antigenemia and evidence of hepatic dysfunction, and to further examine for such in population of heroin users with unique characteristics (i.e., administration of high doses of heroin by three modes).

Liver function tests (alkaline phosphatase, SGOT, SGPT, LDH) were performed on an automated multi-channel analytical instrument (Hycel, Inc., Houston, Texas 77042). Only data relative to the presence or absence of HBAg and HBAb is presented. Further discussion of liver function tests in heroin and non-heroin using population samples is presented in the next section. Sera of 55 non-heroin (N.H.U.) and 123 heroin using (H.U.) soldiers (USARV) were tested.

One of the N.H.U. sample was positive for HBAg and 7 for HBAb. Eight of the H.U. sample were positive for HBAg and 26 for HBAb. Alkaline phosphatase, SGOT and SGPT were significantly greater in the HBAg positive sample of H.U. than in the total N.H.U. sample (test of statistical significance: t-test). No significant differences existed between these tests when the HBAg negative H.U. sample was compared to the N.H.U. sample. SGOT and SGPT were also significantly greater in the HBAg (+) H.U. sample than in the HBAg (-) H.U. sample. HBAb was not associated with significant differences in SGOT, SGPT and alkaline phosphatase either between N.H.U. and H.U. sample or when comparisons were made within groups. LDH was only significantly greater in the HBAb(+) H.U. sample than in the HBAb (-) H.U. sample.

In summary, although SGPT, SGOT, and alkaline phophatase were all higher in the H.U. sample than in the N.H.U. sample, the differences were only significant when comparing the HBAg (+) H.U. sample to the N.H.U. sample. These data and that pertinent to the total H.U. and N.H.U. samples with regard to HBAg and HBAb are being prepared for reporting. The significance of persistent hepatitis B antigenemia and/or hepatic dysfunction based on persistent perturbation of SGOT and SGPT in U.S. heroin users has recently been correlated with persistent or relapsing hepatitis (2, 3, 4, 5).

2. Biochemical Studies in Heroin and Non-Heroin Using Soldiers (USARV)

Reports (6, 7, 8, 9, 10, 11) of perturbations of various clinical biochemical tests during heroin addiction and acute abstinence formulated the background for evaluation of the various clinical biochemical parameters on sera samples of heroin users in USARV. Data from two studies have now been analyzed. The first study (Hycel I) consisted of testing single sera samples obtained from 123 soldiers at admission to a drug treatment center (USARV) for detoxification from heroin dependency, and from 55 self-reported non-heroin using USARV soldiers. The second study (Hycel II) consisted of testing sera taken at 0600 hours, 1000 hours, and 2200 hours on days 0, 1, 2, 3, 5, 7, 9, and 11 of the acute abstinence phase of 10 USARV heroin users (H.U.) and 5 USARV non-heroin users (N.H.U.) admitted to a research ward. The setting of this study is described elsewhere (1, 12). The biochemical determinations were performed by an automated multichannel analytical instrument (Hycel, Inc., Houston, Texas). A brief review of the results of the analysis of these data are presented.

HYCEL I: No significant differences were found between the the N.H.U. sample and H.U. sample in the following tests: CPK, LDH, SGPT, alkaline phosphatase, total protein, and potassium. The following tests varied significantly between the two groups and in the noted direction:

Lower in the H.U. sample vs. the N.H.U. sample:

Calcium: (< .01)
Sodium: (< .01)
Creatinine (< .001)
Uric acid (< .001)
Bun (< .01)
Cholesterol (< .05)

Higher in the H.U. sample vs. the N.H.U. sample:

Phophate (inorganic): (< .001)
SGOT (< .05)
Globulin (< .01)
Alkaline phosphatase (< 0.05 - HBAg (+) H.U. sample vs. N.H.U. only)
SGPT (< 0.02 - HBAg (+) H.U. vs. N.H.U. only)

Comment: The differences in hepatic enzymes (SGOT, SGPT, LDH and alkaline phosphatase in the two samples with note of the differences in subgroups, based on HBAg and HBAb, were noted earlier. Further comment on individual variation within test follows.

The most significant differences between SGOT and SGPT in the samples occurred between N.H.U. sample and the subgroup of H.U. sample who injected heroin intravenously. Serum globulin was significantly higher in intravenous users of heroin vs. the non-intravenous users.

Hycel II: Twenty-two comparisons taken from sera collected at 22 points in time were made for each biochemical test between the two groups (H.U. and N.H.U.). Statistically significant differences (based on Kolmogorov-Smirnov test) were found in the following tests and points in time. Glucose was significantly higher in the H.U. group than the N.H.U. group on day 2 of acute heroin abstinence at 0600 hours only. However, it was elevated over control (N.H.U.) values in 18/22 other times. SGOT was significantly elevated in the H.U. group vs. the N.H.U. group at 0600 hours on days 2, 5, 7, 9, and 11 and at 1000 hours on day 11, and was higher but not significantly, at each of the remaining 16 times. SGPT was significantly elevated in the H.U. group vs. the N.H.U. group in 20 of 22 times, but still elevated above the N.H.U. group in the remaining two times, both of which were at 1000 hours (days 5 & 9).

Although not statistically significant, in greater than 50% of the 22 points in time, the following tests were higher in the H.U. group vs. the N.H.U. group:

BUN: 15/22
LDH: 15/22
Alkaline phosphatase: 16/22

Also, although not significant, the following tests were lower in the H.U. group vs. the N.H.U. group:

Calcium: 15/22
Phosphate: 15/22
Potassium: 17/22
Creatinine: 20/22
Uric Acid: 18/22
Total Protein: 12/22
Globulin: 13/22
Cholesterol: 21/22
CPK: 14/22
Bilirubin (total) 19/22

Comments on Hycel I and II: Noteworthy is that although significant differences of certain tests were noted to exist between the H.U. and N.H.U. groups, few of the actual single test values were pathologically elevated or depressed. Also noteworthy is that: hepatic enzymes SGOT and SGPT were significantly elevated in both H.U. groups when compared to N.H.U. groups; glucose was either significantly elevated or tended toward elevation in the H.U. groups when compared to the N.H.U. groups, and that bilirubin, cholesterol, creatinine, uric acid, potassium and calcium levels tended to be lower in the H.U. groups vs. the N.H.U. groups in both studies. The data and analyses of the Hycel I and II studies are being prepared for reporting in cooperation with the Division of Biochemistry (WRAIR).

3. Serum Immunoglobulins in Heroin and Non-Heroin Using Military Population in USARV

During the past 5 years, several reports (13, 14, 15) have noted increased immunoglobulins to be common in both adolescent and adult heroin addicts in the U.S. In each report, the most commonly recognized serum alteration is polyclonal increase in serum immunoglobulin M (IgM, 19 S macroglobulin). During methadone maintenance or heroin abstinence, high IgM levels have been noted to be much less frequent or previously elevated levels have been observed to return to within normal range. The etiology of primarily IgM elevations in heroin addicts is not known. No definite correlations have been demonstrated between IgM elevations and histories of overt hepatitis, manifest liver disease, hepatitis B antigen, SGOT, SGPT or alkaline phosphatase levels in heroin addicts. Likewise, no frequency correlations with the recognized causes of isolated increase in IgM have been

demonstrated in the samples of heroin addicts studied who were found to have IgM elevations. The cause of IgM elevations in these individuals most frequently proposed is that it is related to increased antigenicity secondary to the wide variety of diluents and contaminants injected intravenously into heroin addicts. The question of whether heroin per se is in anyway directly responsible for polyclonal increases in IgM likewise remains unanswered.

The purpose for evaluating immunoglobulin levels in USARV heroin users is threefold: (1) this population was young and essentially free of diseases commonly associated with hypermacroglobulinemia; 2) heroin was used intravenously in pure form without diluents, (except water); and 3) the use of another route of self-administration of heroin in this population, i.e., nasopulmonary via smoking or sniffing. The sera on 100 heroin dependent soldiers, with no reported or clinically apparent diseases was obtained at time of admission for detoxification. Likewise, sera for control purposes was obtained on 100 apparently healthy non-heroin using soldiers in USARV. Immunoglobulins were determined by the immunodiffusion method of Mancini et al (16). A brief review of the findings follows.

Immunoglobulin M(IgM): The total H.U. group had a significantly higher mean level than the N.H.U. group ($< .01$) although both means were within normal range for IgM. Within the H.U. cohort, the group that used heroin by the nasopulmonary route had a significantly lower level ($< .001$) that did the group that employed the intravenous route. There was no significant difference between IgM values in those who smoked heroin vs. the non-user control group (N.H.U.).

Immunoglobulin G (IgG): The mean value of IgG in the H.U. group was also significantly greater ($< .01$) than that of the N.H.U. group although both values were within normal range. However, mean IgG value for the intravenous users of heroin was, although greater, not significantly elevated when compared to the smokers of heroin.

Immunoglobulin A (IgA): Here again the H.U. group had a significantly higher mean level ($< .001$) than did the N.H.U. group, although again both means were within normal range. The mean value of IgA for the smokers of heroin was slightly higher than that for the intravenous users.

There were no consistent relationship or patterns observed between mean IgA, IgG, IgM levels and daily dose of heroin employed or duration of heroin use. Except for mean IgA levels in the N.H.U. group, the mean values of IgA, IgG and IgM were higher for the subgroups of the H.U. and N.H.U. groups who had been in RVN greater than vs. less than 6 months, although not all differences were significant. Further analysis of this data is in progress in preparation for a report.

4. Expiratory Ventilatory Functions in U.S.A.R.V. Heroin Users

In the last report (1) this study was described in detail. The report has been revised and resubmitted. As noted earlier, decreases in all measured expiratory parameters were found in heroin using soldiers regardless of self-reported modes of administration, durations of use, dosages, or times elapsed since last self-administration. However, of all these self-report variables relating to heroin use, time since last dose might reasonably be expected to have a significant effect on expiratory functions. The failure to note such effects may, at least in part, be attributable to unreliable self-report of time since last dose. In other unpublished work we have noted that soldiers who had denied use of heroin for days or weeks prior to admission, frequently had positive urine tests for opiates. The suspected unreliability of this aspect of self-reporting may be related to the belief that denial of recent use would shorten the period spent at a treatment center. In contrast to the last report, we can no longer clearly assert that the variable of time since last dose prior to study did not have an effect on expiratory ventilatory function.

5. Clinical Observations on High Dose Heroin Tolerance and on Acute Abstinence in Heroin Dependent Soldiers (USARV)

a. Heroin Tolerance

Certain characteristics of heroin use by soldiers in Viet Nam provided a unique model for the study of tolerance and its relation to dependency in young men. The typical heroin-using soldier was young, healthy and took few other drugs than marijuana while using heroin, although he may have used several other illicit drugs prior to initiating heroin use. These soldiers also had a relatively brief total exposure to opiate use when compared to patients treated for heroin use in the United States (17). Heroin in Viet Nam was inexpensive, readily available, of high purity (92-98%) and was distributed in small plastic vials containing 200-300 mg of pure heroin without adulterants (17). Three modes (smoking, sniffing per nasum or intravenous injection) of heroin self-administration were used either singly or in various combinations by this population. Smoking was the most common mode of heroin use and was employed as the sole or primary method by 60% to 65% of the heroin users. Sniffing heroin powder per nasum ("snorting") and intravenous injection were each utilized as the only or primary methods in 5%-10% of heroin-using soldiers admitted to drug treatment centers for detoxification. Various combinations of three methods were used by 25%-30%.

Data pertaining to several demographic variables and illicit drug use histories of 320 heroin-using soldiers admitted to a U.S. Army Republic of Viet Nam (USARV) Drug Treatment Center and selected for study of clinical manifestions of heroin withdrawal are presented in

Table 1. Each soldier was admitted on the basis of a positive urine test for morphine (FRAT confirmed by thin layer chromatography (TLC)). Each one met the following criteria based on self-report, daily use of heroin for a minimum of two months; a last dose of heroin 12 or less hours prior to admission; use of one mode of self-administration of heroin during most of all of the month prior to admission. This population of 320 soldiers included 200 who used heroin by smoking, 60 who took it by sniffing per nasum and 60 who self-administered heroin by intravenous injection. Historical data and a characterization of current patterns of heroin use by the three subgroups (based on mode of use) are presented in Table 2. The mean values of daily dose taken and duration of daily use are presented in Table 3.

The mean daily dose of the subgroup of soldiers who self-adminstered heroin by smoking was 5 vials or approximately 1200 mg per day. Since 70%-75% of heroin used by this mode is destroyed by pryolysis (19), the mean estimated daily dose of heroin absorbed by this group would be in the range of 200-400 mg, indicative of a higher tolerance than that achieved by the typical current intravenous user of "street heroin" in the U.S. Unfortunately, the pharmacokinetics of heroin taken by nasal sniffing have not been adequately studied. Since heroin is readily absorbed by the nasal mucosa and through pulmonary alveoli, one may assume that most of that which enters the nares is absorbed. The mean daily dose of those who took heroin by sniffing was also 5 vials, or approximately 1200 mg per day. The mean daily dose of the soldiers who used heroin intravenously was 6 vials, or approximately 1500 mg per day.

In this group, 16.6% (N = 10) of the intravenous users reported daily use of 10 or more vials, or approximately 2500 mg or more per day; 3.3% (N = 2) reported daily use of greater than 20 vials, or more than 5,000 mg per day. Noteworthy are the remarkable levels of tolerance developed by soldiers in all three subgroups, especially by certain of those who used heroin intravenously. The ability of the individuals in this study to take such high daily dosages of heroin without evidence of overdose certainly supports the well-documented observation that in man, impressively high, if not unlimited, levels of tolerance to certain effects of opioids, such as respiratory depression and lethality, can develop (18).

b. Signs and Symptoms of Acute Heroin Withdrawal in U.S. Soldiers in Viet Nam

The frequency with which signs and symptoms of acute heroin withdrawal were reported in a questionnaire protocol as having occurred during previous withdrawal attempts experienced by 279 of the 320 heroin-using soldiers, described in Tables 1 and 2, are presented in Table 4. The frequency with which certain pre-selected signs and symptoms of opiate withdrawal were observed in this group by a physician after admission to a drug treatment center, is presented in Table 5. In these tables the results are arranged to compare subgroups

which used different modes of self-administration. Generally the subgroup who smoked heroin had the lowest frequency of withdrawal signs and symptoms both by self-report of previous experience (Table 4) and by physician observation in the treatment center (Table 5). The subgroup of intravenous users had the highest frequency of withdrawal signs and symptoms. The frequency of signs and symptoms in the subgroup which self-administered heroin by sniffing is generally between that reported for the other two subgroups.

c. Heroin Withdrawal Intensity in U.S. Soldiers in Viet Nam

Studies of the factors which influence the intensity of the heroin withdrawal syndrome as observed in Viet Nam are in progress. Data and comments on several variables which appear to relate to heroin withdrawal intensity as observed in Viet Nam are presented here. Table 6 presents qualitative clinical estimates of the intensity of withdrawal in the three subgroups of 320 heroin-using soldiers noted earlier (see Tables 1 and 2). These estimates were based on the judgment of the physician in charge. Generally, mild withdrawal was characterized by a mild symptomatology that required no medication or only one or two doses of non-narcotic medication for relief. Withdrawal was described as moderate if symptomatology was of moderate intensity and adequately controlled by non-narcotic agents, usually required on a regular schedule for 24-48 hours during withdrawal. Marked withdrawal was characterized by signs and symptoms of significant intensity and duration, usually consisting of recurrent vomiting and/or diarrhea with abdominal cramps, and/or marked skeletal muscle cramps. These patients were treated with repeated doses of non-narcotic medication. When non-narcotic agents were not adequate to control withdrawal symptomatology, methadone therapy consisting of one to three doses of 10-20 mg during peak intensity of withdrawal was almost always adequate.

As noted in Table 6, generally the degree of intensity of withdrawal, based on qualitative estimates, was greatest in the subgroup who self-administered heroin intravenously and lowest in the subgroup who self-administered heroin by smoking. Although the mean daily dose level of heroin used was somewhat greater in the subgroup who used heroin intravenously than in the other two subgroups (Table 3), these differences are not statistically significant. As approximately only one-fifth of heroin that is smoked is absorbed (19), the mean and median daily heroin doses absorbed by the smokers in this study was an estimated 200 mg (based on mean and median number of 250-mg vials used), in contrast to mean and median daily heroin doses of approximately 1500 mg and 1200 mg, respectively, injected by the intravenous heroin users in this study. The lowest estimated daily heroin dosage absorbed by any one of the 320 heroin-using soldiers studied was approximately 50 mg (i.e., 20% of one 250-mg vial of heroin taken by smoking). The highest estimated daily heroin dosage was over 5000 mg (i.e., use of more than twenty 250-mg vials in intravenous users).

In order to minimize the problem of interpreting the interaction between the route of administration, dosage taken, and dosage absorbed, the relationship between daily dosage and withdrawal severity was examined in detail in that subgroup which used heroin by the intravenous route (see Table 6). The estimated mean daily dose for the 8% (N = 5) who experienced mild withdrawal was 800 mg (based on mean number of 250-mg vials used) compared to an estimated 1400 mg for the 47% (N = 28) with moderate withdrawal and estimated 2000 mg for the 45% (N = 27) with marked withdrawal. These findings suggest that daily heroin dose was a factor in determining withdrawal severity as observed in this group of heroin-dependent soldiers.

Way and co-workers (19), by employing the Himmelsbach scale (8) to quantitate withdrawal intensity, demonstrated a more severe withdrawal syndrome in intravenous users than in smokers of heroin, despite larger daily doses used by the latter group. They attributed this to the smaller daily dose absorbed by those who smoked heroin (an estimated 14% to 30% of total daily dose) and, therefore, to a lower degree of physiological dependency.

Another important characteristic of heroin use in the Viet Nam setting is the relatively brief duration of daily use by most of the heroin-using soldiers at the time of admission for treatment. This is in contrast to the usual duration of use by the typical heroin-dependent US civilians who become involved in treatment for dependency after many months or years of use. Data pertinent to duration of daily heroin use for the three subgroups of heroin-using soldiers in this study are presented in Tables 2 and 3. The shortest duration of daily heroin use in this study was 2 months and the longest was 28 months. Concerning mean duration of daily heroin use (Table 3), only the difference between the subgroup which smoked heroin and the group which used it intravenously is statistically significant ($p = < 0.001$). With regard to duration of daily use and withdrawal intensity, data pertaining to the subgroup who used heroin intravenously are presented (see Table 6). The mean duration of use was 6 months for both the 8% who experienced mild withdrawal and the 47% who experienced moderate withdrawal. The mean duration of use for the 45% who had marked withdrawal was 8 months.

Although the mean values for both daily heroin dosage and duration of daily use in the intravenous users are lowest in the group with mild withdrawal and highest in the group with marked withdrawal, the small number and large variances in the group with mild withdrawal prevent a reliable statistical analysis of these differences. The duration of use was significantly greater ($p = < 0.05$) in the intravenous users who had severe withdrawal compared to those with withdrawal of moderate intensity.

Of the 320 heroin-dependent soldiers in the study, 40% were assessed as having mild withdrawal, one-half of whom were given no medication. Of the 15% of the 320 soldiers who were assessed as

having withdrawal of marked intensity, 56% were from the intravenous use group. Only 8% of the 320 required methadone in addition to non-narcotic agents for relief of withdrawal distress. The signs and symptoms of the heroin withdrawal syndrome in these soldiers differed little from that previously reported in stateside studies. However, the intensity of these signs and symptoms was generally mild. It is remarkable that these young men could take such large doses and yet in most instances, demonstrate a relatively mild withdrawal syndrome. Although a strict rating of this withdrawal using several withdrawal scales is still in progress, it does appear that the intensity of the withdrawal syndrome in these soldiers was similar to that reported by several clinicians (20, 21, 22) who observed a mild heroin withdrawal syndrome in adolescents or young men, also with relatively short histories of heroin use. Previous reports (17, 23, 24, 25) of mild withdrawal in young soldiers in other populations of patients observed in Viet Nam, and the findings from the study population reported above, suggest that other variables besides daily dosage of heroin are important determinates of withdrawal severity.

d. Withdrawal Treatment in U.S. Heroin-Dependent Soldiers in Viet Nam

Table 6 presents a description of types of medication given during heroin withdrawal in the 320 heroin-dependent soldiers described in Tables 1 and 2. The non-narcotic drugs referred to in Table 6 consisted of a variety of agents noted in the previous section on non-narcotic medication for opioid withdrawal used to relieve pain, gastrointestinal compliants, and to treat insomnia and nervousness. When such non-narcotic agents were not effective in ameliorating withdrawal distress, methadone was given, usually in doses of 10 to 20 mg. Seldom were more than three such doses required during the course of withdrawal. In general the smokers of heroin were given the least medication for withdrawal symptoms (see Table 6). The withdrawal in those who used heroin by nasal sniffing was predominantly managed by non-narcotic drugs. Almost all intravenous users of heroin required some medication; however, in spite of their use of large doses of intravenous heroin, only 30% required methadone in addition to non-narcotic agents for relief of withdrawal distress (see Table 6). The mean daily dose for the 30% (N = 18) for the intravenous users who were treated with methadone was 2200 mg (9 vials) compared to 1500 mg (6 vials) for the 68% (N = 41) of the intravenous users treated symptomatically with non-narcotic agents only. Although the mean dose level of the group treated with methadone was higher than that of the group treated by non-narcotic drugs only, this difference was not statistically significant. The mean duration of daily heroin use for the 30% of the intravenous users who received methadone therapy was 9 months compared to 7 months for the intravenous users treated with non-narcotic agents only. The mean duration of daily use of the methadone treated intravenous heroin users was significantly greater ($p = < 0.05$) than the group treated with non-naroctic agents only.

6. Study of Fatal Narcotism and Other Drug Related Deaths in USARV

A study of all autopsy protocols of drug related deaths that occurred in USARV during most of the period that heroin use was epidemic in USARV has been completed (JAN 1970 - JUL 1972). This review has also included alcohol related deaths. The following data were collected: age, race, rank, toxicology data, gross pathologic findings and data on cause and manner of death.

During the 30-month period reviewed, a total of 294 autopsy protocols had evidence of a drug (including alcohol) induced or associated death. Of these, 133 were opiate associated. These deaths have been studied with reference to demographic characteristics, pathologic and toxicologic findings, and with regard to cause and manner of death. These data are being prepared for reporting and are being compared where possible to corresponding variables of a review by Froede (26) on fatal narcotism in military personnel during the period 1918 to 1970.

7. Demographic Characteristics, Military and Drug History Data of USARV Heroin and Non-Heroin Using Soldiers

Data collected by questionnaire on 3484 USARV soldiers (E1 - E6) at DEROS who served in Viet Nam during the heroin epidemic (1971 & 1972) but who denied heroin use is in computer printout form. Frequency analysis of 42 items and cross correlation of each of these against five selected items has been completed. Data relative to 3000 heroin using soldiers (USARV) have been collected employing the same instrument. Approximately 1500 of these questionnaires have been key-punched. These data will be subjected to frequency analysis and cross correlation, and will then be compared to corresponding data of the non-heroin using cohort.

TABLE 1 DEMOGRAPHIC AND ILLICIT DRUG HISTORY DATA
US ARMY HEROIN-DEPENDENT SOLDIERS -- RVN*

N = 320

ITEM	(%)
Age (years)	
18-19	27.5
20-22	59.1
23-25	12.2
26-28	00.9
> 28	00.3
Race	
White	74.1
Black	18.4
Latin-American	5.3
Other	2.2
Residence**	
< 5,000	16.9
5,000-100,000	40.0
100,000-1 million	26.6
> 1 million	16.5
Prior Illicit Drug Use History	
Marijuana	90.6
Amphetamines	70.0
Barbiturates	75.0
Psychedelics	68.4

*Republic of Vietnam
**4 year period prior to military

TABLE 2 HEROIN USE HISTORY vs MODE OF USE
US ARMY HEROIN-DEPENDENT SOLDIERS-RVN*

Item	Smoking N = 200 (%)	Sniffing N = 60 (%)	Injection (IV) N = 60 (%)
Age when first used (years)			
< 15	0.5	1.7	3.3
15-17	7.5	8.3	16.7
18-19	40.0	40.0	43.3
20-21	36.5	33.3	21.7
22-24	14.0	16.7	15.0
> 24	1.5	0.0	0.0
Status when first used			
Civilian	12.0	18.3	28.3
Military (pre-RVN*)	19.0	13.3	23.3
Military (in-RVN*)	69.0	68.3	48.3
Previous withdrawal attempts			
None	20.5	16.6	15.0
1-2	56.5	50.0	41.7
3-6	20.5	28.3	33.3
> 6	2.5	5.0	10.0
Duration of daily use (months)			
3 or less	44.0	25.0	23.3
4-6	34.5	51.7	30.0
7-9	11.0	13.3	26.7
10-12	8.0	5.0	11.7
13 or greater	2.5	5.0	8.3
Average daily dose+ (vials++)			
1-3	37.0	43.3	33.3
4-6	37.5	30.0	40.0
7-9	14.0	13.3	10.0
10-12	6.0	6.7	8.3
13-20	3.5	6.7	5.0
> 20	2.0	0.0	3.3

*Republic of Vietnam
+Average number of vials per day during month prior to admission
++Vial contains approximately 250 milligrams of 92-98% pure heroin

TABLE 3 MEAN VALUES FOR HEROIN DAILY DOSE AND DURATION OF USE VS MODE OF USE

Item	Smoking N = 200 ($\overline{X}$)	Sniffing N = 60 ($\overline{X}$)	Injection (IV) N = 60 ($\overline{X}$)
Dose†: Vials††/day	5	5	6
Duration of steady use (months)	5	6	7

†Average number of vials per day during month prior to admission.
††Vial contains approximately 200 milligrams of 92-98% pure heroin.

TABLE 4 REPORTED SIGNS AND SYMPTOMS DURING PREVIOUS WITHDRAWAL ATTEMPTS VS MODE OF USE

Sign/Symptom	Smoking N = 178 (%)	Sniffing N = 50 (%)	Injection (IV) N = 51 (%)
Rhinorrhea	69.7	78.0	86.3+
Lacrimation	65.2	68.0	84.3+
Sneezing	42.7	56.0	58.8+
Yawning	52.8	54.0	66.7
Diaphoresis	56.2	58.0	66.7
Piloerection	53.4	64.0	72.5
Chilliness	65.7	68.0	78.4
Hot/cold flashes	62.4	68.0	76.5
Muscle cramps	47.8	72.0	62.7
Muscle twitching/ tremors	41.0	64.0*	66.7
Back/leg/joint pains	61.2	70.0	70.6
Restlessness	73.0	76.0	90.2+
Double/blurred vision	18.0	30.0	33.3
Headaches	44.9	36.0	45.1
Dizziness	37.6	40.0	49.0
Restless sleep	71.3	76.0	88.2+
Insomnia	65.2	72.0	80.4+
Abdominal cramps	56.7	64.0	88.2††
Nausea	37.6	54.0	68.6+
Vomiting	25.8	30.0	56.9†
Diarrhea	46.1	54.0	68.6

*Significantly greater than smokers of heroin ($p < 0.05$) Analysis by means of the normal deviate z.

+ Significantly greater than smokers of heroin ($p < 0.05$) Analysis by means of the normal deviate z.

† Significantly greater than smokers and sniffers of heroin ($p < 0.05$) Analysis by means of the normal deviate z.

TABLE 5 OBSERVED AND REPORTED SIGNS AND SYMPTOMS DURING WITHDRAWAL IN TREATMENT CENTER VS MODE OF USE

Sign/Symptom	Smoking N = 200 (%)	Sniffing N = 60 (%)	Injection (IV) N = 60 (%)
Rhinorrhea	56.5	65.0	73.3*
Lacrimation	53.0	60.0	68.3*
Diaphoresis	41.5	40.0	61.7+
Piloerection	42.0	40.0	63.3+
Chilliness	52.0	58.3	70.0*
Hot/cold flashes	45.0	48.3	56.7
Muscle cramps	25.0	35.0	46.7*
Back/leg/joint pains	63.5	73.3	90.0+
Muscle twitching/tremor	26.5	30.0	40.0*
Restlessness	84.0	85.0	96.7*
Double/blurred vision	19.0	10.0	18.3
Headaches	32.0	28.3	31.7
Insomnia	74.5	71.7	81.7
Abdominal cramps	60.5	58.3	93.3+
Nausea	32.5	41.7	55.0*
Vomiting	16.5	25.0	45.0+
Diarrhea	37.0	46.7	48.3
No signs or symptoms	6.0	0.0	0.0

* Significantly greater than smokers of heroin ($p < 0.05$) Analysis by means of the normal deviate z.

+ Significantly greater than smokers and sniffers of heroin ($p < 0.05$) Analysis by means of the normal deviate z.

TABLE 6 SUMMARY OF HEROIN WITHDRAWAL SYNDROME INTENSITY AND TREATMENT vs MODE OF USE

Item	Smoking N = 200 (%)	Sniffing N = 60 (%)	Injection (IV) N = 60 (%)
Estimated degree of withdrawal intensity			
None	6.0	0.0	0.0
Mild	45.0	38.4	8.3
Moderate	42.5	45.0	46.6
Marked	6.5	16.6	45.0
Medication for withdrawal			
None	28.5	16.6	1.6
Non-Narcotic drug(s)	68.5	80.1	68.3
Methadone + non-narcotic drug(s)	3.0	3.3	30.0

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task ØØ Biomedical Factors in Drug Abuse

Work Unit 1Ø6 Clinical and demographic studies of military drug abusers

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6505	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	109
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Neurophysiological Localization of Sites of Action of Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 75	2	121
FISCAL YEAR CURRENT 76	2	71

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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20. PERFORMING ORGANIZATION

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TELEPHONE: 202-576-3457
SOCIAL SECURITY ACCOUNT NUMBER [redacted]

ASSOCIATE INVESTIGATORS
NAME: Koob, G. F., CPT DA
NAME: Martin, G. E., Ph.D.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Heroin; (U) Temperature Regulation; (U) Feeding Behavior; (U) Intracranial Self-Stimulation; (U) Catecholamines; (U) Morphine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Punch individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This research attempts to identify areas of the central nervous system which are most affected by "drugs of abuse". These physiological studies may provide a basic understanding of the mechanisms of drug-induced alterations in the functioning of the nervous system and eventually lead to effective treatment of military personnel using and misusing drugs.

24. (U) The techniques of neurophysiology, neuropharmacology and physiological psychology are used.

25. (U) 74 07 - 75 06 Following last years study of the possible site of action of heroin and morphine in the central nervous system, a more detailed analysis was undertaken of areas in the hypothalamus showing differential effects on heroin administration. Animals receiving dorsal hypothalamic stimulation showed a large facilitatory effect to heroin, whereas animals with ventral hypothalamic stimulation exhibited no facilitatory effect. Another drug acting upon catecholamine metabolism was studied. Clonidine, an alpha-adrenergic agonist, has marked effects upon the cardiovascular system, temperature regulation, feeding, drinking and intracranial self-stimulation (ICSS). These effects were dose dependant: high doses decrease ICSS, feeding and body temperature; low doses increase ICSS, feeding and temperature. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 109 Neurophysiological localization of sites of action of drugs of abuse

Investigators.
Principal: N. H. Spector, Ph.D.
Associate: CPT George F. Koob, MSC; Gregory E. Martin, Ph.D.

DESCRIPTION.

This research has continued to pursue the objective of neurophysiological localization of sites of action of drugs of abuse using a behavioral approach. In this approach, internal neurochemical and neurophysiological changes are assessed using the technique of intracranial self-stimulation (ICSS).

PROGRESS.

Effects of Drugs of Abuse on Intracranial Self-Stimulation: Pharmacological Studies of Neurochemical Substrates.

Intracranial Self-Stimulation, Food and Water Intake, and Catecholamines.

Heroin acts in one respect like amphetamine in facilitating intracranial self-stimulation (Koob, Spector, Meyerhoff, 1975). The action of drugs on reinforcing brain stimulation has been extensively studied and has led to the catecholamine hypothesis of reinforcement (German and Bowden, 1974). Drugs which influence catecholaminergic metabolism have the largest and most selective effects on intracranial self-stimulation (ICSS). In an attempt to delineate further the specific catecholamine involved in ICSS, clonidine, (Starke and Montrel, 1975), a potent α-adrenergic receptor stimulant, was administered to rats maintained in a special chronic preparation. The identification of a neurochemical substrate for reward could have important implications for the hypothesized role of these pathways in the pathophysiology of the abuse of amphetamines and opiates.

Male adult rats with electrodes chronically implanted in the posterior hypothalamus were placed in experimental chambers with access to three levers, one of which activated a brain stimulation circuit from a constant current source, the other two delivered a 90 mg food pellet or a 0.05 ml drop of water. The rats were allowed continuous

access to all levers 24 hours per day on a continuous reinforcement schedule. Stable baseline rates of ICSS were established prior to the intraperitoneal (i.p.) injection of saline or clonidine HCl, at doses of 12.5, 25.0, 50, 100, 200 and 400 μg/kg. During the six hours following the injection, ICSS increased significantly following doses of 12.5, 25.0, and 50.0 μg/kg, with a maximum increase of 82% at 25 μg/kg. At 400 μg/kg, however, ICSS was significantly decreased by 54%. Food and water intake showed maximum increases of 116% and 80%, respectively at 50 μg/kg and maximum decreases of 57% and 91% respectively at 400 μg/kg. At 200 and 400 μg/kg, the decrease in ICSS and in food intake were reflected in the 24 hour totals, while the acute (6 hr) decrease in water intake was not. Following the highest dose, the animals appeared lethargic. The data indicate that the dose-response curve for clonidine is biphasic for these behavioral parameters and that clonidine in excess of 100 μg/kg may produce physiological effects which interfere with performance of a behavioral task. Since these behavioral functions are modified by a noradrenergic stimulant, clonidine, these data support the view that the noradrenergic pathways in the hypothalamus play a role in feeding, drinking and ICSS.

Autonomic Efferents: Temperature Regulation and Clonidine.

When administered peripherally, clonidine exerts an antihypertensive effect via alpha-adrenergic stimulation of the central nervous system (Anden, et al, 1970; Schmitt and Schmitt, 1969; Sherman, et al, 1968). In conjunction with a study of this drug's effect on several behavioral tasks, including feeding, drinking, and responding for intracranial self-stimulation, we examined its action on core temperature in the rat.

Seven male albino rats of the Walter Reed strain weighing between 300-450 gm were used. Each rat was housed individually and maintained on a 12-hour light-dark cycle. Body temperature was measured either by a thermistor inserted 6-8 cm into the rectum or by a chronically indwelling thermistor bead placed surgically in the peritoneal cavity.

A stable baseline temperature was obtained for at least 2 hours before clonidine hydrochloride was administered intraperitoneally in a dose of 12.5, 25, 50, 100, 200, or 400 μg/kg. Previously, a hypothermic response has been reported after the administration of this drug to the anesthetized rat following doses much greater (1-5 mg/kg) than used here (Laverty and Taylor, 1969). In the present experiments, each rat was unanesthetized and free moving. Furthermore, ad lib food and water intake were observed following drug treatment.

At the three highest doses, 100, 200, or 400 μg/kg, administration of clonidine was followed by a mean drop in core temperature of 3.0°,

2.6°, and 3.4°C respectively. However, at two lower doses, 25 and 50 μg/kg, the mean hypothermic response induced by clonidine (-1.7, -1.0°C respectively) was offset by a rise in body temperature in the experiments in which ingestive behavior was also elicited by the compound. As mentioned above, clonidine in a dose of 25 or 50 μg/kg, elicits food ingestion when administered to the rat. When the rat was observed to consume food in these experiments, its temperature rose, often offsetting the fall in temperature observed in the rats given the same drug that did not feed. We have observed that control animals' body temperature rose 0.6 to 2.2°C after they ingested food. This rise has been attributed either to the increased motor activity involved in ingestion, or to the "specific dynamic action" of the food or to both (Strang and McCluggage, 1939; Cormarèche and Spector, 1972). At the 12.5 μg dose a mean net increase in core temperature of only 0.53°C was observed.

These results demonstrate the importance of observing behavioral as well as physiological responses in attempting to evaluate the action of a drug. In addition, clonidine is shown to be a potent agent in inducing hypothermia, but the importance of the dose level chosen to achieve this effect must be emphasized.

The results indicate that clonidine exerts a dose-dependent effect on the rat's body temperature. We are conducting further studies to determine whether this effect is due to the peripheral vasomotor effects of the drug, or to a central adrenergic action on temperature-regulating brain cells. This is the first time that clonidine has been shown to alter core temperature at these dose levels in the unanesthetized rat.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 109 Neurophysiological localization of sites of action of drugs of abuse

Literature Cited.

References:

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Publications:

1. Annau, A., Heffner, R. and Koob, G. F.: Electrical self-stimulation of single and multiple loci: long-term observations. Physiol. Behav. 13: 281-290, 1974.

2. Koob, G. F. and Annau, Z.: Behavioral and neurochemical alterations induced by hypoxia in rats. Amer. J. Physiol. 227: 73-78, 1974.

Other relevant publications listed in report of Department of Neuroendocrinology.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6507	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. ORGN NUMBER	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D.Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	110
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biorhythm Studies in Drug Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	4	240
	CURRENT 76	4	279

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

20. PERFORMING ORGANIZATION

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22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Drug Abuse; (U) Biorhythms; (U) Heroin; (U) Abstinence Syndrome

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Achievement of an understanding of changes in the temporal organization of biological functions attendent upon sustained use of drugs of abuse and upon cessation of drug use in military personnel. Information developed serves to explicate mechanisms of drug action, biomedical consequences of sustained abuse, and post-detoxification readdiction liability. Therapeutic implications are explored.

24. (U) Sophisticated electronic monitoring and bioproduct sampling techniques are employed to generate long, nearly continuous electrophysiological, behavioral, and biochemical measures of biologic functioning during periods of sustained drug use and abstinence. Time series analysis techniques are applied to these data to achieve a full characterization of similarities and differences between normal and drug abusing individuals.

25. (U) 74 07 - 75 06 Electrophysiological, biochemical and behavioral data were obtained from heroin users undergoing complete abstinence and from normal control subjects. A metric to scale the abstinence induced perturbations from normality has been developed and applied to these measures. Abnormalities in biorhythmic functions relating to sleep, activity, and endocrinology are being delineated. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 74 - 30 JUN 75.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task ØØ Biomedical Factors in Drug Abuse

Work Unit 11Ø Biorhythm studies in drug abuse

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Associate: LTC Norman W. Ream, MC; LTC Albert J. Tahmoush, MC; CPT John G. Varni, MSC; Paul Kasper, B.S.; Jeanne C. Stringfellow, B.A.

Description

This work unit is directed at the understanding of changes in the temporal organization of biological functions attendant upon sustained use of drugs of abuse. To date, principal emphasis has been focused on the sequelae of heroin abstinence. The information developed serves to explicate mechanisms of drug action, to delineate the functional consequences of sustained abuse, and to assist in the assessment of post-detoxification readdiction liability. The technology employed involves a variety of techniques for continuously monitoring electrophysiological variables and for sampling behaviors, clinical parameters, and biological fluids. Data are analyzed using a variety of time series statistical procedures.

Progress

1. A Scaling Metric for Assessing Heroin Abstinence

During the past forty years, a number of attempts have been made to develop a metric for scaling the severity of opioid abstinence syndromes (1,2,3,4,5). All are derivative from the pioneering work of Kolb and Himmelsbach (1) and all share the same characteristics and weaknesses. Varying concatenations of signs and symptoms are selected as elements of the scale and each is assigned a weight in accordance with the importance placed upon the element by the originator of the scale. The assigned score values are then summed to derive a single number that is assumed to stand in some direct relationship with abstinence severity.

The weaknesses of these scales are numerous and perhaps fatal in terms of their utility for scientific work. The selection of scale elements and the weights assigned to each are arbitrary in the sense that they depend upon the clinical judgment of the scale's originator. The level of measurement represented by these scales is ordinal, at best. That is to say, one can make responsible statements at the level of more than or less than, but not how much more or less.

As a result of these weaknesses, scales composed of differing elements cannot be compared, one with another, nor can meaningful statistical analyses be performed to assess the significance of observed differences. The approach taken by this laboratory has been to develop a general model for scaling abstinence severity that is free of the weaknesses noted above. This scale is derived from work done by Sacco, Goldfarb, Cowley and Copes in the area of developing quantitative prognoses for shock and trauma patients (6).

The scale is a varietal form of an n-dimensional Euclidean distance metric. This metric is an extension of the familiar Pythagorean theorem of geometry that, in the present model, may be written:

$$d_n = (Z_1^2 + \ldots + Z_n^2)^{\frac{1}{2}} \qquad (a)$$

where d_n is the distance of a point from its origin in an n-dimensional space

Z_n is a transformed value of a sign or symptom chosen for inclusion in the model

Inspection of equation (a) indicates that the value of d_n will be a function not only of the values of Z_n but of the number of symptoms, or dimensions, included in the model.

Dependence of d_n on the number of dimensions included in the model is not desirable since it prevents the direct comparison of models having different dimensionalities. For example, we might wish to compare a model based on cortisol and growth hormone response to abstinence (two dimensions) with one based on systolic blood pressure, oral temperature, and respiration rate (three dimensions). The limitation can be sidestepped by rewriting equation (a) in the following way:

$$d = \left(\frac{Z_1^2 + \ldots + Z_n^2}{n} \right)^{\frac{1}{2}} \qquad (b)$$

The weighting of the sum of the squared Z values by n greatly reduces the dependence of the distance score (d) on the dimensionality of the model.

As indicated above, the values of the signs and symptoms included in the model are transformed. This is done in order to impart certain desirable statistical characteristics to the model and to eliminate problems associated with combining numerical values that might differ greatly in magnitude. For example, a hormone value might be expressed in nanograms/milliliter while a body weight change is expressed in kilograms. In addition, the transformation used converts the value

of scale elements into ratios, i.e., dimensionless quantities, which are necessary if one wishes to combine dimensionally disparate elements like heart rate and hormone secretion.

The transformation employed is the well known standard score of psychometrics. It is defined as:

$$Z = \frac{X - \overline{X}_c}{s_c} \qquad (c)$$

where Z is the standard score

X is the score to be transformed

$\overline{X}_c$ is the mean value of a comparison, or standardization group

s_c is the standard deviation of a comparison, or standardization group.

When a set of scores are thus transformed, the mean of the distribution of transformed scores is equal to zero (0) and the standard distribution is equal to one (1). Since score values are given in terms of standard deviation units, given score values can be related to the normal probability distribution to assess the probability of occurrence of any score.

The expected value of a squared standard score is one (1). Therefore, the expected value for a distance score calculated according to equation b is also one (1). The magnitude of the distance score (d) is directly related to the divergence of the constituent transformed scores from the standardization, or comparison group. Further, if we make the statistically conservative assumption that all of the constituent elements of a distance score are dependent, then the distance scores will be distributed as Chi Square with one (1) degree of freedom. A more liberal assumption would be that all of the constituent elements of a distance score are statistically independent, one from another. In this case, the distance scores would be distributed as the statistic F with n and ∞ degrees of freedom.

The distributional characteristics of the distance score permit the user to conclude reasonably that a given score is not significantly different from the comparison group if the value falls below that given by the F distribution for the appropriate number of degrees of freedom. Similarly, if the distance score exceeds the appropriate Chi Square value, one has reasonable assurance that it is significantly different from the comparison group at the chosen level of

confidence. The region of distance values lying between the required values of Chi Square and F are less amenable to clear interpretation.

The scaling model described above has been applied to data derived from the study of heroin abstinence conducted in the Republic of Viet Nam (7). In the material that follows, no attempt was made to derive a single distance model to describe the abstinence syndrome. Instead, pragmatic criteria were employed to group measures into scales having heuristic utility, e.g., readily measured clinical signs, plasma hormones, performance measures, etc. In each case, the comparison group is provided by our control subjects (n=5) who shared the research ward with the patients. When measures were taken several times each day, time of day has been taken into account in establishing the normative means and standard deviations as a control for biorhythmic variations.

In our preceding Progress Report (8), an analysis of opiate abstinence signs and symptoms scored according to the Himmelsbach system suggested that acute abstinence was not a unitary phenomenon. Rather, it appeared that certain signs or perturbations appeared early in abstinence (day 1) while others appeared later (day 3-4). Three different scales employing the distance model are presented in Figure 1. The first, having as constituents systolic blood presure, diastolic blood pressure, oral temperature, pulse rate, and respiration rate, shows the bimodality described above. The second scale is composed of the plasma hormones testosterone, cortisol, growth hormone, leutinizing hormone, and insulin. The distance scores for this scale exhibit a single peak at 2200 of the first full day of abstinence that coincides with the early peak in the scale for vital signs. The third scale is composed of performance measures. These measures have shown no significant difference from controls in any statistical analysis performed to date. When used in the distance model, no significant trends emerge.

Earlier, we reported that the observed and reported signs and symptoms of withdrawal in the patient group reached peak frequency at 2200 hours on the first full day of abstinence (9). It seems unlikely that the congruence between vital signs, plasma hormones, and clinical signs of acute opiate abstinence is fortuitous. As mentioned earlier, the constituents of the distance model can be altered without changing the fundamental statistical properties of the scale. This has been done with the vital signs scale in an attempt to parcel out the early and late appearing peaks.

Separate distance scores were computed for the clinical parameters of systolic blood pressure and heart rate as well as the respiratory rate and body temperature. The resulting functions of each of these two sets of measures are shown in Figure 2. It is immediately apparent that the early peak in the vital signs scale (Fig. 1) is a function of perturbations in systolic blood pressure and heart rate. The late

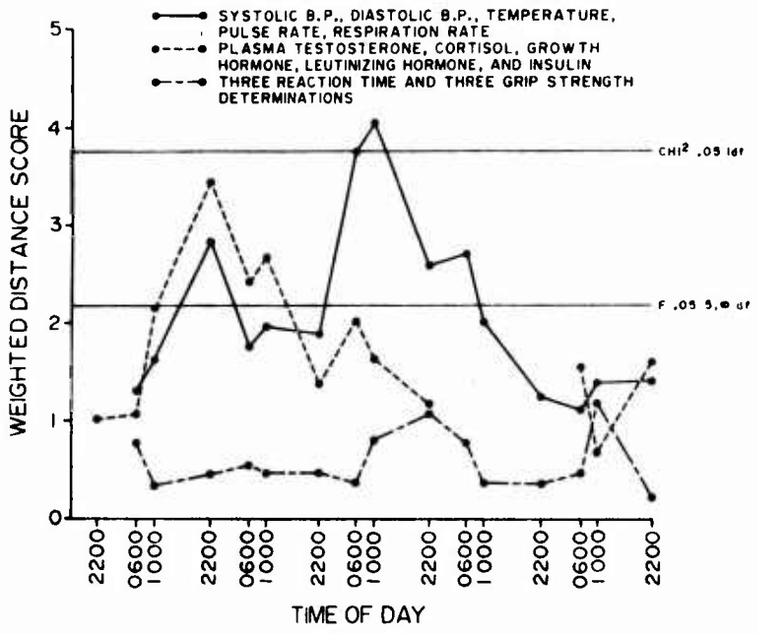

Figure 1. Weighted distance scores for vital signs, plasma hormones and performance. Scores are computed over the first five days of abstinence for ten heroin users.

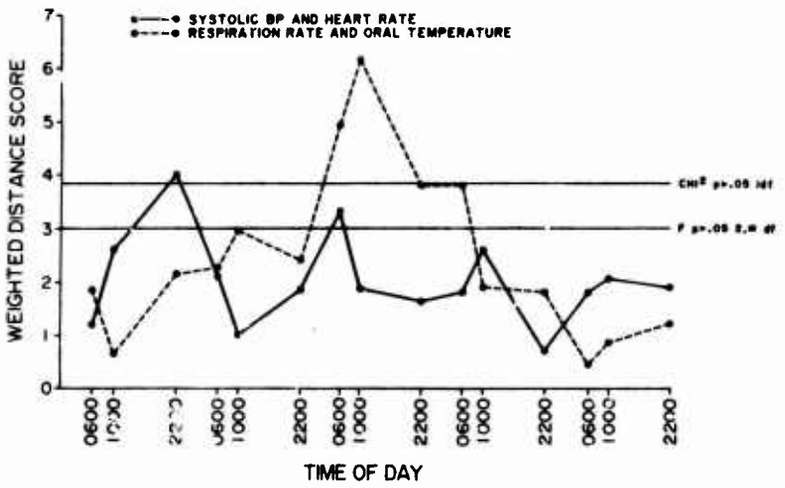

Figure 2. Weighted distance scores for systolic blood pressure and heart rate as well as oral temperature and respiration rate. Scores are computed over the first five days of abstinence for ten heroin users.

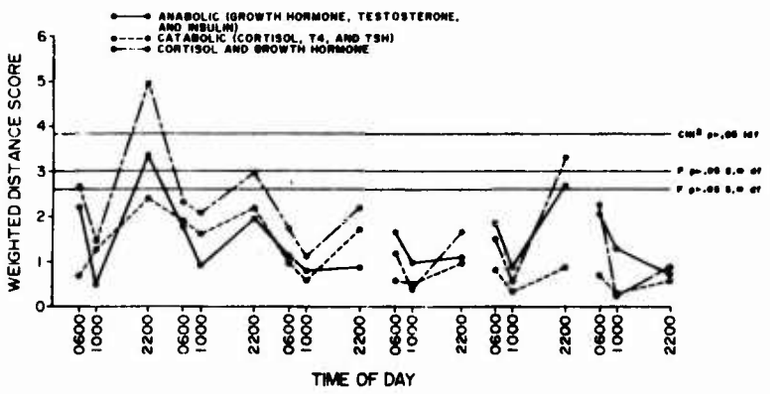

Figure 3. Weighted distance scores for anabolic and catabolic plasma hormones as well as for cortisol and growth hormone considered separately. Scores are computed over days 1, 2, 3, 5, 7, & 9 for ten heroin users.

peak is almost entirely a function of changes in body temperature and respiratory rate.

The plasma hormone distance scale was similarly partitioned into subscales. The criterion employed was the general classification of each of the hormones into anabolic or catabolic mode of action. These data are presented in Figure 3. Initially, only the anabolic scale showed an early peak that reached statistical significant using the liberal F criterion. Examination of the constituents of both the anabolic and catabolic scales indicated that two hormones generally implicated in the endocrinological response to stress contributed most of the variance in each scale. Cortisol and growth hormone were then combined in the third function shown in Figure 3. The sharpening of the peak score at 2200 on day 1 is readily apparent.

These data illustrate the utility of an appropriate scaling model. The present model permits one to make comparisons between physiologically diverse functions in a quantitatively meaningful way. The observation that the easily obtained cardiovascular vital signs covary with the difficult to obtain plasma hormones suggest a redundancy in information that will be potentially useful in constructing a simplified model for clinical application.

The scales reported here have all been constructed on the basis of grouped data. Since the model is equally applicable to individual patients, analyses of individual data are currently underway. These will be used to examine such factors as route of administration, amount of last dose, time since last dose, and length of addiction.

An as yet untested, but potentially powerful aspect of this model of abstinence scaling is its applicability to animal models of addiction. Cross species comparisons present many difficulties, not the least of which arise from differences in basal levels of physiological parameters. Since the model presented here has its conceptual basis in the evaluation of perturbations from normal function, many of these difficulties may be avoidable.

2. Ultradian Variations in Frequency of Eyemovements During Abstinence from Heroin

The existence of rhythmic variations having a period range of fifty to one-hundred minutes is now well established for a several performance and physiological measures (10). However, unlike circadian rhythms which exhibit considerable stability in period while undergoing variation in amplitude, both cycle parameters appear to vary when periodic activity in the ultradian range is examined. The lability of activity in the ultradian band presents two related problems to investigators. First, it must be determined whether the observed period and amplitude variations represent orderly processes, or whether they are more correctly characterized as manifestations of random processes, i.e., noise.

If it is determined that the former is the case, then it must be determined what factors contribute to, or control the period and amplitude variations. The determination of whether we are dealing with real phenomena has, in the past, been constrained by technical limitation in available techniques for time series analysis. For example, spectrum analysis techniques depend upon stability in essential statistical parameters of the time series to produce valid results. Yet, the biological series of interest not only do not exhibit the necessary stability except under unusual conditions, but the instability is precisely the focus of concern and investigation.

The approach to these questions utilized by this laboratory involves a computer-generated filtering procedure known as complex demodulation (11). This technique permits the examination of both the amplitude and phase of a signal located in a bounded area of the frequency spectrum. Both the location and the width of the area are selectable. An important characteristic of this filter is the availability of phase information. If a noise signal is passed through a simple bandpass filter, the output will be a periodic signal whose amplitude is related to the amount of energy in the noise signal that lies within the range of the filter. Simple examination of the output will not permit one to discriminate whether the input signal was composed of noise alone, or signal plus noise. Phase in a noise signal is a random variable. Thus if the phase output of the complex demodulation filter is orderly, one may conclude with some confidence that one is dealing with signal.

The complex demodulation technique has been applied to samples of gross eyemovement data gathered from men undergoing abstinence from heroin in the Republic of Viet Nam. Lateral movement electrooculograms were telemetered continuously from these patients and from a control group for periods ranging up to seven days. The electrooculograms were converted to measures of the frequency of large eyemovements per minute by a simple pattern recognition circuit based on amplitude and duration criteria. Eyemovements under twenty degrees in excusion, e.g., saccades, rapid eyemovements in sleep (REM), and reading movements, were thereby excluded from this analysis.

Earlier work in this laboratory had concentrated attention to a period band centered on ninety minutes, a value derived by extrapolation from the lseep literature dealing with periodicities in REM sleep. However, a preliminary analysis of the eyemovement data suggested that periodicities of much shorter duration were present. Since the complex demodulation technique operates at only one frequency for a single pass over the data, the filtering operation was repeated at each of eleven periods ranging from fifty to one-hundred minutes, in five minute period increments. The technique employed is reminiscent of the earliest days of spectral analysis when a loop of magnetic tape would be played repeatedly and the tuning of an analog filter would be changed between runs through the loop.

Spectrum estimates for each ten minute data epoch were then constructed by combining filter output values for each of the eleven filter passes. In Figure 4, spectrum estimates for the first three abstinence days for one subject are presented. The first spectrum for each day is plotted at 0010 hours and succeeding estimates are plotted in sequence until 2400 hours is reached. Noon, or 1200 hours appears in the middle of the plot for each day.

Little energy appears anywhere in the spectra for Day 1 of abstinence until late evening. By 2400 hours, there is a well defined primary peak at 85 minutes with subsidiary activity at 70 minutes. The activity at 80 minutes continues to build during Day 2, reaching a peak at approximately 1800 hours. There is a rapid decline in activity in this portion of the spectrum during the remainder of the evening. On the other hand, the activity at 70 minutes grows throughout Day 2 accompanied by the development of a subsidiary peak at 60 minutes. During the early morning hours of Day 3, activity at 70 minutes declines and reaches a low level of 0600 hours. The activity at 60 minutes does not reach similar low levels until early in the afternoon of Day 3.

These data are presented as illustrations of one form of output provided by this analysis. The analyses of all patients and controls are not yet complete, a fact largely attributable to technical questions raised by the results generated by the first five subjects. It is not clear, for example, how a double peak like the one that is so apparent at 0010 hours on Day 3 is to be interpreted. Where these multiple peaks have been observed, no simple harmonic relationships have been apparent. Two approaches to increasing our understanding of this and similar questions are underway.

First, complex signals of known frequency and phase composition are being constructed and subjected to the same analysis as the eyemovement time series. The second approach involves an attempt to reconstitute the orginal time series from the outputs of the eleven filter passes. Since the variance of the original series is determinable, it is possible to determine what portion of that variance is accounted for by each of the eleven demodulation periods. An analysis of variance model will be employed to determine the statistical significiance of activity in the various period bands.

For each of the five subjects analyzed to date, the energy in the spectra has been integrated across the eleven periods to arrive at an estimate of the overall level of activity in the ultradian band. Three of the subjects were patients and all showed higher levels of integrated eyemovement activity than did the two controls. There is also a clear tendency for activity to peak during Day 2 and again on Day 4 or 5. The patients also show higher levels of activity at periods shorter than 75 minutes while activity for the controls is concentrated at the slower end of the spectrum.

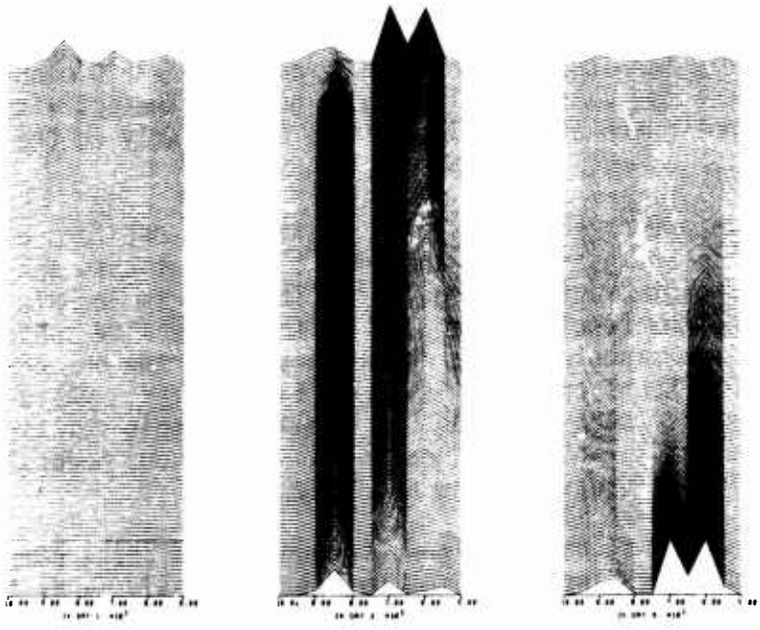

Figure 4. Estimated sequential period spectra for frequency of large eyemovements. Data are those of one heroin user during the first three days of abstinence. Each plot begins at 0000 hours just above the abscissa and ends at 2350 hours at the top of the plot.

Although these interpretations are tentative at this writing, they are consistent with the hypothesis that both the amplitude and the frequency of ultradian rhythms are a direct function of the level or arousal, or stress, experienced by an individual. The hypothesis will be subject to more rigorous testing when direct comparisons are possible between individual abstinence severity scales, and the ultradian analyses of heartrate, eyemovements, and general activity.

3. The Measure of General Activity

During the period when all subjects are undergoing electrophysiological monitoring in the research ward, their behavior was logged on individual subject record sheets. Log entries were made whenever ongoing behavior changed. A simplified activity scale has been developed in order to quantify certain aspects of the behavior of patients and controls. The behavior was divided into five categories and each category was assigned a point value depending upon the level of activity represented by each category. The point values and associated categories were:

0 In bed, obviously asleep
1 In bed, supine and awake
2 Sitting down
3 Standing up, in place or moving
4 Agitated

The point values were assigned on a minute by minute basis and then totalled to provide a ten-minute data epoch to match the eyemovement and heartrate data. All scoring was done by a single investigator with random blind replications by a second investigator to provide a consistency check. In those rare instances where scoring problems arose, the principle investigator was consulted. For any given data epoch, the possible score values ranged from zero to forty.

The simplicity of this scoring system is at once a strength and a weakness. The scoring, while tedious, was unambiguous and accomplished in a comparatively expeditious manner. As a metric, the scoring system is clearly ordinal in nature, i.e., the distance between a score of one and two is not the same as between scores of two and three, nor is it likely that a score of four is in any meaningful sense twice the magnitude of a score of two. However, the scoring of activity is a notoriously ambiguous business and it is highly unlikely that a more elaborate procedure would have produced tangible benefits.

The data for all ten patients and five controls have been entered into computer files and plotted. Preliminary descriptive statistics have been computed and will be reported along with those features of the plotted data that appear significant. Between 2200 hours and midnight of the day of admission to the research ward, the control group had a higher mean activity than the patient group.

Over the next five days, the mean daily activity of the patients exceeds that of the controls with a peak in patient activity appearing on Day 2 of abstinence.

The daily activity pattern of the control group manifests marked consistency. There is a smooth decline in activity after the 2200 hour examination that reaches a nadir between 0300 and 0500 hours. On four of the six evenings examined, the lowest mean activity score attained was zero. The 0600 examination was accompanied by a rapid rise in activity that was immediately followed by a sizeable decrease until the 1000 hour examination. On four of the five days during which the 0600 and 1000 hour decrease could be examined, it fell to a level between "in bed asleep" and "in bed, supine, awake". A third decrease in activity followed the noon meal and lasted until approximately 1600 hours. The post-prandial activity depression yielded scores between "in bed, supine, awake" and "sitting up". The highest levels of daily activity were to be found between 1600 and 2200 hours when the scores ranged between "sitting" and "standing".

The patients never achieved the low night time scores reached by the controls. The maximum night time activity occurred early in the morning on Day 2. There were successive decreases in activity between 2200 and 0600 hours over the next three days. By Day 6, the activity of patients and controls was virtually identical. The decrease in activity following the 0600 examination exhibited by the controls also occurred in the patients. However, the decrease was markedly attenuated on Days 2, 3, and 4. There was no post-prandial decrease in activity in the patients such as that observed in the controls. During the period of peak activity for the controls, scores of the patients are, on the average, slightly higher than those of the controls.

In summary, the nocturnal sleep of the patients is disrupted with maximum disruption occuring early on the second day of abstinence. The patients also exhibit a lessened ability to return to rest after being aroused for an early morning examination. The absence of post-prandial rest completes the picture of generally higher states of arousal in the patients over the entire period of observation. The highest levels of arousal occur late in the evening on Day 1 and early in the morning on Day 2. These data are fully consistent with the application of the distance metric to the endocrinological and cardiovascular data cited earlier.

4. Respiration

During the investigations of heroin withdrawal in Viet Nam, respiration was assessed along with other physiological parameters. Specifically, a single pair of skin electrodes placed over the cardiac apex and gastric antral area were used to record EKG, electrogastrogram and rheopneumogram (RPG). Because this monitoring mode was innovative, a means of standardization had to be devised.

Efforts have been made to compare the resulting, filtered RPG with more traditional measures of respiratory activity. To this end simultaneous recordings were made of the RPG and a signal derived from a thermistor mounted within the mouthpiece of a Collins respirometer. The recordings were made during increasing levels of exercise, with the metabolic costs of the exercise being monitored by determinations of pO_2 and pCO_2. Two basic relationships were suggested. First, the RPG and thermistor measurement provided almost identical respiratory rate information when analyzed by a zero-crossing technique. Both rate measurements increased monotonically with the increasing metabolic cost. Secondly, when both the RPG and thermistor measurements were subjected to spectral analysis, a similar monotonic trend was observed until the metabolic cost approach the highest levels. In this region, the apparent rates diverged, with the RPG's primary spectral component shifting toward a lower frequency.

While this change suggests caution with respect to the application of spectral analysis to respiratory measurements, it indicates that the RPG may be responding to an increasingly complex respiratory pattern of varying depth, to which the thermistor is not sensitive. Use of a narrow-band filter on the RPG has produced a better agreement between the two measures, as would be expected if the above interpretation were correct. Investigation is in progress to determine if such waveforms might be due to differences between abdominal and thoracic components of respiration.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task ØØ Biomedical Factors in Drug Abuse

Work Unit 11Ø Biorhythm studies in drug abuse

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6509	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. / CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	111
b. CONTRIBUTING				
xxxxxxxxxx	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Neuroendocrinology in Drug Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

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e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 75	1.5	105
FISCAL YEAR CURRENT 76	4	380

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Drug Abuse; (U) Psychophysiology; (U) Treatment/Rehabilitation; (U) Neuroendocrinology; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23. (U) Principal objective is to establish the endocrinological consequences of the abuse of psychoactive compounds, including such phenomena as tolerance and withdrawal. The over-all objective is to better specify the impact of drug abuse on the integrated functions of the organism including military performance.

24. (U) Methods include measurements of urinary and plasma hormone levels in humans and non-human primates. Primate models are utilized to substantiate and interpret data obtained from humans and the techniques of experimental psychology are applied to produce critical features of the behavior of drug abuse.

25. (U) 74 07 - 75 06. Initial efforts have focused on documenting the pattern of endocrinological anomalies associated with heroin withdrawal in soldiers which, because of age, health and route of administration, may represent a unique population. It appears that the most striking features of the early withdrawal period are suppressions of cortisol and testosterone and elevation of thyroxine following by a gradual recovery to basal levels. In FY 76 research carried on previously under Project Number 3A762758A833, Work Unit 112, will be reported on Work Unit 111. For a technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 74-30 Jun 75.

*Available to contractors upon originator's approval

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PII Redacted

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 111 Neuroendocrinology in drug abuse

Investigators.
Principal: James L. Meyerhoff, M.D.
Associate: Frank J. Sodetz, MAJ; and John W. Mason, M.D.

Description.

The aim of this work unit is to explore the possibility that relatively distinctive profiles of neuroendocrine responses might be related to various aspects of drug use, with the view that such information, taken together with related work on psychological, neuroanatomical, neurochemical, and autonomic aspects of drug abuse, might eventually facilitate our understanding of the central nervous system mechanisms and perhaps some of the clinical symptomatology involved in drug abuse.

Progress.

1. Organization of Neuroendocrine Responses During Heroin Withdrawal in Human Subjects: All hormonal analyses are now completed in this study of two groups of young men hospitalized during a two-week period following heroin withdrawal in Vietnam. The profile of hormonal changes during the withdrawal period are remarkably similar for the two groups. Following brief, initial suppression, plasma and urinary corticosteroid levels rise substantially to a peak on the second day, while norepinephrine levels are low initially and rise towards normal very gradually. Plasma thyroxine levels are elevated initially and then gradually decline. Plasma testosterone, LH, and insulin levels are low initially and then rise and stabilize within the first few days. Plasma growth hormone levels are high and labile initially, stabilizing by about the fifth day.

Conclusions about the directions of the hormonal changes during the acute phases of withdrawal, as described above, are generally supported both by the longitudinal study of the same individuals over two weeks to a period of clinical recovery and by comparison of withdrawal subjects with a group of control subjects. This rather unusual profile of hormonal responses during heroin withdrawal raises a number of questions regarding underlying mechanisms and possible correlations with clinical symptomatology which suggest the need for further systematic studies along these lines, particularly in laboratory primates.

Chronobiologic analyses of these data are presented in work unit 110, "Biorhythm Studies in Drug Abuse", WRAIR Annual Progress Report 1 Jul 74-30 Jun 75.

2. Organization of Neuroendocrine Responses to Heroin Administration in Monkeys: Pilot experiments have been conducted in 3 monkeys to determine the direction of acute hormonal responses to single injection (0.1 mg/kg) or 3 hour infusions (0.17 mg/kg/hr and 0.50 mg/kg/hr) of heroin through a chronic indwelling venous catheter. Preliminary findings indicate a gradual and marked suppression of plasma cortisol levels over a 3 hour period, followed by marked rebound elevations after cessation of drug administration. There are also rather clear preliminary indications of prolactin and growth hormone elevations during heroin administration. Less consistent are a tendency for total thyroxine to rise slightly and testosterone to rise slightly and then be suppressed the following day. These findings, while highly tentative at this point, suggest that acute heroin administration evokes a broad and unusual profile of neuroendocrine responses and that more conclusive studies along these lines should be conducted to aid in the interpretation of studies of chronic heroin administration and withdrawal and as a foundation for correlative studies comparing neuroendocrine reflections of heroin's action with behavioral, neurochemical, and neural aspects of the drug's action on the brain in a coordinated mechanistic approach.

3. Radioimmunoassay of Opiates in the Plasma of Baboons Self-Administering Heroin: One aspect of the research effort directed at the analysis of the contingencies underlying the acquisition and maintenance of heroin self-administration in baboons has been to document the validity of the baboon as a model for heroin abuse in Man from a physiological and endocrinological perspective. A second aspect of the present program was to provide plasma for the development of the analytical technology for documenting aspects of the pharmacodynamics of self-administered heroin. Both of the issues described above are, and have been, secondary to the analysis of behavioral variables. Therefore, the data obtained to date are incomplete in that samples can only be drawn under conditions which are consistent with the behavioral objectives of a given experiment. However, sufficient data have now been collected both from baboons and from humans undergoing withdrawal to warrant asking selected questions in a more systematic fashion. During the reporting period a radioimmunoassay for opiates was used to evaluate the relationship between heroin intake by baboons self-administering various doses of heroin intravenously. Plasma samples were drawn hourly for thirty consecutive hours from five different animals. The mean hourly rates of infusion in these animals were .053 mg/kg/hr, .151 mg/kg/hr, 1.51 mg/kg/hr, 1.84 mg/kg/hr and 5.1 mg/kg/hr. Generally, hourly variations in heroin intake were reflected in changes in RIA values, however, the phase relationships between these two measures were not perfect. Frequently, changes in RIA values lagged behind changes

in intake by one hour. What was perhaps the most interesting observation from these samples was the regularity of the relationship between overall mean hourly intake levels and overall mean hourly RIA values. In spite of daily variations in intake, hourly variations in intake, differing unit doses and differing mean hourly usage rates, for four of the five animals, mean hourly RIA values were a constant fraction of mean hourly heroin intake. The actual mean hourly RIA values were .35% (mean hourly intake of .152 mg/kg), .34% (mean hourly intake of .151 mg/kg), .30% (mean hourly intake of .053 mg/kg) and .30% (mean hourly intake of 5.1 mg/kg) of the mean hourly intake of heroin. One animal showed an unusually low RIA value in that it was only .14% of mean hourly intake. There are several possible reasons for this discrepancy. For one thing, this animal was a juvenile. For another, this animal, although exposed to our high dose of .5 mg/kg, never showed the long lasting depression of food intake seen in our other high dose animals suggesting that something was different about this animal. Another possibility was that, because the RIA data were collected on a single arbitrarily selected day, the values obtained for all animals were coincidental. In order to investigate this latter possibility a second thirty hour sampling run was completed, this time on the four animals remaining of the original group of five. The samples were collected two months after the original samples. However, during that period all of the animals had been switched to a higher unit dose of 10 mg of heroin/infusion. The new value obtained for the animal with the original RIA at .14% of mean hourly intake of 1.84 mg/kg/hr now was a mean hourly RIA of .17% of its new mean hourly intake of 1.08 mg/kg/hr. However, of the remaining three animals, two had RIA values of .33% and .31% of their new mean hourly intakes which were 1.00 and 1.04 mg/kg/hr. These data seem to indicate rather clearly that the RIA values are a constant fraction of mean hourly intake over a wide range of doses and that the appropriate value is about .33% of mean hourly intake. The one animal with a lower value of .14% for the original series of samples was also lower (.17%) on the second series at a different unit dose. This suggests that there was something consistently different about this animal. The one remaining animal was also lower (.21% versus .35%) on the second series. No data are available as to why this might be so. In three cases, it has been possible to collect samples from animals undergoing withdrawal. In all three cases there has been an elevation (secondary peak) in RIA values lasting several days. The peak has occurred anywhere from 12 to 20 days following the animal's last exposure to the drug. As yet, there can be no explanation of this phenomenon, although there may be something similar occurring in samples of urine taken from humans undergoing withdrawal.

4. Neuroendocrine Aspects of Heroin Self-Administration in Baboons: A number of endocrine measures are available from several different animals. As yet there are insufficient data from which to draw conclusions because most of the observations are unreplicated. The one observation which has been replicated in three animals is a marked suppression in plasma testosterone values during the initial 10 to 15 days of withdrawal. This suppression is unmistakable in that the values obtained probably represent only adrenal testosterone. Reductions from 1500 ng/% to 60-80 ng/% have been observed. Samples are now being processed for other hormones. The extent to which the endocrines of the baboon will approximate the picture in humans cannot yet be determined.

In FY 76 research carried on previously under Project Number 3A762758A833, Work Unit 112, will be reported on Work Unit 111.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6511	75 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORD'R INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	H.Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A76275A833	00	112
b. CONTRIBUTING				
[redacted]	Cards 114F			

11. TITLE (Precede with Security Classification Code)

(U) Neurochemistry of Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 012600 Pharmacology 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 07	75 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	PRECEDING/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	74	4	162
	75	3	160

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Drug Abuse, (U) Neurotransmitters, (U) Self-Stimulation, (U) Cyclic Nucleotides

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To examine the neurochemical mechanism of action and behavioral effects of drugs of abuse, and the neurochemical-neuroanatomical basis for the behavioral effects in a military environment. Drugs studies will include ethanol, hypnotics, opiates, and amphetamines.

24. (U) Systematic examination of contribution of noradrenergic, dopaminergic and serotonergic neural pathways to observed effects of drugs of abuse on intracranial self-stimulation (ICSS). Lesions will be made in cell bodies of origin of pathways and monoamine depletion in forebrain regions will be compared to change in behavioral response to drug. Levels of cyclic adenosine 3'5', monophosphate (cAMP), cyclic guanosine 3'5', monophosphate (cGMP), glutamic acid (GLU) and gamma aminobutyric acid (GABA) will be measured in regions of microwave irradiated brains following acute and chronic administration of and during withdrawal from drugs of abuse. A behavioral procedure which can induce ingestion of 11g/kg/day of ethanol will be used for studies of effect of chronic ingestion of these drugs. Effect of drugs will be studied on release of endogenous catecholamines in brain.

25. (U) 74 07 - 75 06 Two diencephalic loci have been identified which support ICSS but which yield different heroin-induced changes in ICSS. Lesions in locus coeruleus produce an 80 percent increase in ICSS. The microwave inactivation technique has been adapted to mouse brain. Feasibility was demonstrated of assaying glutamic acid in brain following microwave irradiation. Amphetamine produces a dose related release of endogenous dopamine from brain. This research will be continued under Project Number 3A76275A833, Work Unit 111 in succeeding years. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 74-30 Jun 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 112 Neurochemistry of drugs of abuse

Investigators.
Principal: James L. Meyerhoff, M.D.
Associate: G. Jean Balcom, Ph.D.; John W. Holaday, M.S.;
MAJ Robert H. Lenox, MC.

I. DRUGS OF ABUSE ON BRAIN CHEMISTRY AND ON BEHAVIOR

Studies are in progress on the mechanism of action of various drugs of abuse on brain chemistry and on behavior. These include the acute and chronic effects of opiates, stimulants, alcohol, and barbiturates on neurotransmitter chemicals in the brain. Research methods employed include neurochemistry, neuroanatomy, pharmacology, neuroendocrinology and physiological psychology. Specific approaches include:

1. Effects of drugs of abuse on intracranial self-stimulation: neuroanatomical and neurochemical substrate.
2. Effects of drugs of abuse on cyclic nucleotides and neurotransmitters as measured in brain regions following microwave fixation.
   a. acute drug administration.
   b. chronic drug administration.
   c. abrupt withdrawal following chronic administration.
3. Release of endogenous catecholamine from brain.

Effects of amphetamine and other drugs of abuse on intracranial self-stimulation: neuroanatomical and neurochemical substrates.

A facilitative effect of heroin on intracranial self-stimulation (ICSS) has been observed in this laboratory (Koob, Spector and Meyerhoff, 1975). Studies currently in progress are revealing two diencephalic loci which support ICSS but which respond to heroin in different manners: one facilitative and one not. Both types of response are reversed by naloxone pretreatment.

The relationship between intracranial self-stimulation (ICSS) and central brain catecholamine circuits has been intensively investigated in recent years in an attempt to identify the neuronal substrate of reinforcement. Data determining the role and importance of specific neurochemical systems in reinforcement will have important implications for the hypothesized role of these neurochemical

systems in various behavior disorders and drug abuse. The identification of ascending catecholamine pathways through regions previously associated with ICSS generated a new series of mapping studies that have implicated two specific systems as substrates for ICSS, the coeruleocortical norepinephrine pathway and the mesolimbic dopamine pathway. We have examined the effects of selective destruction of both of these systems on ICSS performance produced from posterior hypothalamic electrodes.

Male rats implanted with 3 monopolar electrodes aimed ipsilaterally at the locus coeruleus (LC), ventral medial tegmentum (VMT), and posterior hypothalamus (PH) were trained to lever-press for intracranial self-stimulation (ICSS) at each location. Ten rats were then allowed continuous access to ICSS at the PH electrode only, water and food via 3 levers. After 10 days the rats in the Lesion group (N=5) were subjected to a LC lesion under methoxyflurane anesthesia. Seven days after the LC lesion the rats were subjected to a VMT lesion. The rats in the Sham group (N=5) were subjected to only the anesthesia. The Lesion group had a significant increase in ICSS (24 hr total) when compared to the Sham group after the LC lesion, reaching 170% and 182% of pre-lesion rates on days 6 and 7, respectively. ICSS decreased to pre-lesion rates for 3 days after the VMT lesion. ICSS then returned to above pre-lesion rates. The Lesion group had an 80% and 65% decrease in norepinephrine in the cortex and hippocampus, respectively, on the lesion side when compared to the contralateral (unoperated) side. Dopamine levels decreased 71% and 62% in the olfactory tubercle and the nucleus accumbens, respectively, on the lesion side. The specificity of the behavioral changes was underscored by observing that food and water decreases occurred only during the first 24 hours following the lesion or sham procedure and then returned to control (pre-lesion) levels for the duration of the experiment. These results indicate that other hypothalamic pathways are involved in reinforcing brain stimulation and that the hypothesis suggesting the existence of a simple ascending catecholaminergic reward system should be modified.

The effects of amphetamines and other drugs on ICSS following such lesions are being evaluated to examine the role of such pathways in the drug-induced effects on ICSS. The quantitative assessment of catecholamine depletion in single brain regions of individual rats was possible through the implementation of a recently described assay (Coyle, 1973) of extremely high sensitivity (Koob, Balcom, and Meyerhoff, 1975).

Effects of drugs of abuse on cyclic nucleotides and neurotransmitters as measured in brain regions following microwave fixation.

A number of projects have been initiated to test the effects of drugs of abuse on neurotransmitters and cyclic nucleotides in specific brain regions. A method has been established which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5', monophosphate (cAMP), and cyclic guanosine 3'5' monophosphate (cGMP) in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. The assays employed are the radioimmunoassay of Steiner (1969, 1970) for cyclic nucleotides and the enzymatic method of Graham and Aprison (1966) for GABA and GLU. It is thought that cGMP is responsive to cholinergic transmission (Ferendelli, 1972) and under various conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine (Huang, 1972; Brown, 1972; Kebabian, 1972). Emphasis in the field is shifting to cAMP/cGMP ratios, and the capacity to study both is essential.

Work is proceeding in collaboration with Division of Biometrics and with NIH on development of a 4 parameter computer logistic model for analysis of radioimmunoassay, with a capability of pooling data over many assays. This is a research tool to assess the validity of various statistical models for radioimmunoassay analysis. GABA and GLU are considered to be, respectively, inhibitory and excitatory transmitters (Krnjevic, 1966). The tentative implication (Schumann, 1962) of GABA deficiency in convulsions suggests that it is an important variable to monitor in studies involving administration of or withdrawal from ethanol or barbiturates (Patel, 1973).

In conjunction with the foregoing, studies have demonstrated that the technique of using high-intensity microwave irradiation for enzyme inactivation as indispensable for determining levels of cAMP, cGMP and GABA in brain regions (Lenox, Meyerhoff and Wray, 1974); (Balcom, Lenox and Meyerhoff, 1974). The elimination of artifact has permitted the establishment of new levels of these substances in the regions studied previously. In addition, for many of the regions, the work is unique in that levels have never previously been reported. The use of the microwave fixation technique was evaluated for the measurement of glutamic acid in brain. The technique was found to be compatible with but not necessary for measurement of steady state levels. This will permit further progress toward the goal of the capability of assaying numerous putative neuroactive endogenous compounds from the same brain tissue sample. Glutamic acid levels were assayed in 15 brain

regions. The variation in levels between the regions was not marked - a notable difference between glutamate and other putative neurotransmitters. The regions studied include: cerebellum, brainstem midbrain, inferior colliculi, superior colliculi, substantia nigra, hypothalamus, thalamus, hippocampus, septal nuclei, olfactory tubercle, nucleus accumbens, corpus striatum, amygdala and cortex. A study has been completed on the effect of chronic barbiturate administration withdrawal on levels of glutamic acid in brain regions. Comparison was made with isocalorically maintained controls. No significant differences were observed.

Because of the essential nature of microwave irradiation as a methodological step in many current and planned neurochemical studies, considerable attention is being paid to describing the limitations of this technique and attempting to correct them (Lenox, 1974, 1975a, 1975b). During the reporting period, two significant technical improvements were made in the microwave in vivo enzyme inactivation system. A high power rotary waveguide switch was obtained and tested as a component to the system. This device permits oscilloscopic voltage-standing-wave-ratio tuning of the microwave field to every load in situ just seconds before application of high power irradiation. This results in a significant decrease in variability. A second improvement involves additional modifications which have permitted the successful application of the inactivation system to mice. This consists of placing the load at $\lambda g/2$ into the narrow face of WR340 waveguide modified appropriately to serve as an applicator. This technique has significantly improved uniformity of heating. On theoretical grounds it is thought that uniformity of heating in the rat brain may best be achieved by exposing at frequencies lower than 2450 MHz. Accordingly, an applicator and tuner has been designed and obtained which is optimal for exposures at 985 MHz. Testing of this modification is planned in the near future. Additional work involves the use of circular polarization. A study was completed assessing the temperatures in brains immediately following exposures of rats to microwave irradiation of intensity sufficient to inactivate enzymes in vivo. Techniques studied included exposure of head alone for time intervals of 1.0, 1.5, 2.0 and 2.5 seconds as well as whole-body exposures of 10, 15, 20 and 30 seconds. In each study, the time course of decrease of temperature was monitored. This work has been accomplished with the support and collaboration of the Department of Microwave Research.

Release of endogenous catecholamine from brain.

Previous studies on release of catecholamine from brain tissue have utilized radiolabelled catecholamine, and there was some question

as to whether these entered the endogenous releasable pool. We have successfully demonstrated the potassium-induced release of endogenous norepinephrine from brain. This release is enhanced several-fold by amphetamine. The release is responsive to calcium and temperature. We are currently studying the effect of amphetamine on release of endogenous dopamine from brain slices in vitro. The release is stimulated by amphetamine in a dose related manner. The use of millipore filters permits extremely rapid separation of tissue from incubation medium.

This research will be continued under Project Number 3A76275A833, Work Unit 111 in succeeding years.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 112 Neurochemistry of drugs of abuse

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Publications:

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2. Balcom, G.J., Lenox, R.L., and Meyerhoff, J.L.: Regional gamma-aminobutyric acid levels in rat brain determined after microwave fixation. J. Neurochem. 24:609-613, 1975.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6515	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	113
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with security Classification Code)

(U) Metabolism of Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	3	125
	CURRENT 76	3	127

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Drug Metabolism; (U) Drugs of Abuse; (U) Enzyme Induction; (U) Microsomal Enzymes

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to determine sites, modes and mechanisms of biotransformation of the principal drugs of abuse in man and animal and to develop a suitable animal model to study the influence of other drugs on the biotransformation of drugs of abuse in order to evaluate the impact of these interactions on drug abuse detection and on toxicity of drugs of military importance.

24. (U) Analytical and chromatographic methods will be used to study quantitatively and qualitatively the effects of environmental changes on the metabolism of drugs of abuse in intact animals and isolated organ preparations. Human body fluids will be studied for presence of drug metabolites and other substances which interfere with drug abuse detection methods.

25. (U) 74 07 - 75 06 Work continued on the study of drug treatment on levels of drug metabolizing enzymes and the characterization of hydrolase enzymes of various tissue sources. Assay methods are being developed to measure metabolites in tissues and in biological fluids. It was shown that rats on pyridoxine-deficient diets exhibit less severe withdrawal from morphine than do rats on normal diets. The implications of these findings relating to possible drug metabolism are being investigated. For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval.

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Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 113 Metabolism of drugs of abuse

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Associate: Clarence E. Emery, B.S.; SFC Lovelyn L. Hall; John A. Kintzios, B.S.; Meade R. Drumgold, B.S.; Nell R. Pendleton, B.S.; Ann R. Berman, B.S.; SP5 James P. McGrath, B.S.; Nesbitt D. Brown, B.S.; SP4 Edward J. Michalski.

Progress on this work unit was seriously handicapped by the loss of one principal investigator and one associate investigator who were not replaced because of manpower restrictions. Work was temporarily suspended in order to consolidate and reallocate personnel and resources. New protocols were prepared in order to accommodate and exploit existing expertise and to retrain associate investigators.

Work dealing with the metabolic and humoral responses to tolerance and dependence was suspended. Studies on the interaction of morphine and chloroquine were also suspended.

Work during the latter part of this year was concentrated on the evaluation and standardization of assay methodology for reliable identification and quantification of drugs of abuse and their metabolites in biological materials. These methods were applied to the validation of animal models for use in the study of drug metabolism for prediction of responses in humans. Clinical specimens obtained from WRAMC, USAFE/DADL and from normal volunteers were used to compare with laboratory animal specimens.

Progress is reported for FY 75 in the following areas:

1. Efficacy of commercial RIA to measure metabolites of methaqualone in blood and tissues.

2. The reliability of rat biliary excretion as a model for drug metabolism studies.

3. The use of high pressure liquid chromatography for drug metabolism studies.

4. Plasma levels of drug metabolizing enzymes as an index of enzyme induction.

5. The use of thin layer chromatography for the assay of drugs and their metabolites in biological materials.

1. Efficacy of a commercial RIA to measure metabolites of methaqualone in blood and tissues.

A commercial RIA for methaqualone was studied for its efficacy to measure the major metabolites of the drug in biological materials.

The reported metabolites of methaqualone as reported elsewhere are mono- and di-hydroxy derivatives of either ring or the aliphatic side chain. These compounds are found in urine mostly as conjugates, most likely conjugated with glucuronic acid, although other conjugates cannot be ruled out at this time.

The present evaluation consisted mainly of titration of the RIA with synthetic unconjugated metabolites. In addition, several random urine specimens from a drug screening laboratory were analyzed by GLC. These results were compared with RIA results on the same specimens.

The operating parameters for the assay were also studied to evaluate the potential effects of differing binding and dissociation constants for the different metabolites.

The results of these studies showed that the RIA antibody in the commercial kit successfully detected several major unconjugated metabolites. The 3'-hydroxy- and the 4'-hydroxy metabolites reacted with the antibody with kinetics undistinguishable from methaqualone itself. The 6-hydroxy metabolite reacted with about one-half the affinity of the parent drug and the α-hydroxy metabolite has no substantial affinity for the antibody.

Comparison of RIA and GLC results on large numbers of urine specimens revealed that quantitative values obtained by RIA correlated well with combined levels of metabolites identified by GLC. Only very low levels of unmetabolized drug were noted in the urine specimens. No interfering substances were found in the urine that was studied.

In an attempt to validate the use of the RIA to measure methaqualone or metabolites in tissues and fluids, saline extracts of tissues as well as blood and bile from a suspected methaqualone overdose death were examined by RIA and GLC. The RIA gave low positive values that could not be confirmed by GLC.

Attempts to isolate conjugated drug metabolites or to analyze them directly by chemical analytical means have been unsuccessful. Therefore, RIA results on urine containing metabolites were compared for hydrolyzed and unhydrolyzed preparations. The hydrolyzed preparations gave consistently higher values than the unhydrolyzed materials. These results

strongly indicate that the conjugated metabolites have weaker affinities for the antibody than do the unconjugated metabolites.

2. The reliability of rat biliary excretion as a model for drug metabolism studies.

Many xenobiotic substances that are metabolized in the liver are excreted in bile as highly polar, water soluble substances, usually as glucuronides or ethereal sulfates.

These metabolites may enter into enterohepatic circulation either directly or after hydrolysis by bacterial or intestinal enzyme action. The biliary metabolites may also be excreted with feces.

Because man and rat are species that produce and secrete large quantities of bile, the rat was selected as a potential predictive model for excretion of drugs and their metabolites by man.

During these studies, the animals were anesthetized and the bile duct cannulated after simple laparotomy. Drugs being studied were infused acutely into the portal vein. Bile was collected and analyzed for drugs and their metabolites. Morphine was selected for the model drug because of some familiarity with the analytical methodology and the biochemical pharmacology of morphine in rat and man.

Bile aliquots of 50 µl volume were evaporated to dryness and derivatized by the stepwise addition of 50 µl of dimethyl formamide, 50 µl of acetonitrile, 50 µl of Tris Zil-Z and 100 µl of Regisil plus 10% TMCS. After sealing and heating for 30 minutes on a steam bath, the samples are ready for analysis by GLC, using flame ionization detection.

Under these conditions, glucose, stearic and palmitic acids, and cholesterol serve as internal markers and potentially may serve as internal standards for liver function and bile flow.

Injection of morphine into the portal vein causes the appearance of a peak in bile after a 15 minute delay that corresponds to morphine glucuronide. This peak reaches maximum levels about 30-45 minutes after injection of morphine and gradually declines over the rest of the experiment.

Concomitant with the appearance of the morphine glucuronide peak, the concentration of glucose, cholesterol and palmitic and stearic acids also increased and several small unidentified peaks were resolved on the chromatograms. It is not clear whether these observations signify a specific effect of morphine or whether this is simply a reflection of non-specific biliary concentration.

These preliminary results are consistent with predictions about the usefulness of the model. Further work on the model will be pursued.

3. The use of high pressure liquid chromatography for drug metabolism studies.

High pressure liquid chromatography as an isolation and analytical tool is in its infancy but has already proven its value as a method of great potential to solve difficult problems. One of the most appealing aspects of this method is the potential to analyze unprocessed biological specimens of very small volume and low concentration. Typically, levels of 1 µg/ml of a substance can be detected and quantified in less than 10 µl of serum or other biological fluid or tissue extract.

As a part of any renal excretion study, PAH clearance estimates are needed to estimate renal function. Because of the difficulties with interfering substances in the use of the chemical analytical method, HPLC was applied to this problem on the basis that chromatography would separate the intefering substances and HPLC could be used to validate the methodology.

The results of these studies showed that HPLC gave excellent separation of all related compounds such as paraaminobenzoic acid, acetyl PABA and acetyl PAHA. The method was then applied to a number of specimens obtained from renal function tests and from normal volunteers.

The results of these studies validated HPLC as a method for drug metabolism studies, at least in some cases. It was also noted that the kinetics for acetylation of PAH and PAB appeared to have a bimodal distribution.

Since it is known that there are human phenotypes of acetylation of INH, the hypothesis was proposed that acetylation kinetics for these aromatic amino acids are also distributed in the general population by phenotype. Studies were done on patients and volunteers from WRAMC in which therapeutic doses of procainamide were given. Since this drug is also metabolized by acetylation, the distribution of kinetics of this reaction by phenotype would extend the validation of such a phenomenon as a generality and would be very useful for predictive value. Preliminary results of these studies suggest the phenotype for acetylation of procainamide may have the same distribution as that for PAH acetylation.

The implications of similar phenotypic variables for the acetylation of drugs of abuse are significant because of the stronger

pharmacological activity of some acetylated derivatives of psychoactive drugs. These hypotheses will be investigated.

As a proof of the HPLC method for aromatic amines and their acetylated metabolites, it was necessary to compare it with a conventional method such as Bratton-Marshall. Since the latter method requires the hydrolysis of the acetylated compounds and calculation of difference values, it was necessary to compare results during hydrolysis. The results of these studies gave a strong indication that under the recommended conditions of hydrolysis for the Bratton-Marshall method that less than 100% of the acetylated drugs are hydrolyzed and that the values may be low by as much as 10%. Conversely the acetyl bond is so weak in acid that simple acid extraction of the drug may cause significant hydrolysis again leading to artifact.

The fact that Bratton-Marshall reacts similarly with all aromatic amines is another serious drawback. In summary, these data strongly recommend HPLC as a method of choice for analysis of aromatic amines and clearly demonstrate the utility of HPLC as a valid method for the study of drug metabolism.

4. Plasma levels of drug metabolizing enzymes as an index of enzyme induction.

It has long been recognized that the liver mixed-function oxidase enzymes using cytochrome P-450 as a cofactor are readily induced by xenobiotic substances as well as by intrinsic humoral responses. These effects are usually assessed by observing the effects of drugs and by studying drug metabolism rates.

Abnormal levels of transaminases, phosphatases or other tissue enzymes in plasma are frequently used as indices of pathophysiology or disease. As suggested by these observations, an hypothesis was developed which states that the plasma levels of drug metabolism enzymes might serve as indicators of the relative state of enzyme induction in response to drug therapy or other exposure to environmental materials.

Because of technical difficulties encountered in the development of plasma assays for the mixed function oxidases, a series of hydrolases was selected for the model enzymes to test the hypothesis. The rat was selected as the animal model.

Isoenzyme patterns for the hydrolases, acid phosphatase, 5'-nucleotidase and β-glucuronidase were developed for liver, kidney, spleen, lymph nodes, prostate and thymus. The isoenzyme patterns were similar qualitatively for most tissues that were examined with minor quantitative differences in various bands. These observations were consistent with the current theories that these enzymes exist in lysosomes and carry out tissue non-specific hydrolytic functions. Subcellular

fractionations were accomplished for each of the tissues and the isoenzyme patterns were developed. Surprisingly, the enzymes were localized in fractions other than lysosomal fractions. Specifically, hydrolase enzymes were found in nuclear, mitochondrial and microsomal fractions. Electrophoretic patterns suggested that there were specific banding patterns for each fraction which would tend to argue against simple contamination as an explanation of this observation. The isoenzyme differences also correlated with the non-lysosomal fractions which might explain the quantitative differences observed among the various tissue isoenzyme patterns. These observations require further confirmation and if verified offer many interesting extensions of the hypothesis.

In any case, it was important to the original hypothesis to examine the possible effects of drugs on these hydrolase enzyme levels. Two drugs, phenobarbital, which induces drug metabolizing enzymes, and morphine, which reportedly inhibits some of these enzymes, were given to rats for 3 days. The animals were sacrificed and the tissue hydrolase levels were assayed.

Neither drug affected the levels of acid phosphatase. Phenobarbital caused a 20% decrease in 5'-nucleotidase, and had no effect on β-glucuronidase. Morphine caused a 25% increase in β-glucuronidase levels and had no effect on 5'-nucleotidase levels. These effects were not limited to any of the tissues studied.

Certainly, these results are very preliminary and offer no proof of the hypothesis, but they do suggest that further studies are feasible and that the model deserves further testing for its potential predictive value.

5. The use of thin layer chromatography for the assay of drugs and their metabolites in biological materials.

The success of thin layer chromatography to separate and identify the major metabolites of methaqualone as previously reported gave encouragement to the extension of this method to other drugs of abuse.

The previously reported GLC method for measuring cocaine which used hydrolysis of all compounds to ecgonine and norecgonine offered a means for increasing the sensitivity and separation by TLC. To date, all attempts to develop satisfactory conditions for study of cocaine metabolism have been unsuccessful.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 113 Metabolism of drugs of abuse

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6516	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62758A	3A762758A833	00	114
b. CONTRIBUTING				
~~X~~	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Pharmacokinetics of Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

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c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 75	2	75
	CURRENT 76	6	356

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Lofberg, R.T., PhD

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Drug Excretion; (U) Pharmacodynamics; (U) Pharmacokinetics; (U) Drugs of Abuse; (U) Drug Absorption; (U) Drug Distribution

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to determine the rates and modes of absorption, distribution, biotransformation, binding and excretion of principal drugs of abuse in animals to predict these parameters in military personnel.

24. (U) Biochemical, analytical and pharmacological techniques are employed to assess the influences of diet, environment and chemicals on the pharmacokinetics of drugs of abuse. Correlations of binding and distribution with pharmacodynamic effects will be used to study mechanisms of dependence and tolerance.

25. (U) 74 07 - 75 06 Rats with experimental bile fistulas have been studied as a model for the study of the biliary excretion of drugs in order to evaluate this route of excretion. Methods of analysis of bile for morphine and its metabolites have been developed using gas chromatography of derivatized bile. Morphine glucuronide, but no morphine, has been identified in rat bile; other unidentified metabolites have been detected. Quantitative, synchronous changes in levels of glucose and fatty acids in the bile were noted following morphine administration in the rat. Earlier findings of increased uptake of glucose and conversion of glucose to aspartate, glutamate, and gamma-amino butyrate by brain of morphine tolerant rats were confirmed. The pharmacokinetics of several drugs were studied as model systems in patients with various degrees of renal failure in order to evaluate the role of renal function in drug kinetics. These studies further emphasized the importance of body compartments other than serum in the kinetics of drugs. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 114 Pharmacokinetics of drugs of abuse

Investigators:

Principal: LTC (P) Douglas J. Beach, MSC; Nesbitt D. Brown, B.S.; LTC Gale E. Demaree, MSC; Robert T. Lofberg, Ph.D.; H. Kenneth Sleeman, Ph.D.; Patrick M.L. Siu, Ph.D.

Associate: CPT James A. Cella; Meade R. Drumgold; SP4 Arthur Bogdanove; PFC James W. Nitchkey; Nell R. Pendleton, B.S.; Clarence E. Emery, B.S.; Lovelyn L. Hall; Sylvester West, B.S.; John A. Kintzios, B.S.; Ann R. Berman, B.S.; SP4 James P. McGrath; SP4 Edward J. Michalski.

Progress during FY 75 on this work unit was handicapped by the loss of one principal investigator and two associate investigators who were not replaced because of hiring restrictions. Most work was temporarily suspended in order to allow for consolidation and reorganization of personnel and resources. Experiments designed to study the mechanisms by which chloroquine modifies the kinetics of excretion and tolerance in rats were discontinued. New protocols were developed in order to use existing expertise to the maximum extent and to begin retraining of personnel. Progress on this work unit is reported in the following areas:

1. Standarization and refinement of the WRAIR opiate RIA for pharmacokinetic studies in baboons and humans.

2. Synthesis of spin-labelled opiates for use in binding and immunoassay studies.

3. Synthesis of stable isotope-labelled Δ-9-THC for use in pharmacokinetic studies.

4. Studies of pharmacokinetics in anephric patients.

1. Standarization and refinement of the WRAIR opiate RIA for pharmacokinetic studies in baboons and humans.

The use of RIA for quantification of drugs and metabolites in biological materials is subject to several sources of error. In order to estimate the validity of RIA results in these studies it is important to quantify the error at each source. The identifiable error sources are (1) precision of pipetting of Antibody I,

pipetting of sample, pipetting of labelled antigen, pipetting of Antibody II, (2) binding characteristics for Antibody I, binding characteristics for Antibody II, (3) stability of stored specimens, (4) separation of supernate from precipitate, (5) counting of radioactivity, stability of labelled antigen.

Results of these studies to date reveal that pipetting errors can be held below 1% coefficient of variation at each step with careful manipulation. The pipetting of specimen is the pipetting step subject to the greatest error probably because of the more viscous and less homogeneous nature of this material.

The binding characteristics of the antibodies are the greatest identifiable source of error so far evaluated. Not all sources of interference have been identified; however, the use of detergent to clean equipment clearly interferes with binding in a very unpredictable way. Omitting the use of detergent to clean equipment greatly improves the precision for binding; however, there are still systematic errors which contribute a 10% to 20% coefficient of variation within analytical runs. Variation between runs is even greater; however, this can be compensated by judicious use of quality control samples.

Precise evaluation of the effects of specimen storage is complicated as a component of inter-run variation which appears to be due mostly to variation in binding kinetics. Within this range of uncertainty, however, the effects of long term storage of samples are indistinguishable from inter-run variation.

The error due to separation of supernate and precipitate is indistinguishable from the antibody binding error and in fact may be an inseparable component of the error in binding.

Isotope counting error can be maintained at less than 1% simply by maintaining total counts at greater than 10,000 by adjusting counting time. Periodic clean-up of the labelled antigen also maintains isotopic precision. Changes in this preparation may contribute to inter-run error; however, this component could not be separated from the binding error. Intra-run error can be compensated for by appropriate quality control.

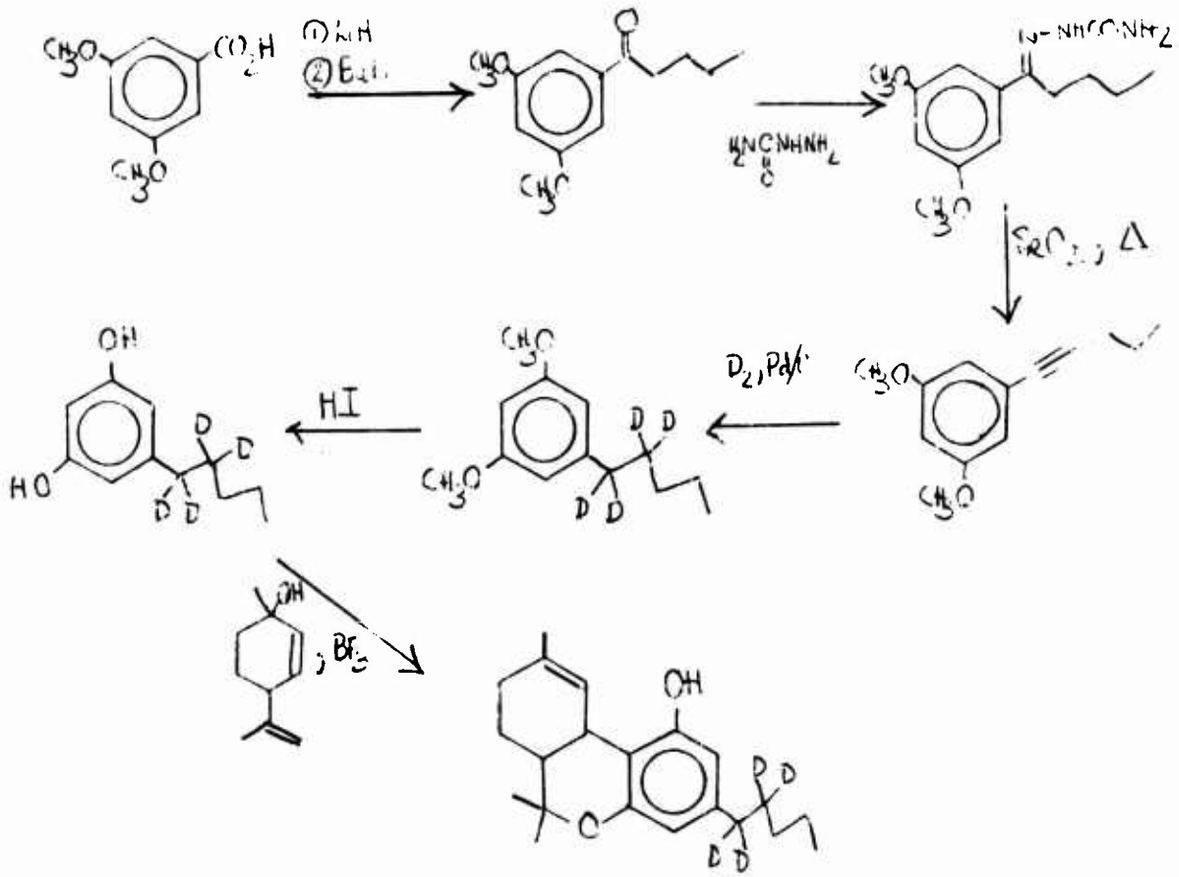
2. Synthesis of spin-labelled opiates for use in binding and immunoassay studies.

The goal of this project was the synthesis of a spin-labelled derivative of morphine which would be both physiologically active and stable. Such a derivative has application in the investigation (both *in vivo* and *in vitro*) of interactions of morphine with its proposed receptors or binding sites. The synthesis of spin-labelled

3. Synthesis of stable isotope-labeled Δ-9-THC for use in pharmacokinetic studies.

The application of selected ion monitoring in combined gas chromatography-mass spectroscopy to analysis of trace components in physiological fluids is facilitated by the use of an internal standard. An isotopically labeled standard is particularly advantageous since it should have extractive and chromatographic properties nearly identical to the parent compound. Moreover this type of standard can lower the detection threshold of the parent by lowering the amount of on-column loss of material.

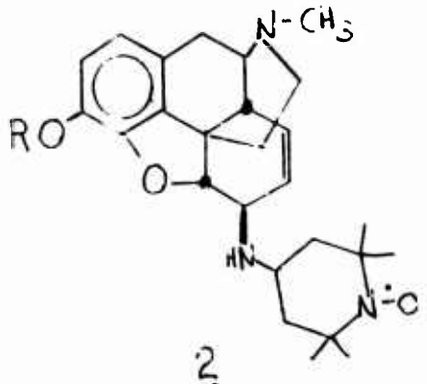
In conjunction with the development of a GC-MS method for the detection and quantification of Δ^9-THC and its metabolites in body fluids a deuterium labeled analogue of the parent drug was desired for use as an internal standard. To avoid interference from natural isotopic peaks (M+n) in the mass spectrum of the parent, a derivative having at least 3-deuterium atoms per molecule was required. Accordingly, Δ^9-THC-d_4, 5, was prepared by the sequence shown in Scheme 2. The experience gained in preparation of this deuterated



5

rearrange to the corresponding 8-isomers. Thus, if rearrangement of the 6-chloro opiates occurred before reaction with the spin label, the product would be that derived from displacement of halogen at the 8-position.

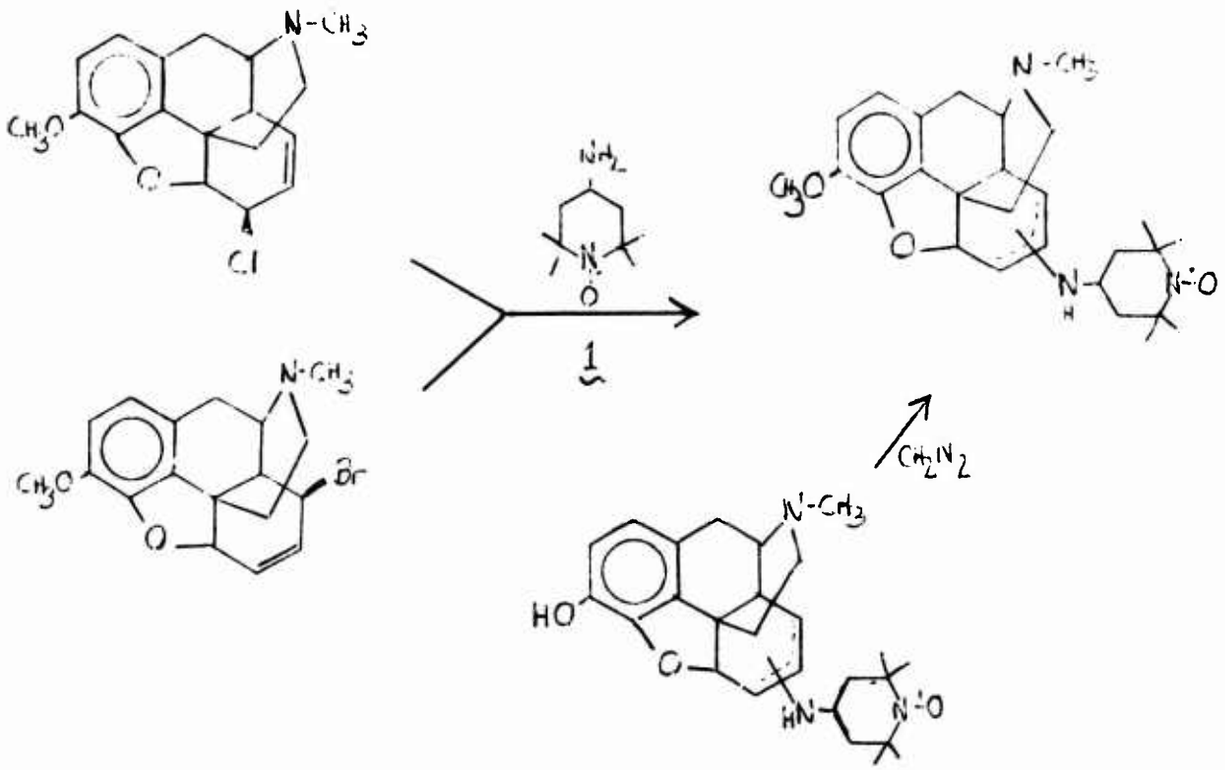
In a control experiment, 6-chlorocodide was subjected to the reaction conditions used to prepare the spin label (DMF, 90°C, 4 hr.) except that the nitroxide was not added. No rearrangement of the halogen was observed under these conditions. However, when chloride ion (as NaCl) was added to the reaction mixture, rapid isomerization to 8-chlorocodide was observed. Presumably a nucleophile is required to initiate the isomerization. Reaction of the spin labeled amine with the rearranged chloride occurs via an S_n2' reaction to afford the 6-spin labeled derivative. Hence, the structure of both spin labeled morphine and codiene is established as having the spin label attached to the 6-position, 2.



2

Samples of both these materials have been submitted to the Arthritis Institute at NIH for testing for FD_{50}, analgesia and binding to guinea pig ileum. Additionally, binding studies will be conducted in these laboratories using ESR Spectroscopy to detect interaction of the labeled compounds with various fractions of rat brain homogenate.

morphine and codeine having the label attached via a carbon-nitrogen amine linkage was accomplished. The problem remaining was to determine the exact position of attachment of the label. Doubt concerning the position of attachment arose from the fact that the same spin labelled derivative was obtained when either 6 or 8 halosubstituted codeines were reacted with the spin label. Moreover, methylation of spin labeled morphine (obtained from reaction of the spin labeled amine, 1, with 6-chloromorphide) yielded a spin labeled codeine identical to the one obtained from displacement of the halocodide isomers.



In the halo opiate series, the 8-halo isomers are more stable than the 6-isomers. Indeed, 6-chloromorphine and 6-chlorocodeine are the only 6-halo isomers available, the bromides and iodides spontaneously

derivative will be applied to the synthesis of deuterated metabolites, in particular 11-hydroxy-Δ^9-THC-d_4 and 11-nor-Δ^9-THC-9-carboxylic acid-d_4.

4. Studies of pharmacokinetics in anephric patients.

Access to clinical specimens from patients in renal failure and who are on repetitive hemodialysis opened a series of studies that are related to three areas of significance: (a) metabolism, distribution and excretion of xenobiotic substances in patients where renal excretion is not significant, (b) the metabolic fate of xenobiotic substances from plastics used in medical catheters and (c) the medical significance of materials leached from plastics into blood.

A gas chromatographic method was developed for analysis of bis-2-diethylhexylphthalate and its metabolites. Internal standards and metabolites have been synthesized. Plasma and tissue specimens have been collected and are stored for analysis.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 114 Pharmacokinetics of drugs of abuse

Literature Cited.

Publications:

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