AD-A009 338

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE. VOLUME II

Walter Reed Army Institute of Research
Washington, D. C.

30 June 1974

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 1973 - 30 June 1974

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 Resources, 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 3A762760A822
MILITARY INTERNAL MEDICINE

Task 00
Military Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6469	74 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 ACC'SS	9. LEVEL OF SUM
73 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	00	120
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Procede 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 06	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 74	9	167
FISCAL YEAR CURRENT 75	9	167

19. RESPONSIBLE DOD ORGANIZATION

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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) 73 07 - 74 06. Prolactin, growth hormone, and thyrotropin release was studied during the psychic stress of fourteen paratroopers undergoing their first jump. All three hormones rose significantly. Seventeen acromegalic patients have been studied before and after transsphenoidal microsurgery. Growth normone was effectively reduced to less then ten nanograms/ml in fourteen and less then five nanograms/ml in eleven of the patients. Transsphenoidal microsurgery is an effective therapy which usually preserves pituitary function. Extensive chromatography studies of serum growth hormone have demonstrated three immunoreactive components of circulating human growth hormone in acromegalics and suggest that at least one of these components is secreted during response to thyrotropin releasing hormone. A resin hemoperfusion technique has been developed which significantly lowers serum thyroid hormones in thyrotoxic dogs and it is believed this should be a useful technique in treatment of life threatening thyroid storm. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*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

PII Redacted

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 120 Metabolic response to disease and injury

Investigators:
Principal: COL Jerry M. Earll, MC
Associates: Marcus Schaaf, M.D.; LTC Leonard Wartofsky, MC; MAJ Jack M. Monchik, 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 therapy. Metabolic balance studies are utilized with 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.

Plasma concentrations of prolactin in humans have been shown to rise in response to a number of stimuli which have in common some element of physical stress, among them surgery under general anesthesia (1,2), endoscopy, and vigorous exercise (1). The role of psychic factors in the prolactin response to stress, however, has not been investigated. Thyrotropin (TSH) secretion in human adults has not been found to be increased by stress of surgery (3), by fever (4), or by exercise (5), but recently it has been reported that small increases in TSH secretion occurred in young men in anticipation of vigorous physical exercise (6). Growth hormone is known to respond to a wide variety of physical stresses, but growth hormone responses to psychic stimuli are less extensively documented. In this study further evidence is provided that psychic stress is associated with release of prolactin and TSH as well as growth hormone. Prolactin, growth hormone, and thyrotropin (TSH) release during the psychic stress of parachute jumping was evaluated in 14 male subjects. Subjects were studied times before and immediately after their first military parachute jump. All three hormones had risen significantly 1 to 14 minutes after the jump, compared to mean levels measured immediately beforehand. Other factors such as cold exposure, physical exertion, and altitude

change appeared unlikely to have produced these elevations. We conclude that prolactin and TSH, like growth hormone, may be released by psychic stress.

Seventeen acromegalic patients were treated by a transsphenoidal approach to the sella turcica using an operating microscope. Serum growth hormone (GH) levels and adrenal and thyroid function tests were measured in 16 patients before and after surgery. Serum GH fell to less than 5 ng/ml in 7 of 9 previously untreated patients, and thyroid and adrenal function was preserved in 8 of these patients. Seven patients previously treated by other modes of therapy did not fare as well. GH levels fell to less than 5 ng/ml in 3, to between 5 and 10 ng/ml in 3 and from 98 to 40 ng/ml in one. Thyroid and adrenal function in previously treated patients was preserved in 4 of 6 cases, but was lost in 2 patients. The results of this study suggest that trannsphenoidal microsurgery is effective and can be considered for the initial therapy in acromegaly.

Galactorrhea is a relatively common symptom with an extensive differential diagnosis. We have studied 28 patients with galactorrhea to determine which tests differentiate normal from abnormal prolactin secretion and separate patients with pituitary tumors from those with galactorrhea of other etiologies. Eight patients had pituitary tumors, 5 had galactorrhea associated with drug therapy (BCP in 4, phenothiazine in 1), 5 had "idiopathic galactorrhea" (normal menses, no drug history, normal skull X-ray, no other illness), and 9 had galactorrhea associated with other conditions (myxedema in 3, menopause in 3, empty sella, renal failure, and sickle cell disease in 1 each). Basal prolactins: highest prolactins were in patients with pituitary tumors (range 31 to 206 ng/ml); basal prolactings were less than 26 ng/ml in all other patients except 2. TRH stimulation tests: women with idiopathic galactorrhea had normal peak responses, while the women with BCP galactorrhea and those with myxedema had significantly greater peak responses; patients with pituitary tumors did not respond to TRH significantly. Chlorpromazine stimulation tests (CST): women with BCP and idiopathic galactorrhea responded normally; 2 patients with myxedema had poor responses and 5 patients with pituitary tumors failed to respond. L-dopa suppression tests: prolactin in all patients was suppressed to 60% or less of basal values. Water load suppression test: contrary to published reports, water was not found to be a consistent suppressor of prolactin in normals or in patients with galactorrhea. It is concluded that a high basal prolactin and failure to respond to either TRH or CST were associated with pituitary tumors. Patients with myxedema and BCP galactorrhea had normal basal prolactin but responded abnormally to TRH or CST. Patients with idiopathic galactorrhea had normal basal prolactin and responded normally to TRH and CST. L-dopa and water loading were not useful in the differential diagnosis of galactorrhea.

Heterogeneity among glandular and circulating forms of human growth hormone (hGH) is well established. It has also been documented that thyrotropin releasing hormone (TRH) may stimulate hGH secretion in acromegalic patients. To further investigate the heterogeneous forms of basal and stimulated circulating hGH in acromegaly, sera obtained from three patients before and after TRH administration were examined by gel chromatography on Sephadex G-100. For comparison, purified pituitary hGH (National Pituitary Agency) was examined under identical conditions. Each sample was co-chromatographed with ^{125}I-hGH serving as an internal standard and with ^{125}I-bovine thyroglobulin (bTG) and ^{125}I to establish the void and total volumes. Unlabeled hGH was measured by immunoassay. All sera (N=8) contained three components of immunoreactive hGH. Peak I material co-eluted in the void volume with ^{125}I-bTG. Peak II material eluted with a $K_{av} \sim 0.2$. Peak III material eluted with a $K_{av}=0.377 \pm 0.006$ SEM, very close to that of the ^{125}I-hGH internal standard ($K_{av}=0.388 \pm 0.002$ SEM, N=14). Peak I material was rechromatographed and co-eluted quantitatively with ^{125}I-bTG. Before TRH, Peak I material comprised $\sim$20-35% of the total hGH, Peak II $\sim$ 12-21% and Peak III $\sim$ 51-60%. After TRH, the concentrations of all three components increased markedly, but only Peak III increased in percentage. Unlabeled pituitary hGH (n=2) also contained three components: Peak I material again co-eluted in the void volume with ^{125}I-bTG; Peak II material eluted with a $K_{av}=0.19$; Peak III material eluted with a $K_{av}=0.37$. Peak I and Peak III materials from chromatograms of both serum and pituitary hGH all behaved similarly when examined by immunochemical dilution. These studies demonstrate three immunoreactive components of circulating hGH in acromegaly and suggest that at least one of these components is secreted in response to TRH. Further investigation will be required to determine if the other components represent products of secretion, peripheral metabolism, or both.

2. Thyroid Metabolism.

Daily administration of thyrotropin releasing hormone (TRH) to normal individuals leads to diminishing Thyrotropin Stimulating Hormone Response (TSH/R) believed due to rising serum T_3 with feedback inhibition of the pituitary. Patients with Primary Hypothyroidism (PH) were studied to test this hypothesis, since their T_3 cannot increase after TRH. Bi-daily TRH (100 µg i.v.) was given to 4 patients for 3 consecutive days, and repeated on a 4th day following oral T_3 (50 µg). TSH/R were unchanged over the first 3 days of serial TRH, and moreover, were unaltered by T3 administration. These observations in PH led us to reexamine TSH/R to TRH in euthyroid subjects given T_3. Other workers have reported that 50 µg T_3 completely blocked TSH/R to TRH in 7 normals. We repeated this experiment in 11 subjects with standard 500 µg i.v. TRH test (STT) on one occasion, and STT 1 hour after oral T_3 on another. Mean peak TSH ($\pm$SE) during control STT was 11.3$\pm$1.7µU/ml. One hour following 50 mcg T_3, TSH/R to TRH was not inhibited, peak TSH averaging 10.4$\pm$1.6 ($p>0.2$), while serum T_3 had risen from a (mean$\pm$SE) basal conc. of 157$\pm$7 to 235$\pm$18 at the time of TRH injection and 342$\pm$37ng% 60 min. later. We have reported that normal subjects given prolonged TRH

infusion (TRH/I) continue to secrete TSH despite elevated T_3 levels (Noel et al JCEM July '74). Continuous TRH/I (1µg/min) for 6 hrs in 5 euthyroid volunteers produced a TSH/R which peaked in 180 min. and then plateaued over the next 3 hours. To ascertain whether this plateau was due to increases in T_3 (observed mean 125→225 ng%), similar infusions were given to 5 patients with PH. TSH peaked within 60-180 mins. and then declined toward basal despite continued TRH/I and the failure of T_3 levels to rise. It is concluded that 1) acute increases in T_3 are not adequate to suppress TSH/R in either euthyroid or hypothyroid subjects; 2) plateaued or declining TSH values in the face of continuing TRH/I are related less to T_3 increases than to other factors governing TSH synthesis and release.

Thyroid storm is a serious and often fatal condition. Present modes of therapy are effective in inhibiting further hormonal release from the thyroid, but do not affect excessive circulating levels of thyroxine (T_4) and triiodothyronine (T_3). We have investigated whether extracorporeal resin hemoperfusion (RH) might remove significant amounts of T_4 and T_3 so that the method could prove clinically useful in thyroid crisis. A similar RH technique has proved effective and safe in man for various drug intoxications. With this technique, blood is perfused through an uncharged Amberlite polystyrene anion-exchange resin with affinity for lipid-soluble materials. Five dogs (35-45 kg) were made thyrotoxic by daily administration of exogenous T_4. They were then subjected to RH utilizing a plastic column (Vol=64 in^3). Serial blood samples from arterial and venous cannulae were obtained during control perfusion (without resin or with inactive resin), and during an experimental period with active resin for a period of two hours. Samples were analyzed for T_4, free T_4 (FT4), T_3, SMA-12, CBC, and electrolytes. No significant changes occured in any parameters during control perfusions. The greatest decreases in T_4, FT_4, and T_3 were observed after initial passes through active resin, with continued RH resulting in progressive but smaller decrements. Final concentrations for T_4, FT_4, and T_3 averaged respectively, 53, 47, and 25% of basal levels. All dogs tolerated RH well, and CBC and blood chemistries did not change significantly. It is concluded that resin hemoperfusion of thyrotoxic dogs has proved a safe, effective means of decreasing serum T_4 and T_3 and holds promise for the treatment of thyroid storm in man.

Low levels of circulating triiodothyronine (T_3) have been described in neonates at birth. The rise in T_3 to normal levels within 48 hours after delivery may be related to enhanced thyroidal secretion secondary to TSH release. The present experiment was designed to examine whether peripheral levels of T_3 might still rise after delivery if thyroidal secretion is blocked, hence suggesting that the source of T_3 could be peripheral deiodination of thyroxine (T_4). Pregnant ewes were injected with 50 µc ^{125}I i.v. in order to label fetal thyroid glands by transplacental transfer of the isotope. Serial measurements of $PB^{125}I$ in the fetus after delivery then served as an index of endogenous hormone

secretion. Three days later, the pregnant ewes were divided into a group given SSKI and a control group. After delivery, lambs of the iodine-treated ewes continued to receive SSKI. At birth, neonatal serum samples for T_4, T_3 and $PB^{125}I$ were drawn at 6 hourly intervals for two days, after which the lambs were sacrificed and their thyroid glands analyzed for T_3 and T_4 content. In 4 control lambs, serum T_3 and $PB^{125}I$ values between time 0 and 48 hours rose significantly, while serum T_4 remained unchanged. In 6 iodine-treated lambs, $PB^{125}I$ values failed to rise while T_4 fell significantly; although mean serum T_3 levels for the group did not change significantly, 3 of 6 animals had an increase in T_3 that averaged 165 ng%. Thyroid gland hydrolysates revealed that iodine-treated lambs had 1.43 times more T_4 and 1.24 times more T_3 per gram thyroid tissue than did thyroid glands of control animals. It is concluded that 1) Enhanced thyroidal secretion (evidenced by rising $PB^{125}I$) contributes to increases in serum T_3 normally seen in neonates after delivery. 2) Stable or increasing T_3 in the presence of inhibition by iodine of thyroidal secretion (confirmed by declining values of $PB^{125}I$ and T_4, as well as increased glandular T_4 and T_3 content) also indicates a peripheral source of T_3 generation, i.e., conversion from T_4.

An oral water load has been reported to decrease serum prolactin (HPr) levels in normal subjects. Since HPr and thyrotropin (TSH) share common control mechanisms, e.g., secretion of both may be stimulated by TRH and suppressed by thyroid hormone, we evaluated whether water loading might similarly affect the concentration of serum TSH. Oral tap water was administered (20 cc/kg over 30 minutes) to 23 normal subjects. There was no significant difference between values for TSH prior to and 90 min. after initiation of water loading. Since it is difficult to assess changes in the concentration of TSH within the normal range, we determined the effect of identical water loads 7 subjects with primary myxedema and high endogenous levels of TSH. Blood was drawn at 30 minute intervals for 1 hour before, and 2 1/2 hours after water loading. TSH did not significantly change at any time after administration of water. A 500 μg i.v. bolus of TRH was administered 120 min. after water loading to determine whether the magnitude of the TSH response might be suppressed or otherwise altered from that observed in the same patients given TRH under control conditions several days earlier. In 4 of 7 subjects, the peak ΔTSH to 500 μg TRH following a water load was slightly higher then that observed to TRH administered during the control period, but the mean Δ peak for the group was not significantly different ($p < 0.1$). These observations suggest that, unlike HPr, TSH secretion does not appear to be influenced by the perturbations in serum osmolality induced by water loading.

Forty-four patients with Graves' disease (diffuse toxic goiter) were selected for long-term therapy with antithyroid drugs over the past 4 years. These patients were followed closely with good control of their thyrotoxicosis for periods ranging from 7 to 46 months, and averaging

17.6 months. During this time, it has been clearly apparent that the incidence of remission after standard courses of either tapazole or propylthiouracil was disappointingly low. Remission was observed in only 4 of 35 (11.4%) patients treated for >12 months, or in 6 of the 44 (13.6%) total patients treated. (Two patients attained a remission after <12 months of therapy). Even by relating all the observed (six) remissions to those patients treated for >12 months (6/35), the remission rate is increased to only 17.1%. Previous reports of remission rates after antithyroid drugs have consistently suggested that permanent remission should be expected in approximately 60% of patients. These earlier studies were reported between 1952 and 1956, a period of time when iodine intake was significantly less than it is today in many areas of the United States. Alexander, et al., (Lancet 2:866, 1965) have shown that relapse in patients with euthyroid Graves' disease was hastened by iodine supplements given in doses which approximate the dietary increases that have now been commonly observed and well documented (Oddie, et al., J. Clin. Endocr. 30:659, 1970). These studies and other recent reports of iodide-induced hyperthyroidism lead to speculation that there may be an inverse relationship between the increasing average daily dietary intake of iodine, and the remission rate which may be anticipated after antithyroid drug therapy in Graves' disease. These results suggest that additional reappraisals of current success with the thionamides at other medical centers are indicated. If these observations are confirmed, criteria for the selection of patients for antithyroid therapy may have to be significantly changed in the future.

3. Calcium.

Although it has been known for many years that hypercalcemia may complicate hyperthyroidism (HT), the true incidence of this association is unclear, with most recent reports suggesting a co-occurence rate of 15-23%. Of greater interest is the paucity of data on measurements of ionized calcium (Ca)++ in HT since (Ca)++ represents the physiologically active portion of the total serum calcium concentration. This communication reports the results of a survey of thyrotoxic patients for the degree and frequency of elevations of total (TCa) and (Ca)++, with measurements of serum immunoreactive parathyroid hormone (PTH) in order to examine the role of the parathyroid glands in this disorder. 47 patients were studied who had both clinical and chemical evidence of HT. The methods employed included TCa by atomic absorption spectroscopy; (Ca)++ by a liquid membrane electrode flowthrough system; and PTH by RIA. TCa was elevated in 29% of patients, while (Ca)++ was elevated in 44%. In an attempt to correlate PTH levels with observed increases in Ca, the patients were categorized into 3 groups. Group I-normal total and ionized calcium; II-elevated ionized and total calcium; III-elevated ionized, normal total calcium. Although not statistically significant, PTH levels were slightly higher in Group I than in Groups II or III, thus appearing to vary inversely with ionic calcium. It is

concluded that there is a high incidence of elevation of both total and ionized calcium in hyperthyroidism which does not appear to be related to hypersecretion of parathyroid hormone.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 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	74 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
73 07 01	D.CHG	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	0C	121
b. CONTRIBUTING				
c. [redacted]	CARDS 144(1)			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of Enteric Disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
C10100 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 74	5	148
FISCAL YEAR CURRENT 75	5	148

19. RESPONSIBLE DOD ORGANIZATION
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NAME:

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 find improved procedures to control diarrheal disease. Present work involves the preparation and testing of oral vaccines against bacillary dysentery, the identification and characterization of agents involved in travelers diarrhea and the virulence of Salmonella. Diarrhea is a significant problem in military personnel operating overseas.

24. (U) Attenuated dysentery strains are being developed. They are being evaluated for safety in several systems and are being tested for potency in monkeys and in man.

25. (U) 73 07-74 06 A new E. coli hybrid expressing the somatic determinants of S. flexneri 2a has been employed as a vaccine in volunteers. Dosages of ten to the tenth cells in volunteers have produced no illness but failed to provide protection to challenge with virulent S. flexneri 2a. Additional hybrids with other shigella serotypes have been prepared. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 73 - 30 June 74.

*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 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 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 enteric infections, particularly those caused by shigellae, salmonellae and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of these parameters, procedures for prevention and control of diarrheal diseases can be devised.

Progress.

1. Genetic control of Shigella somatic antigens and the development of oral living shigella vaccines.

a. One area of research on diarrheal diseases concerns the development of living attenuated organisms for use as oral vaccines against bacillary dysentery. Earlier studies in this department yielded avirulent hybrids and mutants of Shigella flexneri for possible use as vaccine strains (see previous annual reports) in providing protection to challenge with virulent S. flexneri. On the basis of limited human volunteer studies performed by the University of Maryland Vaccine Development Group some of these strains appear to be relatively safe and significantly effective. Nevertheless it is impossible to obviate the theoretical possibility that these strains could revert to a virulent state. To eliminate such complications with the safety of living oral vaccines, we have now taken an alternative approach for preparing possible vaccine candidate strains. In principle, our approach has been to prepare by intergeneric hybridization, avirulent E. coli strains which express the group and type-specific antigens of Shigella rather than their native E. coli antigenic complex. Since the E. coli parent strains employed as recipients are natively avirulent, the problem of reversion to virulence by hybrid vaccine strains is obviated.

2. Construction of Shigella Hfr donor strains.

a. In order to prepare E. coli hybrids with serotypes of various Shigellae, it was essential to construct Shigella donor strains

with the capacity to transfer chromosomal genes. The feasibility of constructing such Hfr donor derivatives is a direct consequence of our previous experience with preparing derivatives of S. flexneri 2a by the method of F-linked terminal marker selection.

b. E. coli K-12 P4X-6, an Hfr which transfers as its terminal marker the lactose utilization genes (lac operon) linked to the F-factor, was mated with shigellae of various serotypes and lac^+ recombinants were selected. Analysis of such recombinants revealed that a small proportion of them had inherited the sex factor along with the lac^+ character. These efficiently transferred chromosomal genes, in crosses with E. coli recipients with a polarity similar to that of P4X-6, i.e. pro-arg-his-gal-lac-F. As far as could be detected by genetic analysis for other unselected markers these shigella donors appeared to have retained only the lac^+-F region of the E. coli donor genome and otherwise exhibited the characteristics typical of the original shigella parent. Through the employment of such genetic manipulations, we have constructed Hfr donor strains of S. flexneri serotypes 1a, 1b, 2a, 3a, 4b, 5, 6 (i.e., group B) and of S. dysenteriae 1 (group A). Extensive efforts to prepare donor derivatives of S. sonnei (group D) have been unsuccessful.

3. Construction of E. coli hybrids which express the various type-specific antigens of S. flexneri.

a. As described in previous annual reports, the genes controlling synthesis of Shigella flexneri 2a group and type-specific antigen can be transferred to Escherichia coli K-12 recipients by conjugation with an S. flexneri Hfr. After mating E. coli with an Hfr strain of S. flexneri 2a and selecting for his^+ recombination, a high proportion of the E. coli hybrids agglutinated in S. flexneri grouping serum. None of these hybrids expressed S. flexneri type-specific antigen 2. When an E. coli his^+ hybrid possessing the S. flexneri group antigen was remated with the same Hfr with selection for pro hybrids, a high proportion now expressed the type-specific antigen as well as the previously inherited group antigen. If such crosses were performed in reverse order (i.e., pro^+ followed by his^+ selection), a different pattern of serological behavior was observed. None of the pro^+ hybrids showed the type-specific antigen. Subsequent mating for his^+ resulted in hybrids with both the group- and type specific antigens. These results showed that genes controlling the synthesis of S. flexneri group antigen (linked to the his locus) and type-specific antigen (linked to the pro locus) are widely separated on the chromosome. Expression of the type-specific antigen depends on the presence of the group antigen.

b. On the basis of these observations with S. flexneri 2a, it was likely that other E. coli hybrids with the different type-specific antigens of S. flexneri could be constructed by similar genetic manipulations. One such E. coli K-12 his^+ group antigen 3,4 hybrid served as the prototype recipient strain for all subsequent crosses to introduce type-specific antigen genes. By mating Hfr strains of S. flexneri serotype 1, 3a, 4 and 5 with the E. coli hybrid recipient capable of expressing S. flexneri group antigens, and selecting for the inheritance of the proline locus, we have recovered E. coli hybrids which express type-specific determinants 1, 2, 3, 4 and 5. Additional genetic and serological studies resulted in the selection of candidate hybrid strains for testing as possible vaccines against dysentery.

c. Similar genetic crosses with an S. flexneri 6 Hfr and a S. dysenteriae 1 Hfr have failed to yield hybrids suitable for consideration as a vaccine.

4. Vaccine trials in conjunction with the University of Maryland Vaccine Testing Program.

a. At the present time, this department has provided Dr. Hornick's group at the University of Maryland with E. coli hybrids expressing the following S. flexneri antigens.

(a) type 1a, 1b
(b) type 2a
(c) type 3
(d) type 4
(e) type 5

Two of these hybrids (those which expresses type factor 2 and type factor 3) have been shown to be safe up to dose levels of $1X10^{11}$ cells in human volunteers. Very recent results of a statistically adequate protection test for vaccine efficacy in human volunteers however, has been discouraging. A previous small pilot protection test had suggested that the E. coli hybrid expressing the somatic antigens of S. flexneri 2a was effective in providing protection to challenge with virulent S. flexneri 2a (see 1973's annual report). On the basis of these observations, a large scale human volunteer experiment was performed during the past year. Three doses of hybrid organisms expressing S. flexneri 2a antigens were administered at weekly intervals in doses of $5X10^{10}$ cells. The vaccine was well tolerated and individuals shed the strain for an average of approximately 5 days. One month after the final dose of vaccine the volunteers were challenged with approximately 140 virulent S. flexneri 2a which was estimated to be an ID_{50}. In the control groups 39 percent of the men developed clinical disease while the attack rate in the vaccine group was 47 percent. Immunologic studies have not yet been completed.

Summary and Conclusions.

1. Hfr donor strains of shigellae capable of transferring extensive segments of their genomes have been constructed for the purpose of preparing E. coli hybrids which express the group and type-specific antigens of various shigella serotypes.

2. Hybrid E. coli strains with shigella serotypes 1a, 1b, 2a, 3, 4 and 5 have been selected for testing as possible oral live vaccines against dysentery.

3. Safety tests have shown such hybrids to be safe up to levels of 10^{11} cells in human volunteers.

4. Human volunteers after receiving three weekly doses of $5X10^{10}$ vaccines cells (serotype 2a) showed no protection to challenge with virulent S. flexneri 2a.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 121 Pathogenesis of enteric diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 0436	74 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
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	02	092
b. CONTRIBUTING	62760A	3A762760A822	00	
c. [redacted]	CARDS 144(f)			CH FR 122

11. TITLE (Precede with Security Classification Code)

(U) Microbial Genetics and Taxonomy

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

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 74	3	138
FISCAL YEAR CURRENT 75	3	157

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Vaccine; (U) Enteric Bacteria; (U) Antigens (U)Virulence; (U) Salmonella; (U) Drug Resistance

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

23. (U) Definition in genetic and molecular terms of the properties of gene transfer antigenicity, and virulence of pathogenic enteric bacteria which because of their disease producing capabilities, are of importance to military medicine concerned with the prevention and treatment of such infections in Army personnel. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and pathogenicity to serve as vaccine strains or as tools to study the infectious process.

24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.

25. (U) 73 07-74 06 Employment of mouse virulent Salmonella typhimurium hybrids expressing various combinations of S. typhosa 9, Vi, and d antigens as challenge organisms in mice immunized with vaccines prepared from these strains suggested that the Salmonella somatic antigens, but not the Vi or flagellar antigen, are important in conferring protection against death in this system. Examination of the molecular nature of six transmissible plasmids conferring a lactose-fermentation capability on clinically isolated Salmonella strains showed that these lac elements were conserved extrachromosomally as supercoiled circular molecules of deoxyribonucleic acid with molecular weights ranging from 30 million to 60 million daltons. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

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

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 122 Microbial genetics and taxonomy

Investigators.

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Description

1. Employment of mouse virulent Salmonella typhimurium hybrids expressing various combinations of S. typhosa 9, Vi, and d antigens as challenge organisms in mice immunized with vaccines prepared from these strains suggested that the Salmonella somatic antigens are important in conferring protection against death in this system.

2. Examination of the molecular nature of six transmissible plasmids conferring a lactose-fermentation capability on clinically isolated Salmonella strains showed that these lac elements were conserved extrachromosomally as supercoiled, circular molecules of deoxyribonucleic acid.

3. A chromosomal locus involved in the expression of the N gene function of bacteriophage lambda has been identified and characterized in Escherichia coli, and in an E. coli x Salmonella typhosa hybrid which blocks action of this phage gene preventing lysis by lambda.

Progress

1. The role of Salmonella typhosa antigens in protection of mice immunized and challenged with S. typhimurium hybrids expressing these antigens

a. In a previous report (Annual Report, WRAIR, 1973) we described the formation of mouse virulent, Salmonella typhimurium hybrids expressing antigens 9, Vi and d, by genetic crosses between a S. typhimurium recipient and a S. typhosa Hfr donor. These hybrids retained the same degree of mouse virulence as their S. typhimurium parent when tested with either C57 black or Swiss white mice. Furthermore, vaccination of mice with S. typhosa vaccines conferred significant protection against challenge by these hybrid strains but not against their S. typhimurium

parent. However, no conclusions were made regarding the protective role of individual S. typhosa antigens. In the present report, further studies with this system are described which suggest that the somatic antigens are of primary importance in conferring protection.

b. All S. typhimurium hybrids used in this study were derived, as described previously (Annual Report, WRAIR, 1973), from matings between the S. typhosa Hfr WR4000 and the mouse virulent S. typhimurium recipient WR5004. The results of protection experiments employing S. typhimurium hybrids H1 (antigenic constitution 9,12: i-1,2), H42 (9,12, Vi: d-1,2) and their S. typhimurium parent strain WR5004 as challenge organisms in variously immunized Swiss white mice are shown in Table 1. As we have seen previously, the vaccine prepared with S. typhosa TY2 protects mice against the challenge of S. typhimurium hybrid H1 but not against their S. typhimurium parent strain, WR5004 (statistically significant at $P < 0.001$). However, both the H1 and H42 vaccines, prepared from hybrids expressing somatic antigen 9, are protective against the H1 hybrid challenge. Although H1 and H42 vaccines appear almost as protective against the WR5005 strain as against the 9,12 challenge strains, the overall pattern suggests that the specificity of the somatic antigen (9 or 4) is the important factor in determining protection against death in this system.

c. The presence or absence of the Vi antigen in the vaccine strain did not affect the outcome in any way which might indicate a specific protection involvement for that antigen with the Vi-expressing challenge organism, H42. On the other hand, it appeared that protection against H42 was generally greater with all vaccines than was the case with the non-Vi-expressing hybrid. In testing the virulence of the H42 hybrid as well as other Vi-expressing hybrids, we have noted that the onset of mouse deaths, at the lower dose levels, is usually delayed by two or three days, as compared with the non-Vi-expressing hybrids. It is possible, therefore, that this circumstance may have some bearing on the greater capacity of the vaccines to protect against challenge with strains expressing the Vi antigen.

d. To examine the role of the Vi antigen in this protection system, two S. typhimurium hybrids, each expressing the Vi antigen, but differing with regard to the somatic antigens, were employed as challenge organisms in mice immunized with vaccines prepared from S. typhosa hybrids expressing various antigenic combinations. The results of these experiments are shown in Table 2. In no instance did the presence of the Vi antigen in a vaccine strain enhance the vaccine's protective capability as compared with one prepared from a non-Vi-expressing hybrid of the same somatic antigenic constitution. Thus, it again appears as if the Vi antigen plays no part in the specificity of protection in this system. Overall, the differences in protection against the antigen 4 or the antigen 9 - expressing challenge strains for a given vaccine in this experiment - were not striking. However, where such differences are observed between these two hybrids, they are seen again to correlate with the specificity of the somatic antigen.

Table 1 - Mouse protection tests employing S. typhimurium parent and hybrid strains as challenge organisms[a]

Immunized with	challenged with		
	S. typhimurium hybrid H1 (9,12:i-1.2)	S. typhimurium hybrid H42 (9,12,Vi:d-1,2)	S. typhimurium WR5004 (4,5,12:i-1,2)
S. typhosa TY2 (9,12,Vi:d)	22/40[b]	28/40	4/40[c]
S. typhimurium hybrid H1 (9,12:i-1,2)	23/40	33/40	19/40
S. typhimurium hybrid H42 (9,12,Vi:d-1,2)	23/40	23/40	23/40
S. typhimurium WR 5004 (4,5,12:i-1,2)	7/39	24/40	37/40[d]
S. typhimurium hybrid H39 (4,5,12,Vi:i-1,2)	7/40	27/40	33/40[d]
Controls (no vaccine)	0/40	1/40	1/40

[a] Swiss white mice (15-18 gms) were inoculated intraperitoneally with 0.5 ml of vaccine (10^9 cells/ml) and challenged 2 weeks later by the same route with 100 LD50's of the various hybrids. Survivors were counted after 21 days.

[b] Survivors/injected

[c] Significantly more protection against challenge H1 than against challenge WR5004 ($P < 0.05$)

[d] Significantly more protection against challenge WR5004 than against challenge H1 ($P < 0.001$)

Table 2 - Mouse protection tests employing S. typhimurium Vi antigen-expressing hybrids as challenge organisms[a]

Immunized with	Challenged with	
	S. typhimurium hybrid H34 (9,12,Vi:i-1,2)	S. typhimurium hybrid H39 (4,5,12,Vi:i-1,2)
S. typhosa hybrid H47 (4,12:d)	29/40[b]	33/40
S. typhosa hybrid H48 (4,12,Vi:d)	20/40	34/39
S. typhosa WR4208 (9,12:d)	26/40	21/40
S. typhosa hybrid H52 (9,12,Vi:d)	25/40	18/40
Controls	4/40	2/40

[a] Swiss white mice (15-18 gms) were inoculated intraperitoneally with 0.5 ml of vaccine (10^9 cells/ml) and challenged 2 weeks later by the same route with 2,500 organisms of the various hybrids (100 LD50's). Survivors were counted after 21 days.

[b] Survivors/injected

2. Isolation and characterization of circular deoxyribonucleic acid obtained from lactose fermenting Salmonella strains

a. We previously examined seven independently isolated, lactose-fermenting Salmonella strains obtained from clinical sources and found that six of these strains were capable of conjugal transfer of the lac character to Salmonella typhosa (Annual Report, WRAIR, 1968). S. typhosa recipients of the lac character were able to transfer this character to Salmonella typhimurium, Proteus mirabilis, and Escherichia coli. Deoxyribonucleic acid (DNA) extracted from Proteus strains containing these transmissible lac elements was examined by cesium chloride density gradient centrifugation, and the DNA associated with four of these elements was observed as separate satellite bands with a guanine + cytosine content of 50% each. The DNA of the other two lac elements was not detectable in the Proteus strains to which they had been transferred. In the present study, using an ethidium bromide-cesium chloride technique, we were able to characterize all six of these lac elements as supercoiled circular molecules of DNA.

b. Four lac elements lac-20, lac-26, lac-28, and lac-50, were transferred to E. coli WR3026 by using S. typhosa WR4204 strains containing these elements as donors. E. coli WR3026 strains containing the other lac elements, lac-22 and lac-32 were obtained by matings with the Salmonella strains which originally contained these elements. Each of the six E. coli WR3026 strains containing a transmissible lac element was tested with E. coli male-specific phage, R-17, and the E. coli female-specific phage, ØII. R-17 was not observed to form plaques on any of these strains and the plating efficiency and plaque morphology of ØII were the same as observed on the E. coli WR3026 F- strain. Thus, the E. coli WR3026 strains containing the lac elements did not respond to either of these phages as do F-containing derivatives of E. coli K-12.

c. Each of the six E. coli WR3026 strains containing a transmissible lac element was examined for the presence of supercoiled, circular DNA molecules by the dye-buoyant density centrifugation method. Supercoiled circular DNA molecules were observed in all of these E. coli strains. Segregants of each of these E. coli WR3026 strains which had lost the ability to utilize lactose were isolated and examined in the same manner as the lactose-fermenting strains. Supercoiled, circular DNA molecules were not observed in any of these strains which had lost the lac^+ character nor were they observed in the recipient E. coli WR3026 strain.

d. Circular DNA isolated by the dye-buoyant density method from each strain containing a lac element was examined in a neutral sucrose gradient. Sedimentation coefficients of the supercoiled DNA were calculated and the molecular weights were determined. The molecular weights, DNA base composition, and calculated number of copies per chromosome for each of the lac elements are shown in Table 3. Also shown in this table are our previous calculations of the percentage of total cellular DNA

Table 3 - Summary of characteristics of lac elements

E. coli WR3026 containing	Base composition of satellite band (% G + C)	DNA in satellite band[a] (%)	Super-coiled circular DNA (%)	Molecular weight of *lac* elements[b]	No. of copies of *lac* elements per chromosome
lac-20	50[a]	0.8	3.57	32×10^6	3.1
lac-22	50	None observed	3.04	36×10^6	2.4
lac-26	50[a]	0.9	2.82	56×10^6	1.4
lac-28	50	None observed	3.97	30×10^6	3.7
lac-32	50[a]	3.0	5.27	40×10^6	3.7
lac-50	50[a]	3.0	3.90	42×10^6	2.6

[a] Obtained from previous studies in *Proteus*

[b] F sex factor in our laboratory was estimated to be 60×10^6 daltons

observed for the four lac elements that were separable in a cesium chloride density gradient from Proteus DNA. The fact that the values obtained in the present study are, in all cases, higher, may indicate that separation of the lac element DNA from Proteus DNA on the basis of G+C composition is less efficient than is isolation as supercoiled, circular molecules by ethidium bromide-cesium chloride gradient centrifugation. An alternative explanation is that there are more copies of the extrachromosomal DNA molecules in the E. coli strains than in Proteus.

e. With the exception of the 56 million molecular weight calculated for the lac-26 element, the molecular weights of the lac elements are significantly smaller than our calculation of 60 million for the molecular weight of the F sex factor isolated from an F^+ derivative of E. coli WR3026. Also, with the smallest of these lac elements, lac-28, having a molecular weight of 30 million, variation in size is apparent among the lac elements themselves. Clearly, however, some of these elements are not distinguishable from one another on a molecular weight basis, e.g., lac-28 and lac-20 (30 versus 32 million molecular weight), lac-32 and lac-50 (40 versus 42 million), or even perhaps lac-22 and lac-50 (36 versus 42 million).

f. In attempting to assess the significance of the size variation among those elements which are distinguishable on this basis, the question arises as to how much of that size represents sex factor DNA and how much might represent added DNA, possibly of chromosomal origin. The finding that none of the E. coli exconjugants containing these lac elements responded to R-17 or ØII as do F-containing K-12 derivatives, or that most of the elements are significantly smaller than F, does not preclude the possibility that, like F, they are, or have been, capable of interaction with the bacterial chromosome. Certainly, the presence on them of the lac determinants does suggest that such an interaction once took place. Thus, despite their isolation from different Salmonella strains at different points in time, it is possible that some, or even all, of these lac elements may be members of the same class of sex factors.

3. Genetic characterization of a bacterial locus involved in the activity of the N function of phage lambda

a. We have previously shown that S. typhosa is unable to adsorb lambda, a well-characterized temperate bacteriophage of E. coli K12. Appropriate E. coli - S. typhosa hybrid strains which had acquired the E. coli K12 genes controlling formation of the receptor substance needed for adsorption of lambda were constructed by matings. Many of these hybrid strains, typified by S. typhosa hybrid WR4255, although now able to adsorb lambda, did not permit plaque formation by this phage. The WR4255 hybrid was lysed, however, by mutants of lambda which bypass the functioning of the N gene of lambda, and by hybrids between phage lambda and phage 21 which have replaced the N gene of lambda. The N gene of coliphage lambda specifies a protein which

regulates the expression of most other lambda functions. This regulation of lambda occurs at the level of transcription, since in the absence of N function, transcription of the lambda genome is significantly reduced. Two classes of bacterial strains have been found to inhibit the action of the N protein of phage lambda. One of these classes is made up of mutant bacteria from E. coli isolated by Dr. D. I. Friedman of the University of Michigan. The other class is represented by E. coli-Salmonella hybrid strains such as S. typhosa hybrid WR4255. A collaborative study was undertaken with Dr. Friedman to determine whether there was a genotype similarity between the E. coli mutant, referred to as Nus, and the S. typhosa WR4255 hybrid which would explain their phenotypic similarity in preventing lambda from plaque formation. Although the Nus mutant, strain K95, and S. typhosa hybrid WR4255, were obtained in uniquely different ways, both bacterial strains share the same major phenotypic characteristics. Each bacterial type does not permit the growth of wild-type lambda phages which are N-gene dependent, but does permit the growth of lambda mutants or lambda hybrids which are N-gene independent. The complete inhibitory effect of the Nus mutant, K95, is conditional, being expressed only at high temperature, 42°C, while the inhibitory effect of WR4255 is observable at all temperatures. Previous studies have shown that the introduction of an additional E. coli chromosomal segment into WR4255 enabled this S. typhosa hybrid to produce lambda phage, suggesting the presence of a lambda replication (λ rep) locus in the segment.

b. In a previous series of experiments, we demonstrated that in the S. typhosa hybrid bacterium, WR4255, a region of the E. coli genome located between minutes 60 and 73 of the 90 minute E. coli chromosome must be present in order for the N gene product of phage lambda to be functional. In these experiments, two E. coli Hfr strains were used which transfer the region of the E. coli chromosome between minutes 60 and 73 from opposite directions; WR2010 which transfers genetic markers in a counterclockwise direction originating at minute 73 and WR2020 which transfers genetic markers in the opposite direction originating at minute 60. Each of these two Hfr strains transfers the information necessary for lambda viability (activity of the N gene product) to S. typhosa hybrid WR4255 as an early marker. The E. coli locus involved in the replication of lambda was referred to as λrep. Using these two Hfr donors in mating experiments with the Nus recipient strain K95, we found that the Nus^+ phenotype (ability support lambda growth at 42°C) is transferred early by both Hfr strains. Therefore, it is concluded that the Nus gene is located between minutes 60 and 73 of the E. coli chromosomal map as is the case for the λrep gene.

c. In order to map the location of the Nus gene more precisely, a number of episome containing strains of E. coli carrying transmissible elements covering various portions of the 60 to 73 minute segment of the K12 chromosome were employed. In this manner the region containing the Nus gene and the λrep gene was narrowed to between minutes 60 to 65 of the K12 chromosome. To define further the map location, we used phage

P1vir to transduce suitable markers in this region. The first marker examined, argG, is located at minute 61 of the K12 chromosome. The phage was grown on a Nus$^-$ strain and used to transduce a Nus$^+$ strain which is argG$^-$. After purification, the argG$^+$ transductants were screened for the presence of the Nus$^-$ gene. The results indicated that the Nus locus is very closely linked (approximately 90% cotransduction) to the argG gene at minute 61 of the K12 chromosome. The phenotypic similarity of the λrep$^-$ and Nus$^-$ alleles, as well as their close linkage, suggested that these markers represent the same locus. We therefore used P1 transduction to map the λrep$^-$ locus of Salmonella. Although it is possible to transduce some markers from S. typhosa hybrid WR4255 to E. coli K12 recipients with P1vir, we were unable to recover any argG$^+$ transductants using an argG$^-$ E. coli recipient. A less direct approach was therefore undertaken to map the location of the λrep marker based on the assumption that λrep may in fact be an equivalent locus to Nus. This procedure involved transferring the arg$^+$ region to an E. coli recipient by conjugation with an appropriate S. typhimurium Hfr strain. A cross was therefore performed between S. typhimurium Hfr WR4021 (transfer orientation o-ilv$^+$-xyl$^+$-argG$^+$...metE..F) and an argG$^-$ E. coli recipient with selection for argG$^+$ recombinants. The small number of argG$^+$ hybrids which appeared were tested after purification for sensitivity to λ, λψcIcI7, λsx, and λbyp. All these argG$^+$ hybrids proved to be insensitive to λ and λcIcI7, but were able to plate λsx and λbyp at 37°C. This pattern is identical to that exhibited by Salmonella hybrid WR4255 and indicates that these exconjugants had acquired the Salmonella λrep$^-$ locus. Transduction studies, using one such argG$^+$ λrep$^-$ E. coli exconjugant, demonstrated that like the Nus allele, the λrep allele cotransduces at a high frequency with the argG locus. In these experiments, lysates of P1vir propagated in the argG$^+$ λrep$^-$ strain were used to transduce an aspB$^-$ Nus$^+$ argG$^-$ recipient WR2045. Analysis of the resulting argG$^+$ transductants revealed that 92% also carried the λrep marker derived from S. typhimurium. However, none of over 100 aspB$^+$ transductants carried the λrep locus nor were any double (aspB$^+$ argG$^+$) transductants recovered. Thus it was impossible for us to order the location of λrep with respect to the aspB and argG loci. We assume that these latter results reflect the genetic inhomology apparent in transduction of genes between E. coli and Salmonella. Nevertheless, it appears likely on the basis of all of the accumulated data that λrep and Nus represent equivalent loci.

Summary

1. Swiss white mice immunized with acetone-killed vaccines prepared from strains of Salmonella typhosa, S. typhimurium, and mouse virulent S. typhimurium hybrids which had acquired, by conjugal genetic transfer, the S. typhosa antigens 9, Vi, and d, were challenged with the S. typhimurium hybrids and with their S. typhimurium parent strain. The overall pattern suggested that the specificity of the somatic antigen (9 or 4) was the important factor in determining protection against death in this system. Vaccines prepared from hybrids expressing the Vi antigen, however, offered no more protection against a Vi expressing challenge organism than did those prepared from non-Vi expressing hybrids of the same antigenic constitution, implying that the Vi antigen played no role in the protection specificity observed in these experiments.

2. Six lac elements, contained originally in clinically isolated, lactose fermenting Salmonella strains, were transferred to a strain of Escherichia coli K12. In this host, each of these elements were shown to be conserved as supercoiled, circular molecules of deozyribonucleic acid. The molecular weights of these molecules, as determined by sucrose density gradient centrifugation, varied from 30 million to 56 million daltons. The calculated number of copies per chromosome varied from 1.4 to 3.7, depending upon the particular lac element examined. All exhibited a guanine plus cytosine composition of 50%. When tested with the E. coli male specific phage, R-17, and the E. coli female specific phage ØII, none of the E. coli K12 strains containing a lac element responded to either phage as do F-containing derivatives of E. coli K12.

3. A locus of the E. coli K12 chromosome involved in the expression of the N gene function of phage lambda has been genetically mapped. The phage specified function controlled by the N gene regulates the subsequent transcription of most of the lambda genome. The bacterial locus involved in N gene expression, called Nus for N utilization substance, maps near minute 61 of the E. coli chromosome based on cotransduction with the argG marker located at minute 61. Two different bacterial variants in which lambda N function is not active have been used in mapping the Nus locus, a mutant of E. coli K12, Nus, and a hybrid bacterium formed by genetic transfer between E. coli and S. typhosa. Although these two bacterial variants exhibit slightly different phenotypes, chromosome transfer studies demonstrate that the same genetic region is involved in the observed N-ineffective phenotype.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 122, Microbial genetics and taxonomy

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6446	74 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 HIST'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 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	3A762760A822	00	123
b. CONTRIBUTING				
c. [redacted]	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 74	4	164
FISCAL YEAR CURRENT 75	4	164

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) Immunopathology;(U)Kidney;(U)Ulcer;(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 kidney 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.

25 (U) 73 07-74 06 Clinico-pathological studies have determined that intestinal spirochetosis is identical in monkey and man, and intestinal spirochetes may become potentially pathogenic if spirochetes extensively invade the gut mucosa. U.S. soldiers developed acute diarrhea with positive shigella stool culture were analyzed on the basis of clinical, bacteriologic, sigmoidoscopic and rectal biopsy findings. Regardless of serotypes, all cases share similar clinical, sigmoidoscopic and histologic features. Studies of experimental amebiosis indicate that amebae penetrate the gut mucosa by the secondary effects on the epithelium mediated presumably by enzymatic activities of degenerating neutrophiles. Studies on pathological conditions of the gut by infection with corona virus and blind loop showed morphologic evidence of alteration of mucosa. Experimentally produced immune complex nephritis studied by histology and FA technique disclosed that the classic complement activation was not required but would be utilized by the alternate complement pathway for mesangial injury in pathogenesis of certain glomerulonephritis. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 73 - 30 Jun 74.

*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 00 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Diseases and Injuries

Investigators.
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Associates: MAJ Robert Colvin, MC: Tatsuo Hase, M. D.; Helen R. Jervis, Dr. Nat. Sc.; LTC Paul K. Hildebrandt, VC.

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 kidney 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, 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 two 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 at WRAIR and WRGH.

I. 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 recent study indicated that the large concentration of epithelial-associated indigeneous microbes resist access of pathogenic bacteria to the intestinal epithelial surface (Savage 1972).

Little is known about "intestinal epithelial cell-associated indigeneous microbes" in mammals including monkey and man (Nelson & Mata 1970). For this reason, we have expanded our previous study on spiral-shaped microbes infested at the surface of the colonic epithelium of the rhesus monkey and man (Takeuchi & Sprinz 1969, Takeuchi & Zeller 1972). In addition, we have studied gastric spirilla in the stomach of rhesus monkeys, another example of epithelial cell associated microbes.

A. Studies of Spiral-shaped Microbes in the Colonic Mucosa of the Monkey and Man

Background

Spiral-shaped microbes are one of the most common components of the indigeneous flora of the large bowel in mammals including monkeys and man. They are fastidious anaerobes and, therefore, difficult or impossible to culture and isolate. Little is known of their structural characteristics and their relation to the host. Our earlier studies on spiral-shaped organisms which characteristically colonize in almost pure culture on the surface of the colonic epithelium (IS: intestinal spirochetosis) of healthy monkeys and man have been expanded.

Our earlier report distinguished what was thought to be spirochetes at the brush border region of the colonic epithelium of this subhuman primate into two structurally different spiral-shaped microbes, spirochetes and flagellated organisms (Takeuchi & Zeller 1972). We have also shown that their massive infestation of the colonic mucosa of monkeys causes only destruction of microvilli and alterations of the apical cytoplasm of the epithelial cell but produces no other structural alterations of the underlying mucosal tissue (Takeuchi & Zeller 1972). By various morphologic techniques, we have studied IS in the vermiform appendices and the colon of human subjects, concluding that IS is identical in both the rhesus monkey and man.

TABLE I. Summary of Human Intestinal Spirochetosis

Case No.	Sex	Age	Location	Degree of Infestation	Histologic Diagnosis	Clinical Diagnosis
1 (WRGH)	M	51	Sigmoid	Localized	Adenomatous polyp	Cholelithiasis Ch. Cholecystitis
2 (WRGH)	M	75	Sigmoid	Diffuse	Adenomatous polyp	Routine sigmoidoscopy
3 (WRGH)	M	36	Sigmoid	Diffuse	Normal	Routine sigmoidoscopy
4 (WRGH)	F	19	Sigmoid	Diffuse	Normal	P.I.D. gonorrhea
5 (NH)	F	17	Appendix	Diffuse	Normal	Endometriosis
6 (NH)	F	25	Appendix	Diffuse	Normal	Ovarian cyst
7 (NH)	F	35	Appendix	Diffuse	Normal	Leiomyoma uteri
8 (NH)	F	36	Appendix	Diffuse	Normal	Leiomyoma uteri
9 (NH)	F	36	Appendix	Diffuse	Normal	Leiomyoma uteri
10 (NH)	F	45	Appendix	Diffuse	Normal	Leiomyoma uteri
11 (NH)	F	45	Appendix	Diffuse	Normal	Leiomyoma uteri
12 (NH)	M	48	Appendix	Diffuse	Acute appendicitis	Acute appendicitis

WRGH: Walter Reed General Hospital; NH: Nyack Hospital

Incidence and Clinical Aspects

Utilizing various morphologic parameters, the incidence of IS was as follows:

Rhesus monkey:

Out of the control population of 221 healthy monkeys, thirty-one (14.0%) were found to be massively infected by spiral organisms in the colon; nineteen (8.5%) were found to be infected in both the colon and rectum, while twelve (5.5%) were diffusely infected from the cecum to the rectum throughout. No spiral organisms were identified in the histologic sections of the small intestine.

Human subjects:

The pertinent information on human IS is summarized in Table I.

Four cases out of 210 colonic specimens and biopsies examined at Walter Reed General Hospital (WRGH) (1.9%) were found to be affected by IS; two of them were associated with adenomatous polyp (cases 1 and 2) and the remaining two cases represented normal colonic mucosa.

There were 8 cases of IS out of 388 appendices (2.1%) removed surgically at Nyack Hospital (NH); only one specimen showed mild acute appendicitis (case 12) and the remaining 7 were normal.

There were no significant G.I. symptoms with the exception of two cases (cases 1 and 12); the former showed a history of epigastric discomfort and dyspepsia and the latter revealed fever and right lower quadrant pain, symptoms which appear to be attributable to cholelithiasis and acute appendicitis, respectively.

Morphologic Characteristics

The histologic as well as ultrastructural characteristics of all 12 cases of human IS, including the acutely inflamed appendix (case 12) and the adenomatous polyp (case 1 and 2), were indistinguishable from those of the monkey IS.

Histology

In paraffin sections stained with H & E, the brush border of the normal colonic epithelium was rather narrow and was uniformly stained by eosin. In contrast, the brush border region infected by spiral organisms was broader than normal and characteristically basophilic (Fig. 1). The spirochete-infected brush border stained with the

Fig. 1 - Surface of colonic mucosa infested with spiral-shaped organisms: human appendix (Case No. 10). A dark hairy fuzz at the brush border region (arrow) depicts multiple spirochetes and flagellated microbes localized on the surface epithelium. In contrast, the brush border of the surface epithelium of the normal colon is regular and thin. H & E. X 400

Giemsa and the Warthin-Starry stains. Gram staining was inconsistent. Spirochetes were never found deep in the crypts. There were no histologic changes in the spirochete-infected mucosa; the epithelial cells appeared unaltered and there was no inflammatory response in the lamina propria with excpetion of the case of diffuse IS associated with acute appendicitis.

Histochemistry

The spiral organisms also stained strongly with PAS and weakly with Alcian Blue, indicating the presence of a large amount of neutral and of a little amount of acid muco-substances. When Alcian Blue was followed by PAS, the organisms showed a strong purple-red color, more intense than that in the goblet cells. When stained with high iron diamine-Alcian Blue sequence, the spirochetes stained only with the Alcian Blue, indicating that the acid component lacked sulfated groups. Tests for the demonstration of various phosphatases and of leucine aminopeptidase carried out on fresh frozen sections did not reveal any specific concentration of these enzymes in the spirochetes. On the other hand, spirochetes showed activity for various dehydrogenases. Frozen sections stained with ORO were negative.

Electron Microscopy

At low magnifications, organisms were easily recognized by their characteristic spiral shaped structure. They were usually localized in the brush border or in the apical cytoplasm of the columnar cells and tended to be present in large numbers. Multiple spiral-shaped organisms frequently almost completely replaced the microvilli (Fig. 2). Most of the fuzzy coat of the glycocalyx was lost and only a thin cap of fuzz was occasionally seen over the tip of the remaining intact microvilli.

Higher magnification, even with the thin sectioning technique, showed that there were two structurally different spiral-shaped organisms, spirochetes and flagellated microbes (Fig. 3). The negative contrast staining technique applied on mucosal scrapings provided structural details of these two organisms.

Intestinal spirochetes had two to six spirals. They measured 3-6 μm in length and 0.2-0.4 μm in diameter, and had gently tapered ends. The spirochete had a central protoplasmic cylinder (cytoplasm) enclosed by a cytoplasmic membrane. Between the cytoplasmic membrane and the cell wall was a space which contained a group of axial fibrils. Some spirochetes showed 12 individual fibrils at the mid-portion and 6 fibrils at each end (6-12-6); others revealed 8 fibrils at the middle and 4 at each end (4-8-4). All axial fibrils wound around the

Fig. 2 - Brush border region of the surface colonic epithelium with massive infestation of spiral organisms: rhesus monkey. Microvilli are completely replaced by numerous organisms which intimately populate the epithelial surface. Well defined spherical masses represent poor fixation and inadequate penetration of embedding media; they are placed only at the fibrillar cytoplasm of flagellated microbes. In contrast, these artefacts are absent in spirochetes (S). Flagellated microbes are predominant. Numerous flagella are evident (arrowheads). X 16,000

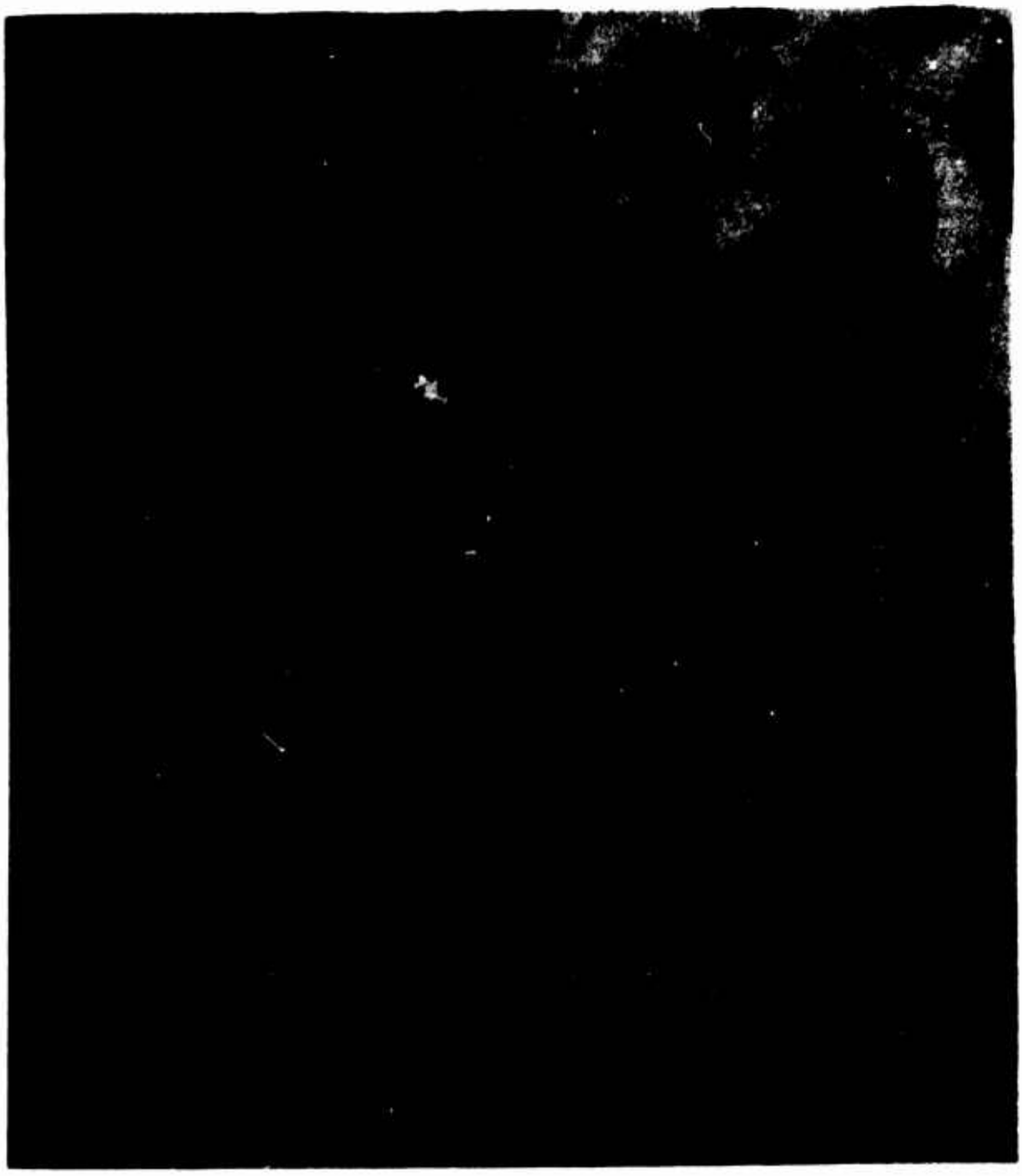


Fig. 3 - Brush border region infested predominantly by spirochetes: human appendix. Most of microvilli have disappeared with a remnant remaining (MV). The penetrating part of organisms shows a close contact with the invaginating host cell membrane. The spirochete consists of a cytoplasmic cylinder, axial fibrils and cell wall (large arrowhead); the cytoplasmic cylinder is enclosed by cytoplasmic membrane (small arrowhead); axial fibrils (semicircular line) between cell wall and cytoplasmic membrane are wound around the cytoplasmic cylinder like a helix. FM: indicates flagellated microbe, with flagella (arrows). X 78,000

cytoplasmic cylinder in a helical form, originating from the terminal disc at each end and extending from one end of the organisms to the other. These structural characteristics are shared by other types of spirochetes. Flagellated microbes were always less frequent than spirochetes and had two to four spirals. They were 4-6 μm long and 0.2-0.4 μm wide. They terminated bluntly, showed a single flagellum originating from each end, and had no axial fibrils between the cytoplasm and the cell wall.

Spirochetes and flagellated microbes were the only two types of organisms identified in the brush border. The ratio between the two types of organisms varied greatly from one case to another in both human subjects and monkeys, but spirochetes were usually predominant. Both organisms could be embedded side by side in the brush border, sometimes penetrating into the apical cytoplasm, and showing a close physical contact with the invaginated laminal plasmalemma of the epithelial cell. Sometimes flagella coiled around the flagellated microbes.

In rare instances, both spirochetes and flagellated microbes were seen penetrating further into the colonic epithelium. In the cytoplasm of columnar cells, they were enclosed by a single membrane, or contained in an autophagic vesicle together with host cytoplasmic components. Otherwise, the remainder of the cyto-components of epithelial cells appeared unaffected by their presence. Both spirochetes and flagellated organisms were occasionally also found in the intercellular space between epithelial cells and in the lamina propria, where both organisms could be noted either free or in macrophages; in the latter case they were enclosed tightly by a single membrane or contained in large phagosomes with other cyto-components. Both spirochetes and flagellated organisms within the epithelium, and even within phagocytic cells, appeared morphologically unaltered. There was no inflammatory response in the lamina propria.

Discussion

The present study has demonstrated the morphologic findings and ultrastructural characteristics of intestinal spirochetosis (IS) in the rhesus monkey and man, from which we have concluded that IS is essentially identical in both species. IS is a common condition (28%) in the healthy rhesus monkey (Macaca mulatta). In contrast, the incidence of this condition in man is far less common (2%) than in the monkey counterpart. Harland and Lee have reported that IS occurred in 10 out of 100 consecutive human rectal biopsies, and was associated with symptoms relating to the bowel or with organic lesions such as carcinoma and multiple polyps of the colon (Harland & Lee 1967). Lee et al. (1971) in their review of light and

electron microscope studies reported that IS occurred in 6.9% of rectal biopsies and 7.8% of the appendices seen and stated that human IS was not consistently associated with G.I. symptoms. Shera (1962) stated that his 53 cases of spirochetosis were associated with "strawberry lesions" of the colon and presented mild to severe diarrhea; the diagnosis was established only by fecal smears which contained large numbers of spirochetes; no spirochetes, however, were demonstrated in histologic sections of strawberry (granular) lesions. In our human cases, with one exception, and in all the monkeys, no bowel symptoms were associated with IS. In the one human case (case 12), the massive spirochete infestation at the brush border of the epithelial cells appeared incidental to acute appendicitis.

In previous ultrastructural studies on human intestinal spirochetosis, only Harland and Lee (1967) and Lee et al. (1971) have observed spiral fibers in the brush border infesting organisms; therefore, they concluded that these were spirochetes, while Gear and Dobbin (1968) did not mention the presence of axial fibers but described them as spirochetes because of their silver positive reaction in mucosal smears. In neither report were the ultrastructural details of the organisms given. Our electron microscope investigations on IS have demonstrated the existence in both monkey and man of two structurally different microorganisms, a true spirochete and a flagellated microbe. Rosebury, in reviewing the classification of spirochetes indigenous to man, found the situation to be one of "near chaos" (1962). As yet, the current classification depends primarily upon the staining characteristics and morphology of the organisms when viewed by light microscopy. Spirochetes in mammals were classified as Borrelia or Teponema, mainly on the basis of their affinity for aniline dye. Later, Listgarten and Socransky (1965) used the electron microscope as an aid in a taxonomic differentiation of oral spirochetes based on size of the protoplasmic cylinder, number of axial fibers and structure of outer envelope. On the basis of staining characteristics for aniline dye, the intestinal spirochetes belong to the genus Borrelia. On the basis of ultrastructural characteristics, we were able to conclude that the brush border spirochetes are morphologically identical to oral spirochetes and their major cell type is that of Borrelia vincentii (Listgarten & Socransky 1965). However, it can not be concluded from their ultrastructural characteristics alone that both oral and intestinal spirochetes are identical organisms.

It has been suggested that the constant association of flagellated microbes and spirochetes signifies a possibility that intestinal spirochetosis is a variant of Vincent's angina (20). Vincent's angina, identified also as fusospirochetal disease, is known as one of the

most complicated symbiotic infections and is said to be caused by both spirochetes and fusiform bacteria acting in symbiosis with other types of anaerobic bacteria (Smith 1932). Under the phase microscopy, fusiform bacteria can be easily distinguished by their characteristic shape from other enteric microbes. The polar flagellation is absent in published electron micrographs of Fusobacterium fusiforme and Fusobacterium pleomorphum (Takagi 1962). The exact identification and taxonomic differentiation of flagellated microbes, however, must await successful culture methods and biologic and serologic testing.

The brush border of the intestine is an important region from the physicochemical and immunological point of view. Rich in various enzymes, the surface of the microvilli is coated by a fuzzy outer layer composed of a mucopolysaccharide complex and covered by immunoglobulin A. The present study calls attention to the effect of massive microbe infestation at this important region of the colonic mucosa. Spiral organisms do invade host cells, but only their upper three cyto-components - glycocalyx, microvilli and terminal web, while the other host cellular structures remain unchanged. This implies that cell structures might be selectively destroyed without apparent ill effect to the rest of the cell. In rare instances, spirochetes penetrate deeper into the epithelial cytoplasm beyond the upper three components and are capable of invading the lamina propria. Yet, the effect of penetration if self-limited and elicits no inflammatory response. In the colon of normal rats (Davis et al. 1972) and dogs (Leach et al. 1973) spiral organisms are present above the microvilli and can penetrate the epithelial cells without eliciting a cellular response.

Conclusion

Intestinal spirochetosis (IS) is the infestation of the gut mucosa by spiral-shaped organisms. The incidence of IS is 28% in monkeys and 2% in human subjects. IS is not associated with any G.I. symptoms. In both primates, the morphologic characteristics and the relationship to the host are indistinguishable. Spiral organisms stained with H & E appear as broad basophilic haze on the colonic surface and are strongly positive by Warthin-Starry (silver) stain. Spiral-shaped organisms include two structurally different organisms, spirochetes and flagellated microbes. They intimately populate the brush border region of the surface of the colonic epithelium. They are absent in the crypt and in the small intestine. Infestation by spirochetes produces no alterations of cyto-components of the underlying host structures. Under as yet undetermined conditions,

spirochetes are capable of penetrating beyond the brush border region into the epithelial cytoplasm and also into the lamina propria. Although this occurrence is very rare, it is possible for mucosal penetration, when it occurs in large numbers, to cause structural alteration and clinical symptoms in hosts.

B. Studies of Gastric Spirilla with Mucosa of the Stomach of the Rhesus Monkey

The presence of spirilla (GS) in gastric mucosa has long been recognized in various animal species including monkey and man. GS are fastidious anaerobes and have not been cultured even with strict anaerobic conditions. As a result, little is known about the entity and the condition is often overlooked. Using histology, thin sectioning, negative staining and shadow casting techniques by EM, we studied structure of GS and their host relationship in healthy rhesus monkeys. In paraffin sections stained by H & E, GS may be mistaken for strands of mucus. GS are Gram-positive and best demonstrated by silver stains. In thick sections of Epon-embedded tissue, GS are recognized as "corkscrews" with up to 12 coils. They are 8um long and 0.8um wide. GS have characteristic polar flagella. They concentrate in isthmus and are found less frequently in neck and base of gastric glands, while they are rare or totally absent in gastric lumen. GS are closely associated with parietal cells (Fig. 4) and are capable of penetrating into parietal cytoplasm. Preliminary studies have indicated that in either intra- or extra-cellular locations, GS elicit neither changes of host cyto-components nor inflammatory response.

To date GS have been found in the stomach of all of 33 monkeys examined. The precise role of GS infestations in the gastric mucosa is being investigated under normal and pathological conditions.

Fig. 4 - Gastric spirilla (GS) in glandular lumen of isthmus, stomach, healthy rhesus monkey. A spirillum is in a direct contact of microvilli of a parietal cell (PC). Mucus granules are in a process of discharge from a mucus neck cell (arrows). Mucus granules (MG).

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 ameba. 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 ameba, 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 ameba and the subsequent changes of the mucosal tissue surrounding invading ameba. Some believe the ameba penetrates 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. However, definitive information concerning the specific mechanism by which invasion takes place remains undocumented.

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.

The present investigation was undertaken to clarify the mode of tissue invasion of E. histolytica from gut lumen into lamina propria through cecal epithelium and how cytoplasmic components of host cells respond to this penetration.

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 the cecum. Each section was divided into two pieces and processed for light and electron microscopy.

Observations

Light microscopic observations of paraffin sections of the ceca from infected guinea pigs showed many trophozoites, massed focally in the lumen in association with cellular debris and eosinophilic exudate, and on or near the surface (interglandular) epithelium; fewer were in the crypts lumen (glandular). Even slight mucosal pathologic changes such as mucin discharge occurred always in conjunction with the presence of amebae. Unless the proliferation of amebae in the cecum was massive, there was no generalized mucosal response to the infection.

The exact position of the amebae in relation to the epithelial elements and the tissue response to them were better revealed in one μ sections of Epon-embedded material. Amebae were seen in more or less close contact with the surface (Fig. 5) or the crypt epithelium (Fig. 6). Elsewhere one or more amebae could be seen between epithelial cells (Fig. 8), most commonly within the surface interglandular epithelium. If multiple sites were involved, the amebae were seen equally within the interglandular and glandular epithelium. Surface epithelial cells in proximity of the intraepithelial amebae were often cuboidal rather than columnar, with indistinct brush borders (Figs. 5, 6).

Fig. 5 - An ameba (AM) is away from the epithelial barrier while other amebae contact the interglandular epithelium (arrows). In the gut lumen, cell debris are mixed with mucus strands. X 650

Fig. 6 - An ameba is in the crypt lumen showing a close contact with the brush border of crypt epithelial cells (arrow). X 550.

Fig. 7 - An ameba is seen between cells of the interglandular epithelium and another is in contact with the epithelial cells. The epithelial cells are cuboidal and irregular. The brush border is indistinct. In the glandular lumen an ameba (3) is close to the epithelial surface while another (4) contacts epithelial cells. X 460

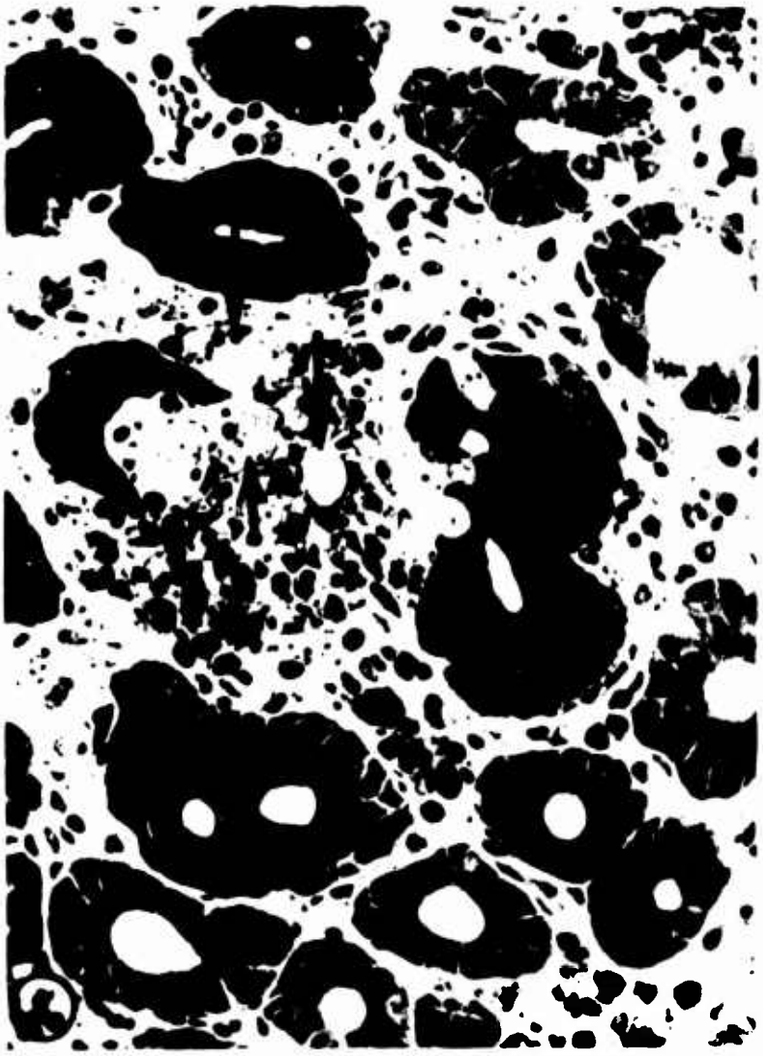


Fig. 8 - Amebae (small arrows) are within the crypt epithelium, or in the lamina propria. Another ameba had already invaded the sub-epithelial tissue (large arrow). X 350

Aggregations of bacteria were present in the gut lumen but none were identified within the mucosa unless they were associated with intramucosal amebae. Goblet cells were few in number, severely discharged, and occasionally absent. Crypt glands were often distorted with dilated lumina. Polymorphonuclear leukocytes (PMN) infiltrated around the sites closely associated with amebae. Extravasated erythrocytes were present in the lamina propria and occasionally within the epithelial lining.

Electron microscopic observations confirmed the light microscopic findings and revealed further details. If no amebae were seen in the general vicinity of the mucosal epithelium in the infected guinea pigs, the epithelium was the same as in the control. When the amebae, however, were associated with a certain area of the mucosa, even if not in contact with the surface epithelium, epithelial cells sometimes showed changes including reduction in number and height of microvilli, accumulation of membrane-bound lipid droplets and occasional autophagic vesicles. However, other cyto-components of epithelial cells, including the glycocalyx, remained unaltered.

When the amebae were closer to the surface epithelium, the glycocalyx overlying the microvilli became obscure or disappeared, while dense material was present between the organism and the altered microvilli (Fig. 9). Such material was absent from the microvilli in the immediate vicinity and from the remaining membrane of the advancing ameba. Concurrently, a variety of cytoplasmic changes were seen in the epithelial cells and the microvilli shortened and became sparse. The fibrillar cores of microvilli and terminal web became obscure and lipid droplets increased in number. The mitochondria were often swollen with an opaque matrix and often deranged cristae. Meanwhile, both rough and smooth ER were dilated. PMN emigrated from the capillary lumen and appeared in the underlying lamina propria and sometimes migrated through the epithelium. In contrast, proximity of an ameba to the brush border of epithelial cells in the crypts was associated with a lesser degree of alterations of host cyto-components limited mainly to the microvilli. Even when the ameba was in direct contact with the flattened microvillous surface, cytoplasmic alterations of these epithelial cells were minimal or absent (Fig. 10). Interaction between epithelial cells and amebae, when these were located between epithelial cells, however, elicited the same responses of host cytoplasmic components regardless of the location With the amebae in close apposition, microvilli completely disappeared and epithelium was limited by a smooth luminal plasma membrane. When this occurred, the apical cytoplasm, lacking cell organelles (Fig. 11), bulged and projected toward the lumen. These cells, which were in very close proximity to amebae, were seen as detached from adjacent cells,

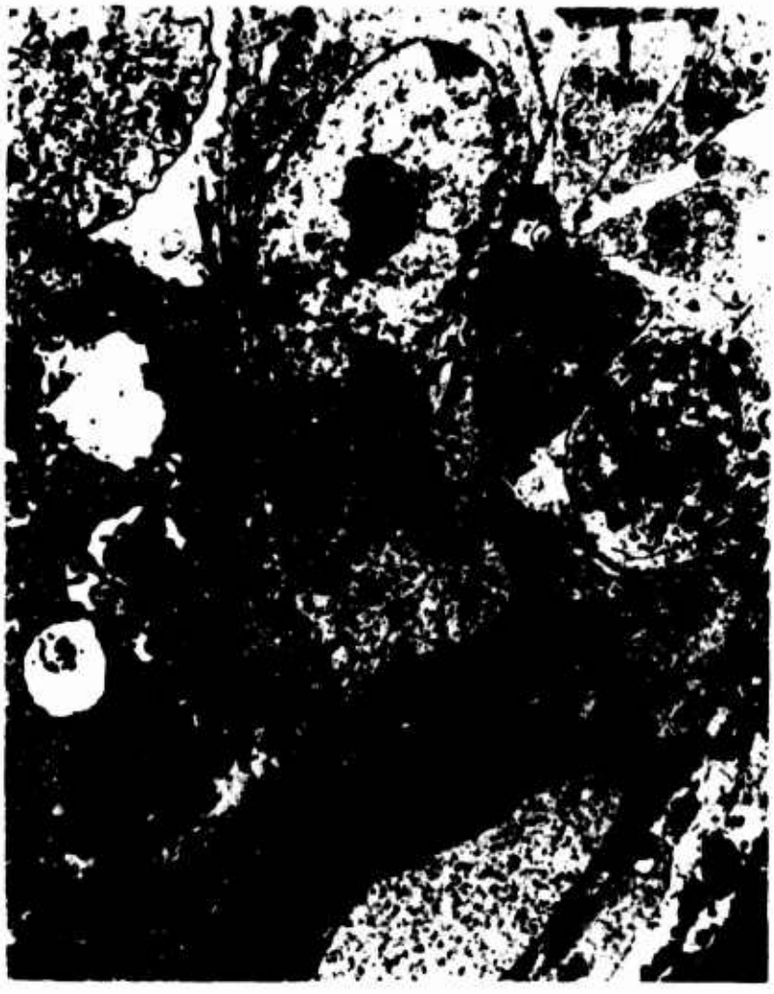


Fig. 9 - Two amebae lie close to microvilli of surface epithelial cells. Note dense material between amebae and the short, sparse microvilli (arrows). Epithelial cells are cuboidal and their cytoplasmic components show swollen mitochondria, lipid droplets (arrow heads) and dilated ER. A portion of a PMN is in the subepithelial region near the basal lamina (dotted line). F: Fibroblast. L: Lymphocyte. Refer to Fig. 15-1. X 7200

Fig. 10 - Ameba in the crypt lumen shows contact with the microvilli of epithelial cells. Microvilli are short and sparse (right half), while those at lower left for the most part have disappeared with occasinal remnants remaining. At upper left, the microvillous surface is flat and in contact with the cell membrane of the ameba (arrows). Otherwise, cyto-components of host cells are unaltered. Dense material is absent. Note, at bottom, multiple invaginations of the cell membrane of the organism. E: Phagocytosed erythrocyte. FV: Food Vacuole. Corresponds Fig. 15-3. X 8400

Fig. 11 - Amebae (AM) in contact with the luminal surface of interglandular epithelial cells (EP). Varying degree of cytoplasmic changes ranges from nearly normal in one cell (EP1) to severe alterations (EP4). Microvilli and terminal web for the most part have disappeared with a remnant remaining (large arrow) in a host cell (EP2). The cell membrane of amebae is thicker and denser than the host plasmalemma. AM1 has adhered to the projected apical cytoplasm of host cell (EP3). Note spectrum of mitochondrial changes (small arrows) within a single cell (EP4). X 10200

Fig. 12 - Several epithelial cells extruding from the surface epithelial lining next to an ameba. Note a variety of cytoplasmic changes in extruding cells. Extruding cells have been separated from the basal lamina (dotted line) producing spaces that are filled with an erythrocyte and PMN. Spectrum of degeneration of nuclear material in PMN ranges from near normal (1), to condensation of nucleoplasm and cytoplasm with extrusion of granules into extracellular space (2, 3, 4) to lysis of cell membrane with release of cyto-components (5). The luminal membrane is partially disrupted (arrow) in close proximity to lysing PMN with cytoplasmic fragments (5) (Fig. 15-5). X 4100

Fig. 13 - Higher magnification of a section adjacent to Figure 7 shows a portion of ameba cytoplasm (AM1), (apparently a pseudopodium), extending from ameba AM2 toward the basal lamina (dotted line). The basal cytoplasm of a host cell lacks cellular organelles and shows cytoplasmic projection (arrow) toward the pseudopodium (AM1). Note close contact between membrane of host cell (EP4) and ameba (AM1). Increasing degeneration process ranging in degree from EP1 to EP4 is evident. M: altered mitochondria. A third ameba (AM3) is in the lumen. LD: Lipid Droplet. X 9100

Fig. 14 - In the epithelial amebae (AM1 and 2), AM2 is beyond the intercellular space and basal lamina (dotted line) within the lamina propria, while AM1 is between epithelial cells. Extruding epithelial cell (EX1) shows V-shaped cytoplasmic projections (CP), one of which (CP1) adheres to AM1 and the other (CP2) contacts the ameba in the lamina propria (AM2). Surrounded by extruded cells (EX2 and 3) and CP1 and 2 are extracellularly discharged granules and cyto-components of degenerating PMN (2-4). A normal PMN (1) is present in the lamina propria. X 6100

either from the basal lamina (basement membrane) or from both basal lamina and lateral plasmalemma of adjoining cells, thus producing spaces between epithelial cells (Fig. 12). In detaching cells, the cytoplasmic alterations described above became more severe. Mitochondria were most severely affected. The cristae were further deranged and electron opaque spherical granules became evident in the matrix (Figs. 11, 13). At this stage, increasing numbers of PMN and rare macrophages were found in the epithelium, filling the intercellular spaces (Fig. 7). PMN showed a variety of degenerative processes including depletion of glycogen particles, condensation of cytoplasm and nucleoplasm, pinching off of cytoplasm and disruption of cell membrane, resulting eventually in release of cytoplasmic contents (Figs. 12, 13). This occurred most frequently in PMN which were in direct contact with amebae.

Epithelial cells separated lastly from adjacent cells at the intercellular tight junction; at this stage the amebae were seen between adjoining cells. Occasionally amebae were found within the epithelium at the level of the intercellular tight junction but extending pseudopodia to the basement membrane (Fig. 13), or in the extracellular space of the lamina propria beyond the basal lamina (Fig. 14).

Discussion

On the basis of the present studies, it was possible to establish the probable mode of E. histolytica penetration in the cecal mucosa of the conventionalized guinea pig which, we believe, may well represent what happens in the natural human host. Contributing to the value of this investigation was the choice of a germ free experimental host inoculated with E. histolytica and the enteric flora from a human case of symptomatic amebiasis. Several years' experience with this model has indicated its superiority over conventional animals for study of enteric amebic disease. Conventional animal models have floras characteristically different from that encountered in human patients, and it has been shown that different bacteria may influence appreciably the many facets of enteric amebic disease (15).

Under the conditions of this experiment, penetration of amebae in the cecal mucosa was asynchronous and proceeded at different rates in different animals and even in different areas of the cecum of a single animal. The sequence of phenomena which occurred during the process of penetration was reconstructed from hundreds of individual observations on the interplay of amebae with the mucosal epithelium, as amebae were found increasingly closer to and finally into the epithelium. Changes observed in the epithelial cells followed a consistent pattern which formed the basis for our interpretation

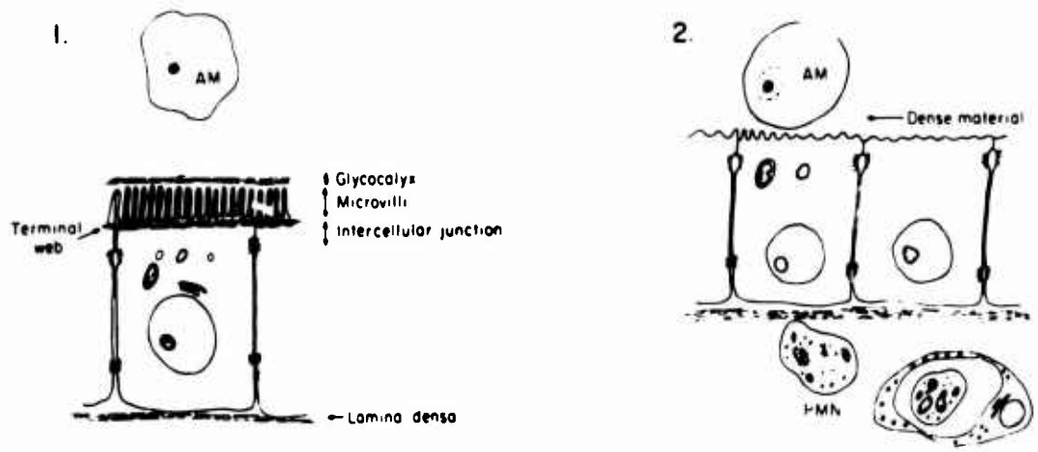
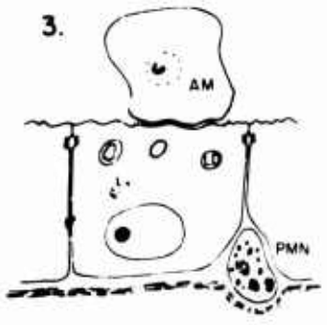
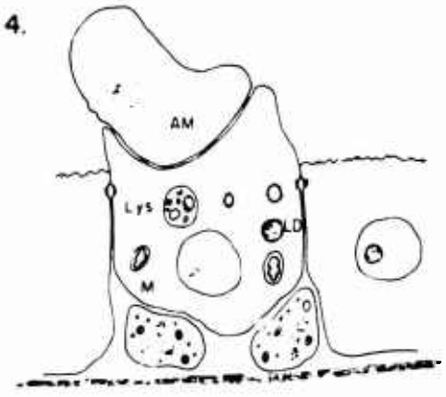


Fig. 15 - A schema, based on results of present studies, illustrating penetration of cecal epithelium of guinea pigs by trophozoites of Entamoeba histolytica following intracecal inoculation of amebae and enteric flora from human case of amebic colitis.

1. With ameba (AM) in the gut lumen away from the brush border, epithelial cells occasionally show lipid droplets and shortening of microvilli. Otherwise host cyto-components remain unaltered. The brush border consists of glycocalyx, microvilli and terminal web which ends at the level of the intercellular junctional complex. Mitochondria and endoplasmic reticulum are present in the apical cytoplasm of the epithelial cell.

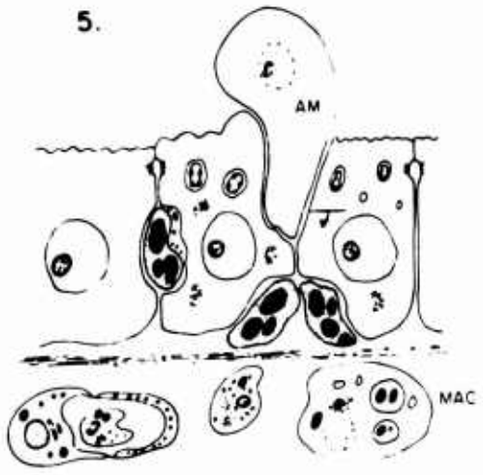
2. As the ameba comes closer to the brush border, microvilli become shortened and irregular. Lipid droplets are common in the epithelial cytoplasm. Dense amorphous material emerges between the ameba and microvilli. Polymorphonuclear leukocytes (PMN) migrate from the capillary into the lamina propria beneath the epithelium.

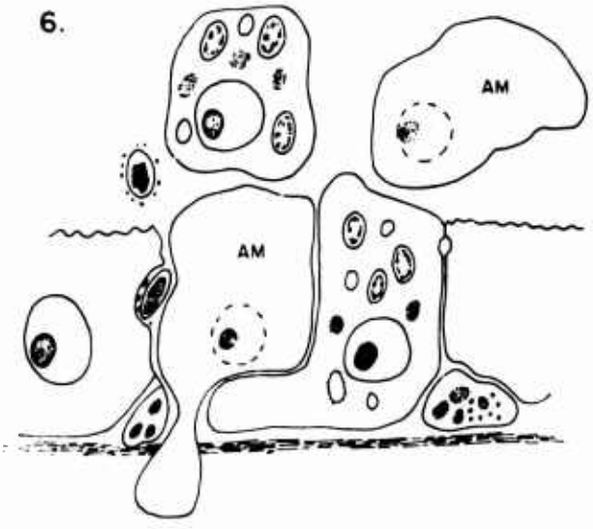




3. As the ameba advances further, the microvilli become further altered and transformed into a smooth luminal plasmalemma while the dense material disappears. Meanwhile, PMN begin to migrate into the intercellular space of the epithelium. LD: Lipid Droplet.

4. Further progression of the ameba results in its direct contact with the projecting apical cytoplasm of the epithelial cell. Mitochondria are swollen, ER dilated, and lipoid droplets increase in number. Autophagic vacuoles (AV) become common. PMN move through the interepithelial space which remains structurally unaltered.





5. The pseudopodia of ameba penetrate into the epithelium by separating the intercellular junction. Cytoplasmic alterations of epithelial cells become evident, and PMN in the vicinity of the ameba begin to show degeneration.

6. The intercellular ameba moves further by pseudopodia and reaches the lamina propria through the basal lamina (basement membrane) while extrusion of epithelial cells into the lumen is completed.

of the process of penetration as illustrated in Fig. 15. As the amebae approached the cecal surface, the epithelial cells underwent profound cytoplasmic changes. First the microvilli were shortened (Fig. 15-1, -2, -3) and lipid droplets in increasing numbers formed in the apical cytoplasm. These observations paralleled previous observations on enteric infections with pathogenic bacteria (Takeuchi et al. 1965; Takeuchi 1967; Takeuchi, Sprinz and Formal 1968). Fat seen in colonic epithelial cells of monkeys infected by Shigella flexneri occurred before penetration of epithelium by the bacterium and was considered to represent a functional disturbance of intracellular metabolism in host cells by the focal toxic effect of the luminal Shigella (Takeuchi, Formal and Sprinz 1968). This, we believe, is pertinent to the present ameba study; toxic substance(s), a product of the ameba, may be responsible for early focal changes in epithelial cells.

The brush border of intestinal epithelium is important from physicochemical and immunological points of view; recently it has been described as coated with secretory immunoglobulin A. The present study demonstrated the impact of E. histolytica on this important region of the large intestine. As the ameba came close to epithelial cells, electron dense material developed and localized between the advancing portion of organisms and the microvilli (Fig. 15-2). Since goblet cells in the vicinity were discharged, the material was not likely to be mucus, but appeared to be glycocalyx, produced in excess by the epithelial cell in the vicinity of the organism. It is possible, however, that the dense material could be a secretory product of amebae, or derive from both amebic and epithelial sources. No attempt was made to chemically define the material.

As a first step in the process of epithelial penetration, the ameba adhered to the luminal plasmalemma of the cytoplasmic projections of epithelial cells (Fig. 15-3, -4). In these cells, microvilli, their internal fibrillar cores and the terminal web had disappeared, indicating that structural support of the luminal surface of host cells was virtually lost. As a result, the ameba was capable of establishing a very close contact with the cell surface. It is conceivable that separation of adjoining cells might occur by the ameboid activity of the organisms exerting pressure on clearly damaged epithelial cells (Fig. 15-5). Cell separation, however, was observed to begin at the basal lamina before any evidence of amebic penetration and tight junctions dissolution, and might be based on a different mechanism. To this effect the osmiophilic granules in the mitochondria of epithelial cells in contact with

penetrating amebae were of considerable interest. These granules bore a striking resemblance to intramitochondrial granules which have been described in myocardial cells injured irreversibly by a transient ischemia (Jennings et al. 1971) and also in hepatocytes under the toxic effect of carbon tetrachloride poisoning (Reynolds 1965). Both studies provided evidence that they were calcium dependent granules and that their emergence was a morphological expression of accelerated influx of calcium ion into mitochondria which indicated alteration of intracellular distribution of calcium ion. In our studies, it is interesting to note that the process of epithelial cells separation was associated with the presence of these granules, suggesting that changes in calcium metabolism and attendant loss of calcium at the tight junctions, which are calcium dependent, might be responsible for the failure of the junctional complexes at the site of penetration.

The present studies provided additional morphologic evidence for chemotaxis of leukocytes in early enteric infections with bacteria and protozoa, in particular during the stage of epithelial penetration by pathogens about which little is known. In an EM study of salmonella-produced acute enteritis in guinea pigs, it has been shown that penetration of ileal epithelial cell by Salmonella typhimurium produced a series of alterations in epithelial cytoplasm and elicited an inflammatory response in the lamina propria (Takeuchi and Sprinz 1967). On the other hand, ultrastructural observations on colonic epithelium-normal flora relationships in rats and rhesus monkeys showed that intestinal spirochetes (part of the normal flora in these species) were capable of penetrating the colonic epithelium without eliciting an acute inflammatory response (Takeuchi and Zeller 1972; Davis et al. 1972). It is thus suggested that only passage of the enteric barrier by virulent microbes is capable of producing acute inflammation in the lamina propria (Takeuchi & Sprinz 1967). In the guinea pig amebiasis, focal acute inflammation occurred in the lamina propria before epithelial penetration by the pathogen (Fig. 9). PMN migrated from capillaries and began to accumulate in the sub-epithelial tissue in the immediate vicinity of luminal amebae.

Epithelial penetration by E. histolytica elicited a series of degenerative changes in transepithelially migrating PMN. This occurred in PMN in the immediate vicinity of the ameba both with and without direct contact. It has been reported that such contact occurring either in vivo or in vitro results in lysis of PMN (Griffin 1971; Jeruminta & Kradolfer 1964) but not in disruption of the cell membrane of the ameba contacting the PMN (Griffin 1971; Cherez 1972).

Rich in various enzymes, including lysosomal hydrolases, collagenases, protease and esterases (Weissman et al. 1972; Jaroff 1972; Lazarus 1972), lysates of PMN are capable of degrading such protein-polysaccharide complex structures as basement membrane and cartilage (Jaroff 1972). Whatever the precise mechanism of PMN lysis might be, the lysates of PMN would probably contribute to changes in host tissue including separation of cells and facilitate the further desquamation of epithelial cells.

The present investigation revealed a mechanism of mucosal penetration by E. histolytica which had not been previously suggested and provided no data to support previously held theories on this subject (Ratcliff 1932; Dobell & O'Conner 1921; Rees 1929).

E. histolytica seems to occupy a unique position among enteric protozoa capable of penetrating the enteric epithelial barrier. These include Eimeria sp. in the rabbit and rat, Hexamita muris and Giardia lamblia in humans. Although the mode of penetration from gut lumen to the epithelium by these protozoa is not clear, when they penetrate they are identified within the epithelial cell. In contrast, E. histolytica passes through the junctional complex and appears to move actively by pseudopodia within the intercellular space, reaching the lamina propria.

Conclusion

Germ free 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 mode of penetration of cecal epithelium by the ameba was examined by light and electron microscopy. Initially the ameba penetrated the interglandular epithelium. Later, it penetrated equally the glandular and interglandular epithelial barrier. As the ameba approached the brush border, the microvilli became shortened, irregular, and eventually disappeared. Dense material appeared between the advancing ameba and microvilli. As the ameba progressed further, the apical portion of the epithelial cytoplasm projected into the lumen contacting the organism, thus becoming detached from adjoining cells. This produced spaces between epithelial cells through which amebae invaded interepithelial spaces. There were marked alterations of cytoplasmic components of epithelial cells. Polymorphonuclear leukocytes migrated into the epithelium filling these spaces; these often showed a variety of degenerative processes. Amebae, utilizing their pseudopodia, moved further through the intercellular spaces and reached the lamina propria.

Further studies on cellular responses of mesenchymal cells and microcirculation in the colonic mucosa subsequent to the epithelial penetration by ameba 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. Clinical, Bacteriologic, Sigmoidoscopic, and Rectal Biopsy Studies of Acute Shigellosis in American Soldiers

We have been studying clinical, bacteriologic, sigmoidoscopic and rectal biopsy findings on 33 American soldiers who developed acute diarrhea and positive Shigella stool cultures while stationed in Vietnam in 1970.

Fecal bacteriology showed: 15 Sh. Flexneri, 13 Sh. sonnei, 4 Sh. dysenteriae, and 2 Sh. bodyii cases. In 5 cases, several serotypes of pathogenic E. coli were also cultured. All cases exhibited sudden onset of watery diarrhea, and a majority abdominal cramps often with vomiting. Leukocytosis was seen in 16 cases. Hematocrit was within normal range.

Sigmoidoscopy showed consistent edema and hyperemia. Bleeding was present in 25 cases and ulceration in 22, often associated with purulent exudate. Increased number of WBC were always present in fecal smears, but did not correlate with severity of lesions.

Histopathology represented an acute colitis with changes in epithelial cells, with desquamation, mucosal hemorrhage and active mucus secretion (Fig. 16, 17, 18, 19, 20). Intraepithelial penetration by Shigellae was seen in 27 cases (Fig. 21). Regardless of serotype of Shigellae, all cases shared similar clinical and sigmoidoscopic features. Histologic features were indistinguishable from those of experimental shigellosis in subhuman primates (Takeuchi, Formal & Sprinz 1968).

Fig. 16 - Rectal mucosa 18 hrs. after onset of symptoms; 8 hrs. after beginning of diarrhea. Notice the thickened mucosa, with increased cellularity in the lamina and edema. The epithelial covering is complete but shows focal piling of epithelial cells (arrow). H & E. X 114

Fig. 17 - Detail of Figure 16 showing RBC extravasation in the lamina propria (arrow) H & E X 190

Fig. 18 - Rectal mucosa 4 days after onset of symptoms. Over 12 hrs. after beginning of diarrhea. Microulcerations in the epithelium with acute cellular exudate (arrows). H & E. X 475

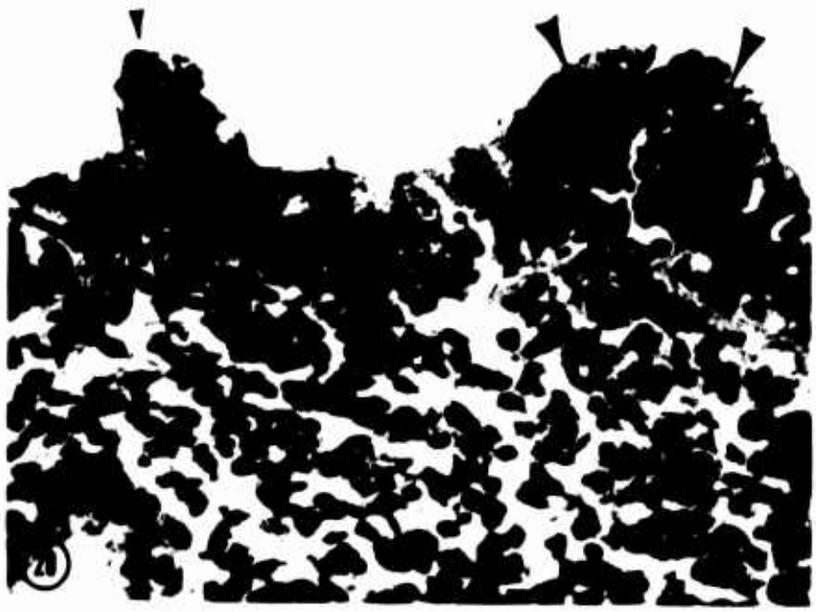


Fig. 20 - Rectal mucosa 18 hrs. after onset of diarrhea. Notice RBC exudation through the epithelium (large arrows) and epithelial cells piling (small arrow). H & E. X 475

Fig. 21 - Rectal mucosa 12 hrs. after onset of diarrhea. Shigella organisms in necrotic epithelial cells (small arrows) and in lamina propria (large arrow). Giemsa X 1000

C. Histopathologic Studies on the Small Intestine of the Dog Infected with Corona Virus

Little is known about the pathology of the bowel infected with enteroviruses. Recent studies on the small intestinal biopsies from human subjects infected with Norwalk agents (non-bacterial gastroenteritis: traveler's disease) have indicated that the lesion of the small gut represents nonspecific acute enteritis characterized by alteration of villi and villi-crypt ratio, degeneration of epithelial cells and acute inflammatory response, but failed to determine the site of viral replication in the gut mucosa (Trier et al. 1973). The corona virus isolated by Dept. of Vet. Microbiology, WRAIR, produces a severe acute enteritis in the lower small intestine of 2- to 7-day-old upppies characterized by diarrhea, shortening of villi, discharge of mucin and flattening and vacuolization of epithelial cells, therefore, provides an excellent model for clarifying studies of various unsolved problems of viral enteritis in man. In collaboration with Dept. of Vet. Microbiology, we have initiated light and electron microscopic determination on lesions and the site of viral replication in the small intestine with the corona virus (see Annual Report, 73-74, Dept. of Vet. Microbiology, WRAIR).

III. IMMUNOPATHOLOGIC STUDIES ON GLOMERULONEPHRITIS OF THE MONKEY INFECTED WITH TRYPANOSOMES

Background

Immune complex glomerulonephritis in man is thought to be an immunologic complication following a variety of infections, such as nephritogenic streptococcal infections, quartan malaria, acute staphylococcal endocarditis, and secondary syphilis. Immunoglobulins and components of complement have been found in renal glomeruli of patients who developed glomerulonephritis as a result of such infections. Experimental work has implied that antigens interact with specific antibodies forming complexes in the circulation which are deposited in the glomerular capillaries. These complexes activate complement through the classic pathway leading to the release of mediators some of which have the capacity to cause local inflammation which results in glomerular injury (Dixon 1963). Endogenous antigens may also react with antibodies and lead to the development of glomerulonephritis. Thus, in the glomerulonephritis seen in patients with systemic lupus nephritis, antinuclear antibodies have been found in the kidneys (Kirshan & Kapland 1967), and host immunoglobulins, nuclear antigens and complement have been demonstrated in a characteristic lumpy pattern in the glomeruli (Koffler et al. 1967). Recently properdin, a component of the alternate pathway of complement activation, has also been demonstrated in the glomeruli of patients with acute post-streptococcal glomerulonephritis, membrano-proliferative glomerulonephritis, and the glomeruli of lupus erythematosus patients, implying that activation of complement and subsequent damage may be initiated by other less-understood mechanisms (Westberg et al. 1971).

Immunopathologic studies of protozoan diseases have proven to be of considerable interest in regard to the pathogenesis of glomerulonephritis in man and monkeys infected with Plasmodia (Ward 1969), and in rats infected with Babesia (Annable & Ward 1973). In malaria and babesiosis, the glomerulonephritis is associated with glomerular deposits containing immunoglobulins, antigens originating from the infective agent, and complement proteins. This is compatible with the concept that in each case the glomerulonephritis is induced by immune complexes entrapped from the circulation.

The present studies were begun after finding that monkeys infected with Trypanosoma rhodesiense developed chemical and histologic evidence of renal failure and glomerulonephritis (Sadun et al. 1973).

Morphologic changes similar to those described in human cases of membranoproliferative glomerulonephritis (West et al. 1965) were observed in the kidneys of some of the infected animals.

Experimental Animals: Nineteen young male monkeys (Macaca mulatta) originating from India were used in this study. After a 30-day quarantine and period of conditioning, the animals were placed in individual cages and given a diet of Purina Monkey Chow, fresh fruit and water. Sixteen monkeys were each inoculated intravenously with approximately 10,000 Trypanosoma rhodesiense (EATRO No. 1886 strain) contained in 0.5 ml of phosphate-buffered saline-glucose (PSG) solution. Three control animals received 0.5 ml of phosphate-buffered saline-glucose solution only. The trypanosomes for the inocula were separated from infected rat blood in a DEAE-cellulose column, and washed twice by centrifugation in PSG solution. Fresh preparations of blood obtained by ear puncture were examined 5 times each week to determine the course of parasitemia. Blood for serum collection was taken from the femoral veins of the monkeys after they had been sedated with intramuscular Sernylan (0.5 mg/kg). The sera were stored at -70° C until used.

Biopsies: Renal wedge biopsies were taken at 14, 30 and 50 days following inoculation of trypanosomes. Uninfected control monkeys were subjected to biopsy at the same time intervals. For biopsy, the animals were anesthetized with intravenous pentobarbital (50 mg/kg) and an incision was made through the flank using sterile operating procedures. Wedges of renal cortex were removed and immediately divided into three parts. One portion was fixed and processed for electron microscopy (EM). A second portion was quick-frozen for immunohistochemical studies by placing the tissue in isopentane quenched in liquid nitrogen. A third portion was fixed and processed for light microscopy (LM) in 10% neutral buffered formalin.

Immunofluorescent Studies (IF): Fresh frozen renal cortical tissue was processed according to earlier descriptions. Gel double diffusion tests showed antibodies to human IgG, IgA, IgM and C4 properdin gave a strong cross-reaction to the heterologous monkey serum protein. Accordingly, antibodies to the human proteins were used for immunofluorescence studies. The only exception was the use of antibodies to monkey C3 prepared in rabbits. In IF studies the direct technique was employed for all proteins except properdin. The usual controls to insure specificity of reactions were employed (Ward et al. 1969). For the detection of properdin the indirect IF technique was employed. The tissue sections were first incubated with rabbit antibody to human properdin, then washed and incubated with fluorescein tagged sheep antibody to rabbit IgG.

Serologic Measurements for CH_{50}, C3 and C4: Serum CH_{50} levels were assayed with sensitized sheep red cells according to the technique of Kent and Fife (1963). Assays of complement components C3 and C4 were based on the cross-reactions of antibodies between human and monkey proteins. The single radial immunodiffusion technique of Mancini et al. (1965), as modified by Yount et al. (1967), was used. Human antibodies and standards for C3 and C4 were obtained from Hyland Laboratories and Meloy Laboratories. Serum albumin was determined by protein-electrophoresis in cellulose acetate.

Results

Serum Complement and Renal Deposits in Non-Infected Monkeys: The serial serum complement levels in three control animals had a mean value of 138 ± 28 CH_{50} units. Renal biopsies from the control animals obtained at three different intervals (days 14, 30, 50) failed to show the presence of any protein deposits in the kidneys.

Serum Complement and Renal Biopsies in Infected Monkeys: The details of serum complement as well as the normal range in each of four infected animals are given in Figures 22-25 (Numbers 801, 997, 812 and 970 respectively). Between 20 and 30 days after inoculation with the infective agent (15-25 days after detectable parasitemia), serum complement levels began to fall in each of the animals. In animals 801, 812 and 970 there was no detectable hemolytic activity on at least one occasion during this period. The CH_{50} levels in monkey No. 997 persisted at a 50% level for at least 10 days (Fig. 24). There was a persistent parasitemia during this time In one infected animal (No. 970) (Fig. 25), the CH_{50} level fell to undetectable levels at 18 days, but then returned to hypernormal or normal range. Also demonstrated in Figures 1 to 4 are the findings of the renal biopsies. Positive deposits consisting of one or more of the proteins listed above were found at some time during the hypocomplementemic period.

Morphologic Changes in Glomeruli: Renal biopsies obtained from the three control animals revealed normal cellularity without evidence of exudation, proliferation, or sclerosis (Figs. 26, 27).

Fifteen of the 16 infected animals showed glomerular abnormalities. The common feature consisted of mesangial cell proliferation, endothelial swelling, the presence of few to many polymorphonuclear leucocytes, variable mesangial sclerosis, and focal basement membrane thickening and duplication. In four infected animals in which

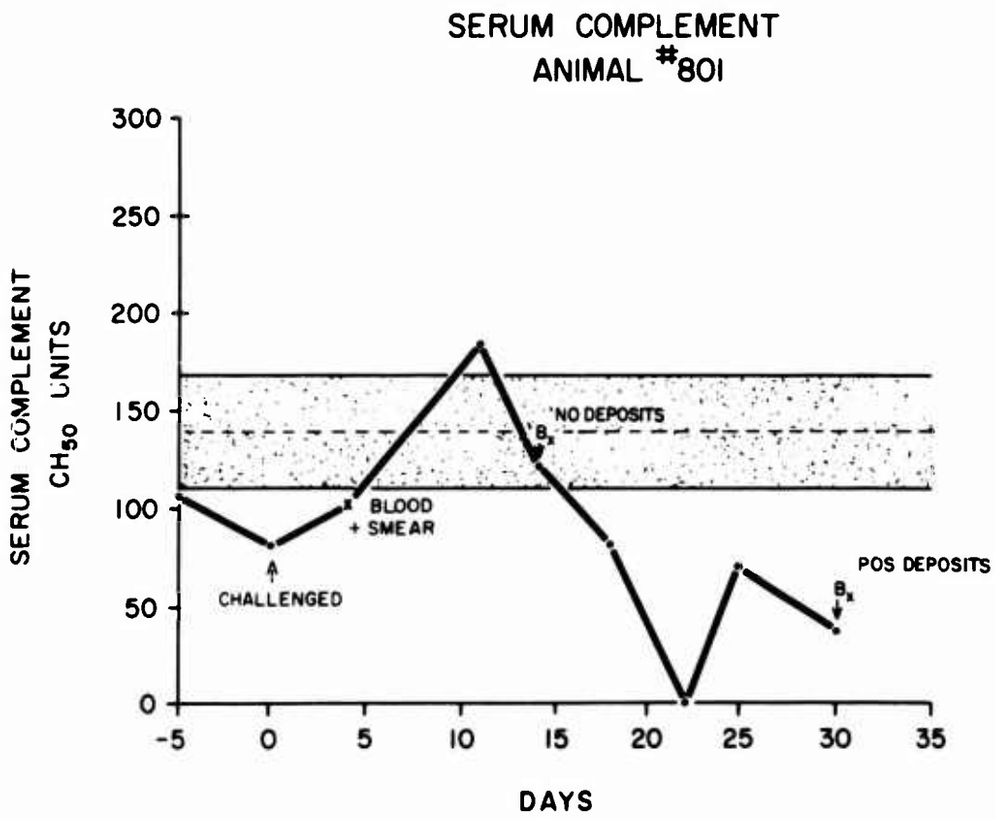


Fig. 22 - Sequential serum complement levels in animal No. 801. Arrows indicate time of renal biopsy (14 and 30 days post-inoculation of trypanosomes) and immunohistochemical resuits. Interrupted line and shaded area indicates mean ± S.E. of the control values for serum complement (CH_{50}).

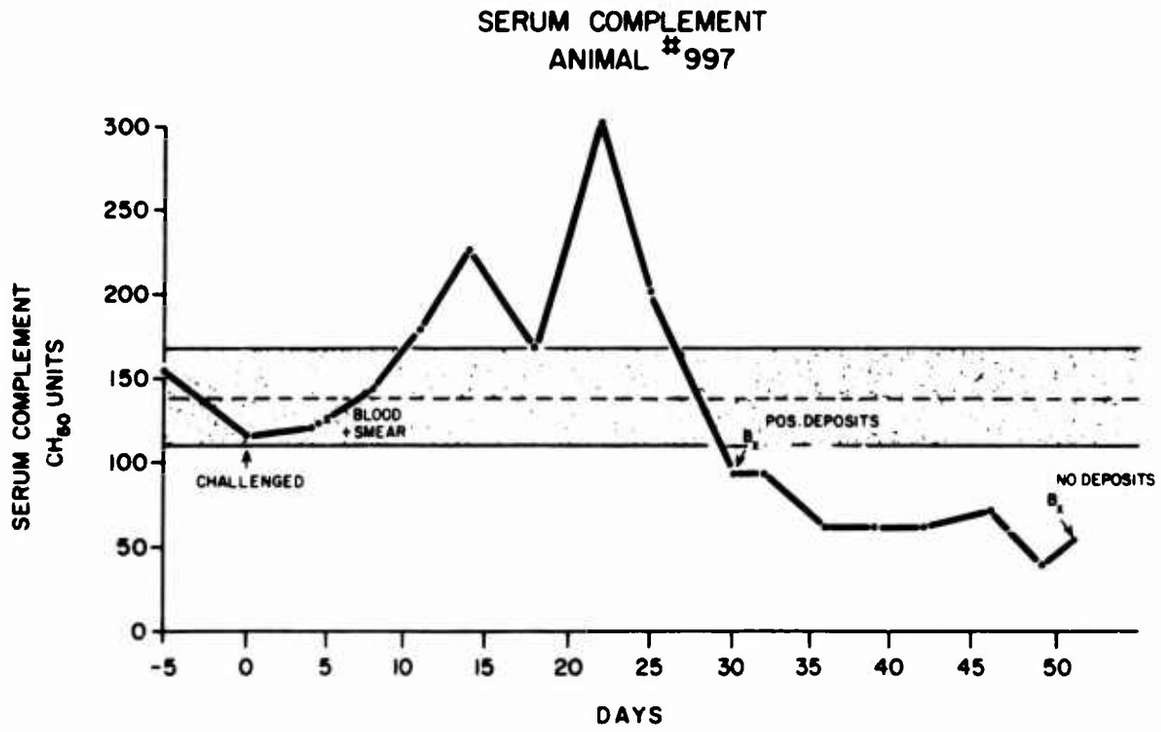


Fig. 23 - Sequential serum complement levels in animal No. 997. Arrows indicate time of renal biopsy (30 and 51 days post-inoculation of trypanosomes) and the immunohistochemical results. Interrupted line and shaded area indicate mean S.E. of the control values for serum complement ± (CH_{50}).

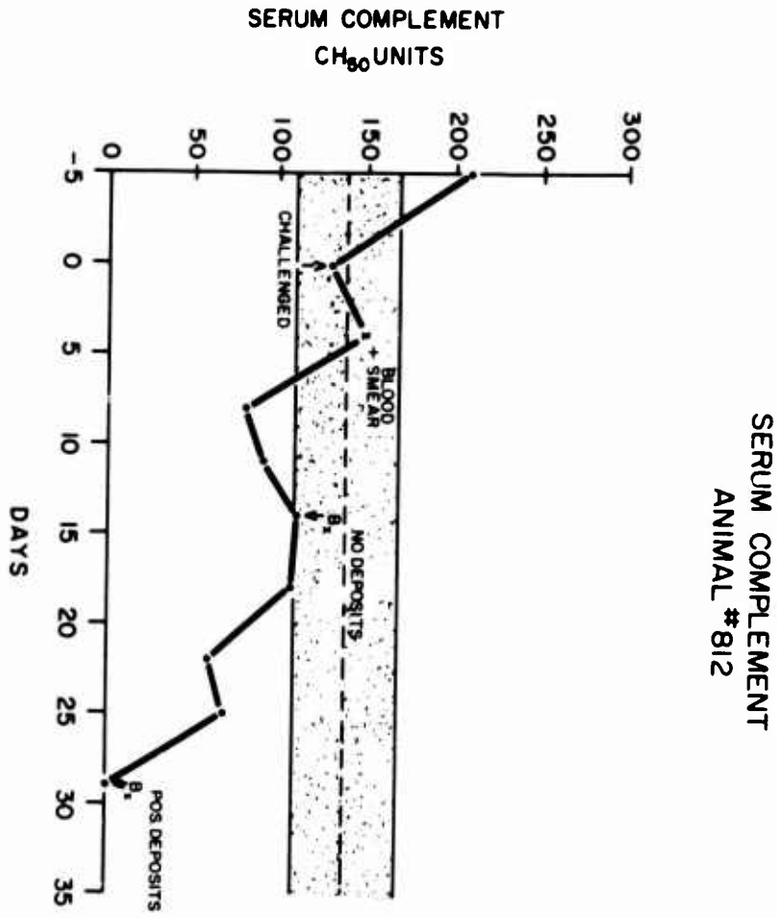


Fig. 24 - Sequential serum complement levels in animal No. 812. Arrows indicate time of renal biopsy (14 and 29 days post-inoculation of trypanosomes) and immunohistochemical results. Interrupted line and shaded area indicate mean ± S.E. of the control values for serum complement (CH_{50}).

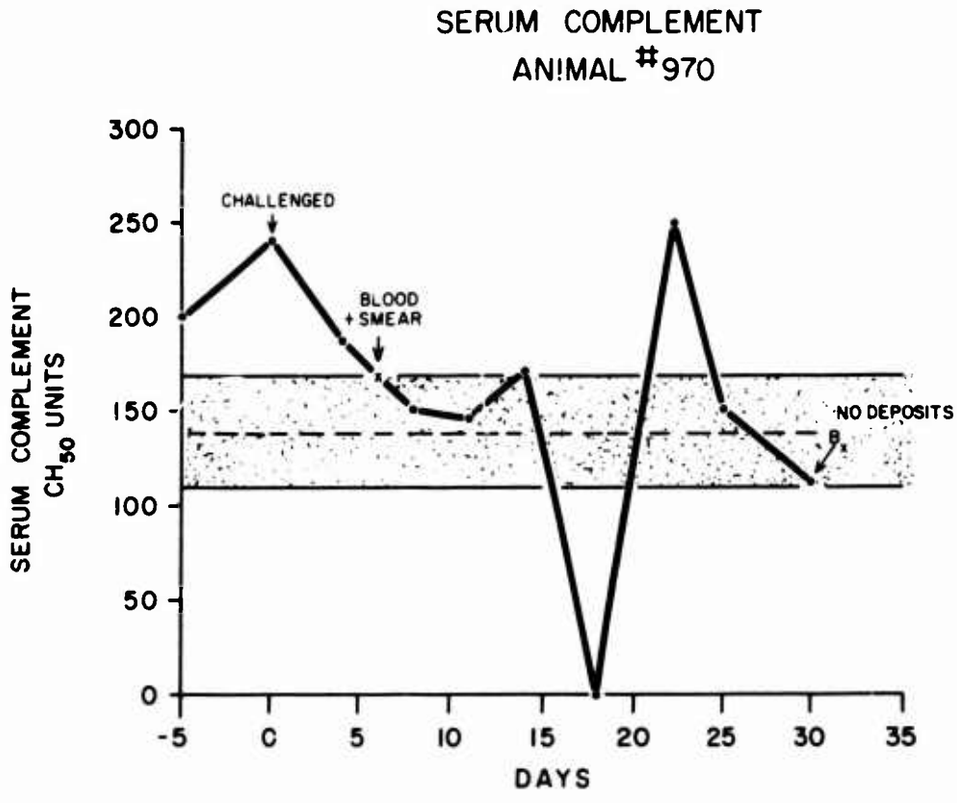


Fig. 25 - Sequential serum complement levels in animal No. 970. Arrows indicate the time of renal biopsy (30 days post-inoculation of trypanosomes) and immunohistochemical results. Interrupted line and shaded area indicate the mean ± S.E. of the control values for serum complement (CH_{50}).

TABLE 2

Percent changes in selected serum proteins and deposits in glomeruli 30 days after inoculation

Animal no.	Serum				Renal deposits					
	Albumin (%) *	CH50 (%)*	C4(%)*	C3(%)*	Properdin	C3	C4	IgG	IgM	IgA
801 - infected	46	41	135	45	4+	3+	0	0	Trace	0
997 - infected	49	68	122	27	4+	3+	0	0	2+	0
812 - infected	51	12	19	22	4+	3+	0	0	2+	0
970 - infected	62	57	29	72	0	0	0	0	0	0
952C - noninfected	91	135	208	115	0	0	0	0	0	0
966C - noninfected	84	104	95	98	0	0	0	0	0	0
976C - noninfected	ND†	95	112	106	0	0	0	0	0	0

* Percentage of preinfection value.
† ND, not determined.

serologic studies were done, three (801, 997, 812) showed glomerular changes associated with depression of serum complement and protein deposits within the glomeruli. The specific pathologic changes of each of these animals are presented below:

Animal 801 was first subjected to biopsy on the 14th day (Fig. 22). The LM observations of this biopsy were essentially normal except for rare focal areas of mesangial sclerosis. EM observations confirmed this finding and revealed essentially normal capillary loops without evidence of abnormal deposits. The second biopsy obtained on the 30th day, at a time when the serum CH_{50} was 41% of the pre-infective level, showed definite focal mesangial hypercellularity with increased mesangial sclerosis and focal basement membrane thickening (Fig. 28). Electron microscopy revealed widening of the central mesangial areas which contained increased numbers of mesangial cell processes (Fig. 29). Surrounding the mesangial cells, the mesangial matrix was characterized by numerous electron lucent defects. Within these areas of rarefaction were irregular electron dense deposits (Fig. 29). Focally this change extended out into the basement membrane of the capillary loops at a point where the capillaries attach to the central mesangium.

Animal 997 was first biopsied on the 30th day at a time when the CH_{50} level was depressed to 68% of preinfection values (Fig. 23). This biopsy revealed diffuse glomerular hypercellularity, numerous polymorphonuclear leukocytes in the capillaries, and obliteration of the capillary lumina (Fig. 30). EM studies revealed focal swelling of the endothelial cells, and plugging of the glomerular capillary lumina with polymorphonuclear leukocytes, mononuclear cells, and occasional macrophages containing secondary lysosomes. The mesangial areas were widened and contained electron lucent areas similar to those described in animal 801. A second biopsy obtained on the 51st day following 15 days of depression of the CH_{50} serum levels exhibited a persistent hypercellularity of the mesangium which, in addition, showed increased PAS positive mesangial mature material (Fig. 31). Methenamine silver and PAS stains showed that the capillary membrane was thickened and focally duplicated. Many capillary loops appeared obliterated (Figs. 31, 32). EM observations disclosed focal areas of capillary wall thickening with processes of the mesangial cells extending out into the peripheral capillary wall (Fig. 32). In these areas, there was production of new basement lamina which corresponds to the picture of peripherally duplicated basement membranes seen with the PAS and MS stains by LM. The capillaries were focally occluded by swollen endothelial cells, mononuclear cells and an occasional polymorphonuclear leukocyte (Fig. 32). Occasional electron dense subepithelial deposits were

Fig. 26 - Biopsy from control rhesus monkey showing normocellular glomerulus with open capillary loops limited by delicate basement laminae. PAS, X 520.

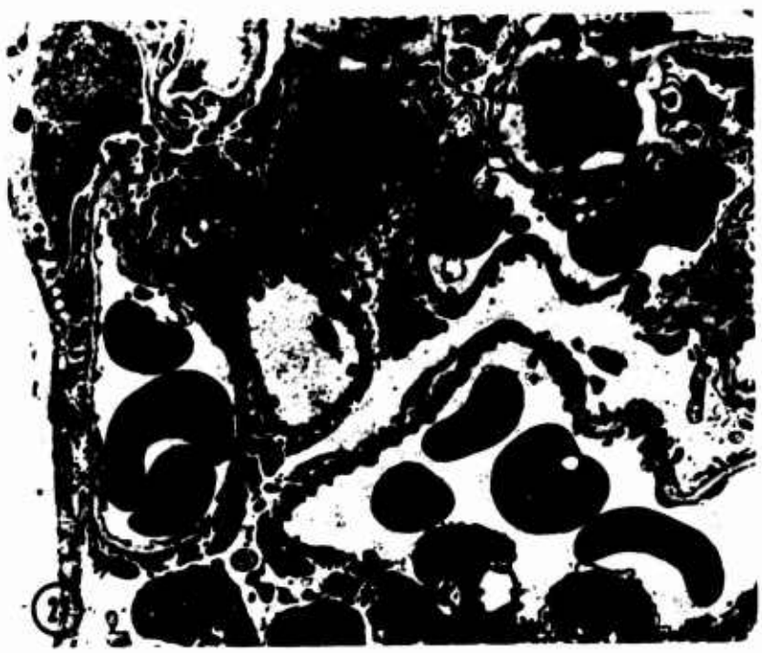


Fig. 27 - Electronmicrograph of normal control. Note appearance of mesangial areas containing mesangial cells (arrows) surrounded by a compact mesangial matrix with an electron density similar to that of the basement membrane. X 1,300.

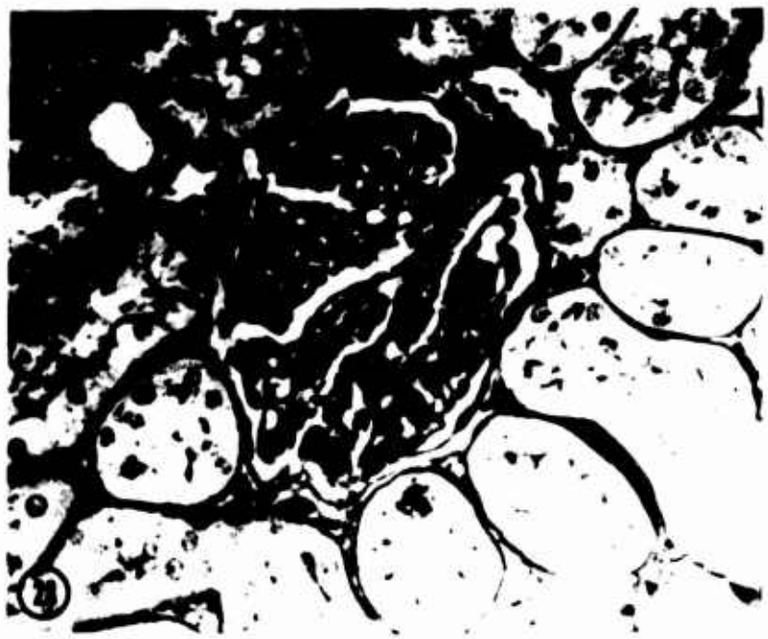


Fig. 28 - Renal biopsy of animal No. 801 taken 30 days post-inoculation of trypanosomes. Note general hypercellularity of the glomerular lobules and obliteration of capillary lumens. 3 μ section stained with PAS, X 550.

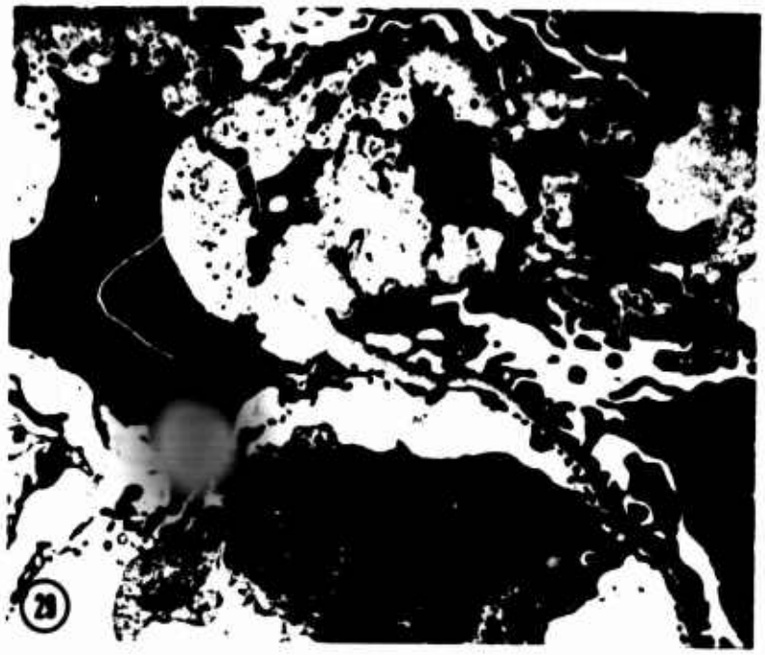


Fig. 29 - Renal biopsy of animal No. 801 taken 30 days post-inoculation of trypanosomes. Note rarefraction of mesangial matrix in areas surrounding mesangial cells. Numerous fine cytoplasmic processes of the mesangial cells extend into these areas. In addition, numerous electron dense, punctate material is seen within the mesangial area. X 2,900.

Fig. 30 - Renal biopsy of animal No. 997. Taken 30 days post-inoculation of trypanosomes, at time of depressed serum complement. Note marked hypercellularity of glomerular tufts, polymorphonuclear leukocytes, and obliteration of capillary lumina. PAS, X 500.

Fig. 31 - Renal biopsy of animal No. 997. Taken 51 days post-inoculation of trypanosomes. Note thickened and duplicated basement membranes (arrow), mesangial sclerosis, and obliteration of capillary lumina. Note also the focal interstitial inflammatory infiltrates. PAS, X 500.

Fig. 32 - Renal biopsy obtained in animal No. 997 51 days post-inoculation with trypanosomes. Note obliteration of capillary lumina, some of which contain polymorphonuclear leukocytes (p). There is focal reduplication of basement membrane (arrow). There is swelling of podocytes with loss of filtration sites focally and rare focal subepithelial deposits (d). X 1,700.

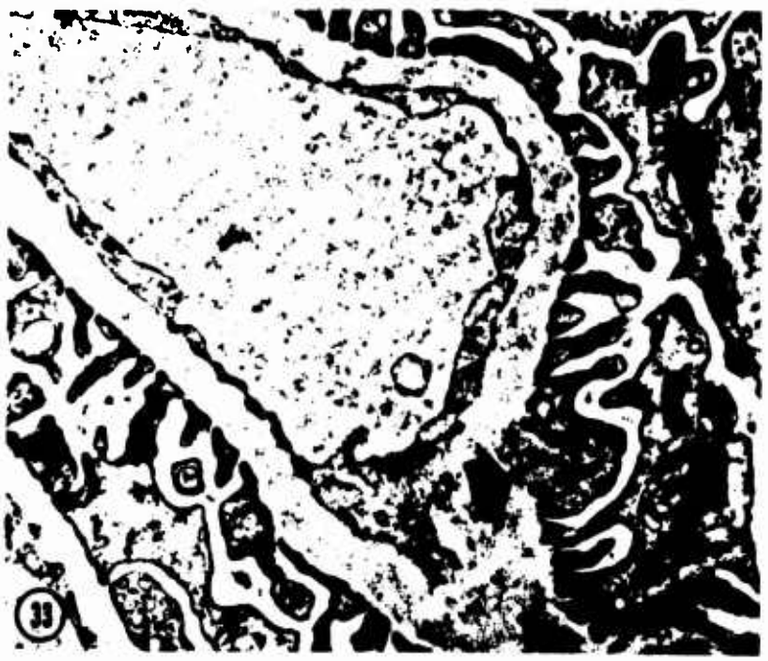


Fig. 3? - Renal biopsy from animal No. 909 51 days post-inoculation with trypanosomes. Arrow indicates sub-epithelial deposit in area of slightly tangential section of capillary wall. X 22,000.

seen (Figs. 32, 33) as well as rare subepithelial deposits.

The first biopsy obtained from animal 812 on the 14th day at a time when the CH_{50} level was in the normal range showed a rare focus of mesangial sclerosis but no evidence of active disease. A second biopsy obtained on the 29th day when the CH_{50} level had been depressed for 7 days, revealed a definite, diffuse mesangial hypercellularity and wrinkling and focal thickening of basement membrane. Electron microscopy confirmed widening of the mesangial regions and increased numbers of mesangial cellular processes. In addition, the mesangial matrix contained electron lucent areas which contained irregular electron dense material.

A biopsy obtained on the 30th day of infection from animal 970 revealed essentially normal glomeruli with focal areas of slight mesangial hypercellularity. The CH_{50} level was within the normal control range at this time although it was 72% of this animal's preinfective value. No glomerular deposits were seen.

Correlated Studies of Serological and Renal Biopsy Observations:
Four infected monkeys and three uninfected animals were studied in detail with respect to serological changes of whole complement, levels of C3 and C4 as determined by radial immunodiffusion, and protein deposits in renal glomeruli. The data are summarized in Table 1. The four infected animals were biopsied on the 30th day (after inoculation with trypanosomes) at which time their CH_{50} values were 41%, 68%, 12% and 72% of the pre-infection values determined in the same animals. Three uninfected control animals sampled also on the 30th day had complement levels of 135%, 104% and 95% of pre-infection values. The antigenic assay for C3 indicated severe depression in the C3 levels (45%, 27% and 22%) in three infected animals (Table 2). This was correlated with depressed CH_{50} levels and C3 deposits in the glomeruli as determined by immunofluorescence. Two animals showed depression of C4 levels but C4 deposits were not present in the glomeruli. The C3 and C4 levels in the uninfected controls were close to the preinfection values.

Immunofluorescent Patterns of Glomerular Deposits:
The various proteins detected in glomerular deposits have been summarized in Table 1. The pattern of fluorescence was one of a diffuse stain, limited to glomeruli, involving both glomerular capillary loops and mesangial areas. In some cases discrete granular patterns of fluorescence were seen. There was no evidence of smooth, linear fluorescence. Typical patterns of fluorescence are shown in Figure 34; the properdin reaction (Pro) was completely blocked (XPro)

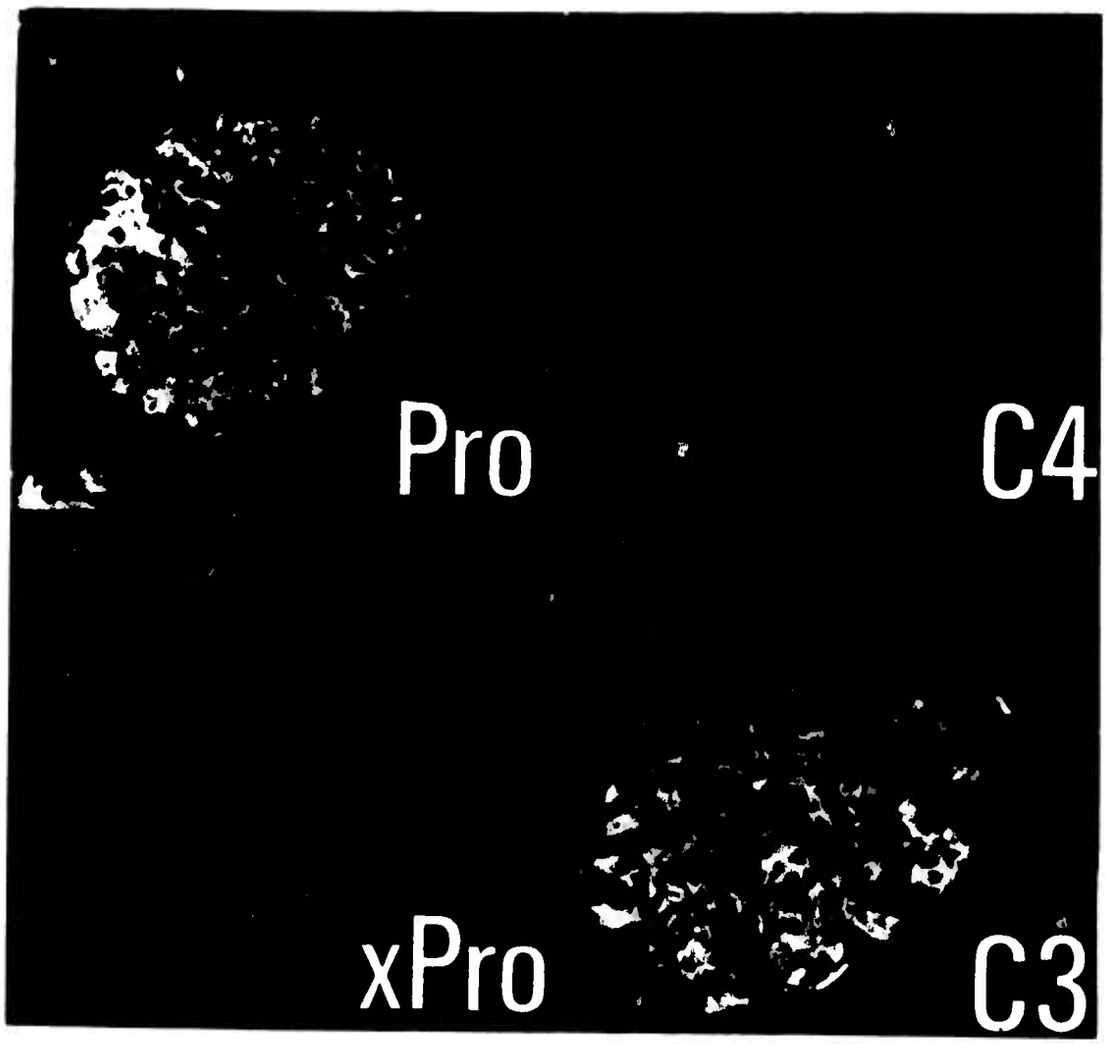


Fig. 34 - Immunohistochemical findings in a renal biopsy from animal No. 997, 30 days post-inoculation of trypanosomes. Upper left panel reveals a 4+ reaction with indirect technique using rabbit serum containing anti-properdin antibody (Pro). Lower left shows blockage of the reaction by prior absorption with purified human properdin (xPro). Upper right shows negative staining in C4. Lower right reveals 3+ staining for C3.

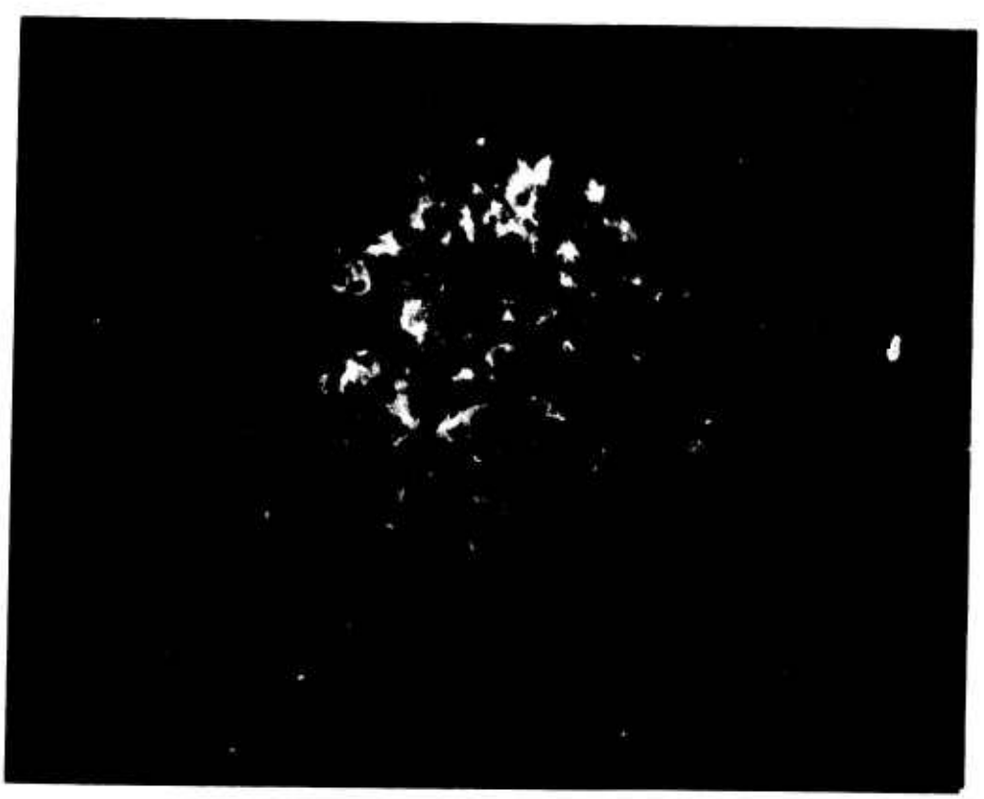


Fig. 35 - Renal biopsy from animal No. 997 showing deposits of IgM in a granular distribution.

by previous absorption of the antiserum with purified human properdin. Deposits of IgM were seen in one monkey (Fig. 35). The granular appearance of the deposits was particularly apparent. Considerable deposition in mesangial areas was evident.

Discussion

Glomerulonephritis develops in the course of experimental trypanosomiasis in monkeys. The earliest lesions are characterized as proliferative and show variable degress of increased numbers of mesangial cells, swelling of endothelial cells, and variable margination of polymorphonuclear leukocytes. Later in the evolution of the process there is mesangial sclerosis and duplication of capillary basement membranes which in several cases clearly resemble a membranoproliferative type of glomerulonephritis (West et al. 1965).

Glomerulonephritis has been implicated in at least two other parasitic diseases. Kibukamusoke reported a high incidence of diffuse proliferative and membranoproliferative glomerular lesions in adults and children with nephrotic syndrome in Uganda and provided extensive evidence suggesting a malarial etiology in many of the cases. A spectrum of glomerular abnormalities has been found in selected patients with hepatosplenic schistosomiasis resembling the lesions of membranoproliferative glomerulonephritis (Andrade et al. 1971). It should be emphasized, however, that the pathogenesis of schistosomal nephropathy is not yet understood and that the possible role of immune complexes in schistosomiasis is still unresolved (Cavallo 1972).

The immunohistochemical evidence presented in this study points to an immunopathologic basis for the glomerulonephritis. The granular pattern of immunofluorescence would indicate the likelihood of an immune complex-type of nephritis rather than a nephrotoxic mechanism. The great rise in total serum IgM levels on human infections with African trypanosomes is well documented. Therefore, additional studies were conducted to determine the presence of anti-trypanosomal IgM antibodies in infected monkeys. The results which will be published elsewhere show a consistent increase in specific IgM antibodies during the time in which the glomerular deposits develop. The presence of properdin and C3 in the glomerular deposits of all animals studied suggested that the alternate (properdin) pathway was activated. The serologic data indicated that the basis for the acquired hypocomplementemia is in some animals related to a change in the C3 but not the C4 levels. The explanation for a low C4 in two animals but absence of C4 deposits in the glomeruli in these animals was not apparent. A second study has indicated that in at

least some animals C4 deposits were in the glomeruli (unpublished). It is probable that both the classic and alternate pathway operate in the pathogenesis of this lesion. The agent(s) responsible for activating complement has not been defined. Indeed, it is not evident if the activating agent resided in the glomerular deposits, or if the complement pathway was activated within the blood stream with subsequent deposition of altered proteins in glomeruli. On the basis of these studies it would seem that trypanosomal infection in monkeys is particularly predisposed to initiating activation of the alternate pathway of complement.

In the context of glomerulonephritis in man it should be pointed out that there are two general situations in which reactant proteins of the alternate pathway (properdin) have been demonstrated in glomerular deposits. On one hand, a combination of reactant proteins of both the classical (immunoglobulin and C4) and the alternate (properdin deposits) pathways have been activated. This agrees with the current concept that activation of the classical pathway can result in activation of the alternate pathway through release of the C3b fragment from C3 and sequential interactions of complement proteins beyond this point (Ruddy et al. 1972; Muller-Eberhard 1972).

A second disease reported in humans which seems relevant to these studies is the syndrome of hypocomplementemic glomerulonephritis. This disease is associated with a membranoproliferative glomerulitis, deposits of properdin in renal glomeruli, and persistently low C3 levels in serum with relatively normal levels of C1, C4 and C2 (Sadun et al. 1973; Michael et al. 1969). Most recent investigations suggest that this disease may be a unique syndrome insofar as the renal disease appears to be associated with a more or less specific activation of the alternate complement pathway (Ruley et al. 1973). There is, as yet, no information that would pinpoint the reaction product responsible for the morphologic and functional alterations in the glomeruli.

T. rhodesiense infection in monkeys results in glomerulonephritis which in certain respects resembles the hypocomplementemic glomerulonephritis of humans. In view of these findings it would be of interst to carefully study the renal structure and function of patients infected with African trypanosomiasis. Likewise this experimental infection could prove useful in studying the evolution and pathogenesis of glomerulonephritis involving the alternate pathway (properdin) of complement activation.

IV. STUDIES ON MICROAGGREGATES IN STORED BLOOD

Background

Storage of whole blood for transfusion induces the formation of cellular aggregates, which are a cause for concern because of their ability to pass through standard donor blood filters and to enter the pulmonary circulation of the recipient (Swank 1961). Jenevein and Weiss (Jenevein & Weiss 1964) identified emboli in small pulmonary vessels of transfused patients and showed these emboli to be the same, histochemically, as aggregates seen in donor blood. Belyakov, Kartashevsky and Rumyantsev (1968) believed that aggregates, which they called microclots, produce microemboli and vascular spasm in the recipient. Swank has shown an elevation of pulmonary artery pressure (Hissen & Swank 1965) and an increase in alveolar dead space (Swank & Edwards 1966) following the transfusion of blood rich in aggregates.

Earlier studies by light microscopy have indicated that these aggregates consist of nuclear and cellular debris, leukocytes, erythrocytes and fibrin (Swank 1961; Walter & Button 1966; Moslely & Doty 1970; McNamara 1971). Solis (1972), in determining the size and number of aggregates on a particle size analyzer, and Swank, in measuring screen filtration pressure (Swank 1961), have suggested that platelets and white blood cells are the major contributors to the formation of cellular aggregates. In this study, electron microscopy has been utilized to determine the composition of these aggregates, the time and frequency of their appearance, and their evolution in the course of storage of whole blood.

Collection of Blood and Preparation of Buffy Coat

The blood of healthy human donors was collected in Fenwall plastic bags containing ACD, formula A, using normal blood bank procedures. All blood was kept for 60-90 minutes at room temperature before proceeding with further preparations. Since the type of blood container, the frequency of sampling and methods of blood suspension and centrifugation (O'Brien 1968) could possibly influence aggregates formation and composition, the treatment of blood after collection was varied in three ways:

Group 1: Blood was stored in the plastic collection bags at 4-6° C and aliquots were withdrawn sequentially for study during a 21 day period.

Group 2: Blood was stored as for Group 1 but only one aliquot was removed from each bag at the end of the first and second week of storage.

Group 3: Blood was transferred to and stored in sterile plastic test tubes. This blood was not disturbed in any way; the buffy coat was allowed to form by spontaneous settling and was sampled at the end of the first and second week of storage.

For Groups 1 & 2: At the time that a sample of blood was desired for study, the Fenwall bags were suspended 15 times within a 30 second time period. In order to obtain a buffy coat rich in both platelets and leukocytes, aliquots of blood were transferred to plastic or siliconized test tubes, centrifuged at 5000-7000 g for 6-8 minutes. The platelet poor plasma was pipetted off gently and the buffy coat was overlaid with 2.5% glutaraldehyde solution in .1M phosphate buffer at pH 7.2. Since the temperature may affect platelet aggregation (Kattlove & Alexander 1971), some of the above aliquots were maintained at a constant temperature - either at room temperature (25 C), or at 4-6 C until fixation was completed. To eliminate the possibility that platelet aggregation might have been induced by centrifugation, some aliquots of blood were fixed in 0.1% glutaraldehyde, as described above, prior to centrifugation as well as in 2.5% glutaraldehyde after centrifugation. Moreover the buffy coat, which formed spontaneously, in Group 3, was fixed as described above, without centrifugation.

Techniques for Morphologic Studies

In each case the buffy coat was fixed as described for 2 hours and then removed in its entirety from the test tubes, cut in strips and washed in the .1M phosphate buffer at pH 7.2. The strips were post-fixed in 1% osmium tetroxide in phosphate buffer for 1 hour, then dehydrated and embedded in Epon 812. For light microscopy (LM), sections were cut from Epon embedded material at 1-2μ, stained with toluidine blue or methylene blue-azure II. For electron microscopy (EM), ultra thin sections were stained and examined with an HU-IIC electron microscope.

Results

Fresh Blood: With LM, the buffy coat showed distinct free platelets in the upper layer and unaltered individual leukocytes in the lower layer (Fig. 36). With EM, all platelets, whatever the mode of preparation, showed pseudopods, abundant glycogen, random distribution of intact granules and a narrow surface connection system (SCS)

Fig. 36 - The buffy coat has two layers; the platelet layer (PL) shows free platelets, while the white cell layer (WCL) contains numerous unaltered leukocytes. Day 0: 1 micron Epon section stained with methylene blue - Azure II. X 590.

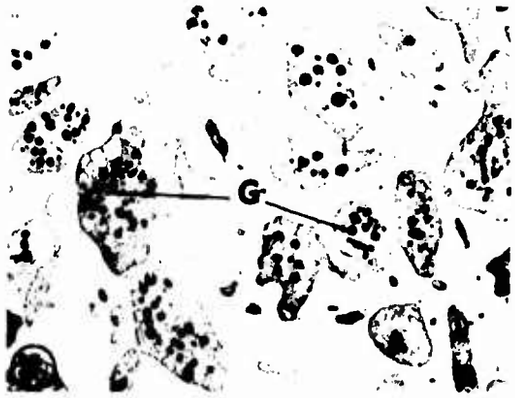


Fig. 37 - The free platelets have intact granules (G) throughout the platelet layer. Cytoplasmic projections (pseudopods) are abundant. Day 0: X 3,600.

(Fig. 37). The contents of the latter were characterized by their amorphous appearance, identical to that of plasma protein, and by the absence, with few exceptions, of other components. Platelets fixed at room temperature were lentiform with a well formed microtubular system, while cold fixation caused rounding of platelets and loss of microtubules.

Stored Blood: The cellular aggregates which develop during blood storage were classified in this study into three distinct types according to their composition: Simple platelet aggregates (SPA), Complex platelet aggregates (CPA), and Neutrophil-platelet aggregates (NPA). Fibrin, other blood elements or plasma protein did not contribute to their formation.

By LM, the first type of aggregate, SPA, began to appear as platelet clusters at 24 hours of storage. By EM, SPA were composed of a small number of rounded platelets which approximated each other over a limited surface area (Fig. 38). Platelet membranes were intact, although occasionally they merged with that of an adjoining platelet (Figs. 38, 39). With storage, platelet granules underwent gradual and progressive changes. By the first day of storage, granules, often centrally located, released their content into the hyaloplasm or in the surface connecting system (SCS). In the first instance, the released material appeared as a round granular mass resembling a "bull's-eye" many times larger than the original granules and staining less intensely (Fig. 38). The bull's-eyes were never membrane bound but at their periphery occasionally showed arrays of filamentous material (Fig. 39). In the latter instance, release in the SCS, the granules assumed a variety of appearances (Figs. 38, 39). On occasion, granules and hyaloplasmic contents were seen in transit from inside the platelets into the SCS (Fig. 45). The progressive decrease in the number of granules in aggregated platelets, as storage time was prolonged, indicated that the discharge of granules was a continuous process. By the end of the third week of storage, few granules remained in the platelets.

The second type of aggregate, CPA, first seen on LM on the third day of storage, appeared to be larger than SPA. By EM the aggregates were seen to be composed of densely packed platelets. In the peripheral portions of the CPA, platelets were large and saccular with sparse organelles (Figs. 40, 41, 42) and had distinct plasma membranes. In the central portion of the aggregates, the platelets were reduced in volume and crammed with organelles (Figs. 40, 41). Their plasma membranes either formed numerous pseudopodia (Figs. 40, 41) or were obscured or absent (Fig. 42). The latter occurred more frequently as storage time was prolonged. Granule discharge in the

Fig. 38 - Platelets are predominantly in the form of simple platelet aggregates which are characterized by loose approximation of intact plasma membranes (large arrows). However, at some site of contact, plasma membranes are closely opposed (small arrows). Many platelets show bull's-eye (BE) formation, resulting from the release of granule contents into the cytoplasm. The surface connecting system (SCS) is dilated. Day 1: X 9,000.

Fig. 39 - This simple platelet aggregate has a greater platelet surface approximation than that seen in Fig. 3. Filaments (F) are seen around an early bull's-eye. The dilated surface connecting system (SCS) contains a finely granular material, whose texture is identical to plasma, and also denser particulate material. (g) granules; (GLY) glycogen particles. Day 3: X 16,400.

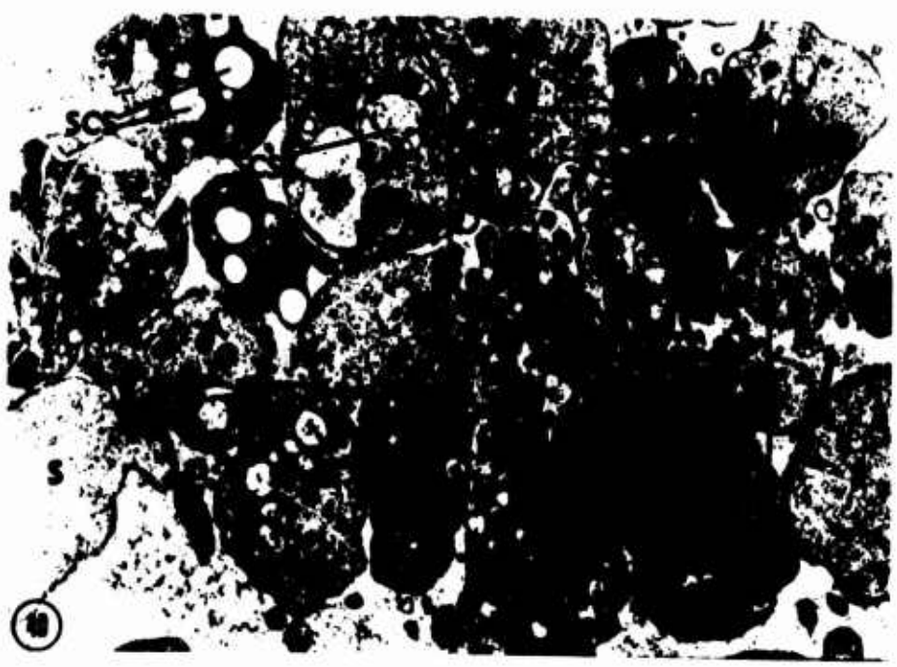


Fig. 40 - An early complex platelet aggregate. Platelets are crowded together and the surface membranes are extensively approximated or fused (square). In the periphery, platelets are saccular. (S). Pseudopods are closely intertwined in the center (square). As in simple platelet aggregates, the surface connecting system (SCS) is dilated. Day 4: X 10,000.

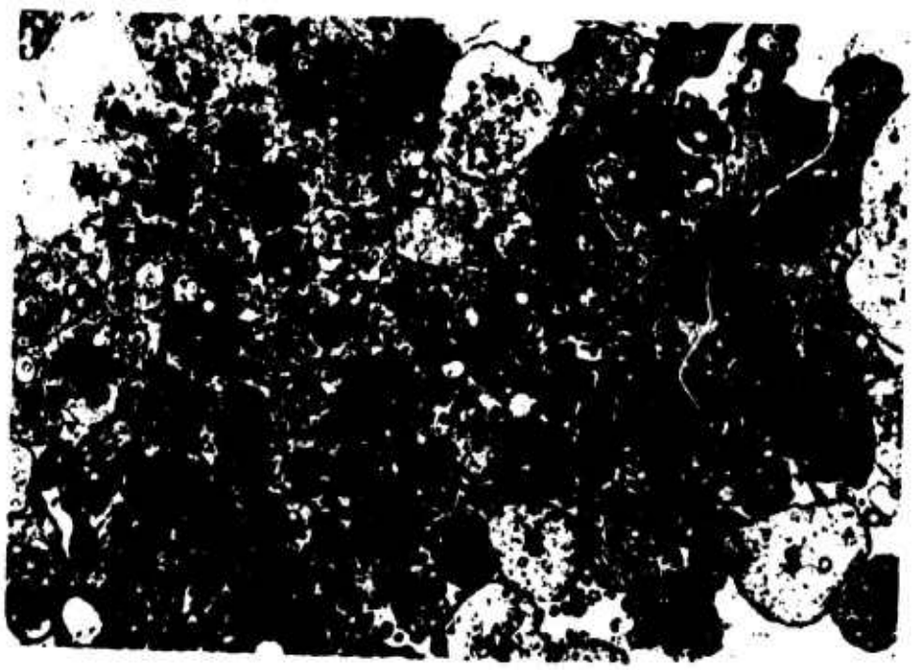


Fig. 41 - This is a more developed complex platelet aggregate than that seen in Fig. 5. Saccular platelets (S) are seen in the periphery. Platelets (M) demonstrate a migration of the bull's-eye (arrows) to the center of the aggregate. Day 12: X 8,000.

CPA resembled that observed in SPA. In addition, with longer storage time, the bull's-eyes were found more frequently toward the center of the aggregates (Fig. 41), the peripheral saccular platelets became depleted and the amount of platelet-free dense material in the central part of the aggregates increased (Fig. 42).

The third type of aggregate, NPA, first seen by LM on the third or fourth day of storage, was larger than the SPA and CPA. By EM, it consisted of individual or aggregated platelets, SPA and CPA, bound to extruded degenerated neutrophil nuclei. The sequence of events of neutrophil degeneration which culminated in the formation of these aggregates appeared to be as follows: LM observations demonstrated the formation of a distinct perinuclear space (Fig. 43) which in EM corresponded to an irregular widening of the double nuclear membranes (Fig. 44) together with changes in the distribution of both euchromatin and heterochromatin (Figs. 44 and 45). The nuclear and plasma membrane ruptured and the nuclear material was released from the cell (Figs. 44, 45). It became ill defined and faint staining on LM (Fig. 43) but remained easily identifiable by EM (Figs. 44, 45). The extruding nuclear material appeared to flow and adhere to the surfaces of platelets, often engulfing them (Figs. 45, 46). In the NPA, there were no intact neutrophils, other types of leukocytes or erythrocytes.

The mode of storage and frequency of handling of blood influenced the formation of NPA (Table 3). Undisturbed blood (Gropu 3) had the fewest NPA despite the fact that it contained an abundant amount of degenerated neutrophil nuclei. More NPA were present in blood which was handled only once (Group 2) and still more were present in blood which had been repeatedly handled (Group 1).

All three types of aggregates, once formed, could persist during the course of blood storage. However, generally, there was an evolution from free platelets into SPA and CPA and later into NPA, diagrammed in Fig. 12. Most aggregates at the end of storage were NPA or CPA and very few free platelets remained.

Discussion

The present study has clearly defined the origin of the particulate material accumulating in stored blood. Sequential LM and EM observations carried out throughout the normal 21 days' storage period have provided evidence that only two blood elements - platelets and degenerate neutrophils - form this material and no other cellular or non-cellular components of the peripheral blood such as fat, fibrin, denatured protein, red blood cells, intact neutrophils or other types of leukocytes participate.

Fig. 42 - This is a late complex platelet aggregate. Platelets have disintegrated extensively in the center of the aggregate. Many platelets appear to have no granules while others have many crowded together (square). Peripheral saccular platelets (S), devoid of granules, pseudopodal formations and dilated surface connecting systems are seen. Bull's-eye (BE). Day 12: X 8,000.

Fig. 43 - Neutrophils are in the process of degeneration and show perinuclear swelling (small arrows). The degenerate nuclear material extruded from neutrophils (large arrows) stains less intensely than the nuclei of unaltered cells. Day 4: 1 micron Epon section stained with methylene blue-azure II X 640.

Fig. 44 - Degenerating neutrophil, showing relatively well preserved granules (G). Nuclear material (NM) is in the process of extruding and spreading over the neutrophil surface. The double membrane of the nuclear envelope is irregularly widened (NE). Day 4: X 10,000.

Fig. 45 - Neutrophil-platelet aggregate. One platelet (EP) of a simple platelet aggregate (P, P) is totally engulfed by nuclear material which has identifiable areas of euchromatin (E) and heterochromatin (H). In the lower right corner, the cytoplasmic contents of a platelet are seen entering the surface connecting system (small arrow). (G) granules of neutrophil. Day 6: X 16,000.

Fig. 46 - Neutrophil-platelet aggregate. Nuclear material (NM) extruded from two degenerating neutrophils has adhered to a complex platelet aggregate which is centrally located. At the bottom of the figure, the plasma membrane of an intact eosinophil is closely apposed to the saccular surface of a platelet. Eosinophils do not degenerate or become part of NPA. Day 6: X 14,000.

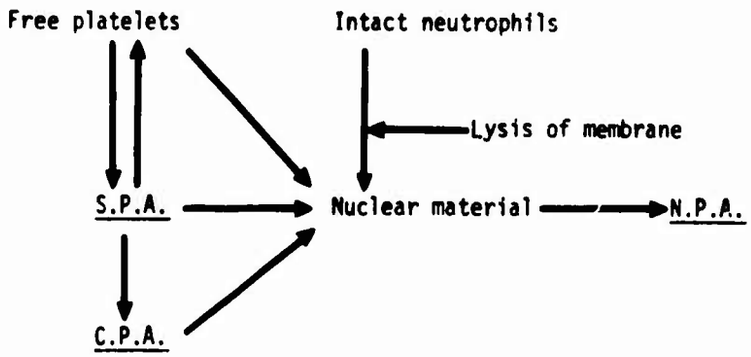


Fig. 47 - Schematic representation of the formation of the three types of aggregates. Free platelets give rise to simple platelet aggregates (SPA) which in turn progress to complex platelet aggregates (CPA) or can be dissociated into free platelets. Nuclear material derived from neutrophils can form neutrophil-platelet aggregates (NPA) upon contact with free or aggregated platelets.

TABLE 3

TIME AND STORAGE MODE IN RELATION TO AGGREGATE TYPE AND TOTAL % OF PLATELETS AGGREGATED

Days of Storage	Mode of Storage	Simple Platelet Aggregate (S.P.A.)	Complex Platelet Aggregate (C.P.A.)	Neutrophil Platelet Aggregate (N.P.A.)	% of Platelet in Aggregate
0	group 1	0	0	0	0
1	"	++	0	0	10
3-4	"	++	++	+	30
6	"	++	+++	++	60
	group 2	++	+++	+	60
	group 3	++	+++	+	50
11-12	group 1	++	+++	++++	60
	group 3	++	++++	++	60
14-16	group 1	++	+++	++++	50
	group 2	++	++++	++	50
17-19	group 1	++	++	++++	50
21	group 1	++	++	++++	50

CODE: 0 = not present; + = rare; ++ = present, but not abundant; +++ = abundant; ++++ = predominant type of aggregate, far more than all others combined. % platelets in aggregate is the percent of the total platelet population that has entered any one of the 3 different types of aggregates.

Furthermore, three different types of aggregates have been identified and the evolution of one type into another has been recognized as well as the alterations of platelets and neutrophils responsible for this evolution. The cellular aggregates found early in stored blood are composed only of platelets clumped together first in a loose form, the SPA, and then, in an irreversible form, the CPA; within 3-4 days of storage, degenerating nuclear material from neutrophils is added to these aggregates cementing together free platelets, SPA and CPA, and resulting in still larger aggregates, the NPA. Careful examination of numerous electron micrographs did not reveal any other component.

The first two types of aggregates, SPA and CPA, are composed solely of platelets. They have been previously recognized to form sequentially in vitro (Rodman et al. 1962; Sirma & Kenze 1968) and in vivo (Jergensen et al. 1967; Takeuchi & Sprinz 1967); in the past they have been termed loose reversible aggregates and consolidated or contracted platelets respectively. Since SPA are loose and reversible under circumstances where granule release has not occurred, they are considered less obstructive than the CPA which cannot separate into component parts. Since the release of platelet granule contents begins in blood storage within 24 hours, the ease with which these aggregates may be reversible in vivo may be open to question. One may conservatively assume that within 3 days of blood collection, and probably before, platelets have formed aggregates which would obstruct the pulmonary vasculature of the recipient.

It is of importance to note the early appearance of a third type of aggregate, the NPA, which is irreversible and remarkably larger than both the SPA and CPA. This type of aggregate has not been previously described and, therefore, deserves close attention. Only the degenerated nuclear material from neutrophils interacts and adheres to platelets plasma membranes. This nuclear material does not penetrate or "lyse" the platelet plasma membrane, since many platelets, although completely surrounded by nuclear material, appear absolutely intact as shown in Figures 10 and 11. By contrast, platelet plasma membranes are readily destroyed by fusion and/or attrition with other platelets in the centers of CPA or by penetrating fibrin fibrils during blood coagulation (Rodman et al. 1966).

Since the prerequisite for platelet-nuclei interaction is dependent upon both degeneration and extrusion of nuclear material from the neutrophils, the question arises: what causes the nucleus to extrude, swell and adhere? Since extrusion is preceded by dilatation of the space between the double membrane of the nuclear envelope,

it is possible that this dilatation exerts enough pressure upon the membrane to cause its rupture. Nuclear swelling which follows extrusion may represent a loosening of the very dense molecular packing of nucleoprotein in situ (Pardon et al. 1967). The cause of sticking between swollen and extruded nuclear material and the plasma membrane of the platelet could be a charge effect between the negatively charged surface of platelets (Madoff et al. 1964) and positively charged aspects of histones dissociated from nucleic acid. Whatever the physicochemical character of the bond may be, it is a unique one and does not involve other available plasma membranes of intact leukocytes or red blood cells. The appearance of neutrophils in the NPA brings to mind the role of neutrophils in thrombosis, since in arterial thrombosis leukocytes occur more frequently than chance trapping would allow and are aligned onto the surface of platelet masses (Banks & Mitchell 1973).

The cause for the rapid transformation of platelet aggregates in cold blood storage from the loose reversible type to the compact and irreversible one was not investigated in this study. However, there are many possible and not unexpected contributing factors. Exposure of platelets to cold (Zucker & Borrelli 1960) without the intervention of thrombin promotes platelet aggregation. Alternatively, these results could be mediated by thrombin concentrations too low to generate fibrin (Zucker & Borrelli 1959) or by adenosine diphosphate released from red blood cells (34). Whatever the initial cause of platelet aggregation in storage, there is a self-perpetuating mechanism for its progression, represented by the release of platelet granules. In this study, the release appeared to be a continuous event leading to exhaustion. The granules released into the blood might have an effect not only upon platelets in storage but also upon recipient platelets in vivo.

Based upon findings presented here, a reconsideration of terminology is in order. In the past, a variety of names such as amorphous debris, nuclear or cellular debris, fibrin, clots, microaggregates of amorphous material were used and reflected the confusion about the unclarified origin and composition of this material. The word "clot" implies the presence of fibrin in the aggregates but this is absent. The term "amorphous debris" should also be discarded since it reflects the limitations of light microscopy rather than true composition. The term "nuclear debris" has a modicum of truth but is misleading because it neglects the invariable attachment of platelets to the extruded nuclei of neutrophils and the distinct and unique interaction between platelets and degenerate nuclear material. Since the platelets in CPA and NPA have invariably undergone the release reaction, and are irreversibly adherent to each other, the terms platelet thrombi and neutrophil-platelet thrombi are commendable,

especially since the platelet behavior in the blood bag resembles in vivo arterial thrombosis. However, reservation of the term "thrombosis" exclusively for in vivo phenomena speaks against the use of such term for an in vitro storage event.

We, therefore, recommend the terms Simple Platelet Aggregates, Complex Platelet Aggregates and Neutrophil-Platelet Aggregates because they specifically describe the cellular origin of these materials, connotate their origin in vitro, and imply their embolic character when infused into the recipient's vasculature.

Conclusion

Storage of whole blood under normal blood bank conditions induces the formation of injurious particulate material. By electron microscopy the origin and composition of three distinct types of cellular microaggregates have been recognized and termed Simple Platelet Aggregates (SPA), Complex Platelet Aggregates (CPA) and Neutrophil Platelet Aggregates (NPA). The first type to form, the SPA, may be reversible, is composed only of platelets and appears by 24 hours of storage. CPA form from the SPA and are composed of densely compacted platelets which are irreversibly fused. Later, extruded nuclear material of neutrophils adheres to free and aggregated platelets to form the NPA. The interaction of platelets and nuclear material is irreversible, unique, and has not been previously described. Other components of blood such as red cells, other types of leukocytes and fibrin never participate in microaggregate formation.

From this study it is that we recommend the following terms: Simple Platelet Aggregates, Complex Platelet Aggregates and Neutrophil-Platelet Aggregates which connotate specifically the cellular origin and imply their possible embolic character when transfused into the recipient's vasculature.

V. INTERDEPARTMENTAL COLLABORATIVE RESEARCH PROJECTS

Experimental Schistosomiasis (See Annual Report 73-74 from Dept. of Immunology & Medical Zoology).

1. Characterization of Surface Antigen of Cercariae of S. Mansoni: By means of in vitro immunofluorescent techniques (FA) on living worms, we have demonstrated that sera from infected rats with Schistosoma mansoni contain antibody (IgG) which reacts specifically with the surface of adult worms, cercariae and schistosomules. Further studies have been carried out in characterizing the specific surface antigen of cercariae.

2. Penetration of Cercariae Into the Skin: The natural means of entrance of cercariae in man is through the intact epidermis. When cercariae are similarly applied onto the skin of an immunized rhesus monkey, an intense local inflammatory response ensues which in turn resists further infection. We have investigated the immunopathologic features of this cutaneous reaction in the skin of normal and immunized rhesus monkeys with S. mansoni infection. Preliminary results have indicated that the skin lesion represents that of delayed hypersensitivity reaction. Further studies on the skin lesion by FA, light and electron microscopy are being studied in defining this characteristic cutaneous response.

3. Glomerulonephritis in Schistosomiasis: Because of the persistent presence of antigen (worm) in the blood and a pronounced host antibody response in Schistosomiasis, deposition of immune complexes in the renal glomerulus might be expected. Using FA techniques, preliminary studies have shown that rhesus λM and C3 granular deposit are increased in the glomeruli of infected rhesus monkeys with S. mansoni. EM studies are planned to clarify the nature of glomerular lesion in S. mansoni infection in rhesus.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Diseases and Injuries

Literature Cited.

Publications:

1. Takeuchi, A.: Electron microscope studies on acute enteritis produced experimentally in guinea pigs. Proceedings of the 6th Intern. Symposium on Food-borne Diseases, W.A.V.F.H., pp 45, 1973.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA0[illegible] 6463	74 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
73 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	3A762760A822	00	126
b. CONTRIBUTING				
c. [illegible]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Infectious Hepatitis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 600 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 74	1	256
FISCAL YEAR CURRENT 75	1	256

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Knodell, MAJ R.G.

NAME: Flannery, LTC E.P. DA

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 hyperimmune gamma globulin prevents transfusional hepatitis. Collaborative efforts are in progress to develop immunologic methods for the detection of hepatitis A antigens and antibody.

25. (U) 73 07 - 74 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 285 patients have volunteered for this study, which compares two types of gamma globulin and a placebo solution. Collaborative research with other institutions has been undertaken to isolate antigens associated with hepatitis A for the development of useful immunologic methods for the identification of the etiologic agent of this disease. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* 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 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 126 Infectious hepatitis

Investigators.
Principal: Marcel E. Conrad, COL, MC
Associate: Eugene P. Flannery, LTC, MC
Robert G. Knodell, MAJ, MC

Description: To determine if gamma globulin with a high HBAb titer provides protection against transfusional hepatitis and whether transfused blood with HBAg only by radioimmune assay but not by other methods of testing causes hepatitis.

Biological specimens obtained in volunteer studies of infectious hepatitis at Illinois State Penitentiary in collaboration with Dr. Joseph D. Boggs of Northwestern University are maintained for distribution to investigators attempting to isolate the etiologic agent of hepatitis, develop antibody tests to identify patients with the disease, and develop an animal model of the disease that would obviate the need for human studies.

Results: During recent years there has been a high incidence of hepatitis among patients hospitalized at WRAMC undergoing cardiac bypass surgery. Estimates of the incidence of hepatitis in this group based upon the detection of elevated transaminase determinations 3 months after surgery are 10 to 20 percent of patients. It is believed that this is caused by the requirement to use 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 HBAb 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 and 6 months after surgery. The blood specimen is tested for HBAg, HBAb, SGOT, and serum bilirubin determinations. In addition, a history is obtained from each patient at intervals after surgery. All blood used for transfusion is tested by radioimmune assay for HBAg and HBAb.

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 HBAb 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 soldiers 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.

This is a collaborative double blind study being conducted at Walter Reed Army Medical Center and at Letterman Army Medical Center. Since the inception of the study, 284 patients undergoing cardiac bypass surgery at either Walter Reed Army Medical Center or Letterman Army Medical Center have volunteered as participants in the study. Each patient has received under code either 10 ml of high titer HBAb 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 months after surgery. Only seven patients have had clinical icteric hepatitis. Only 20% of patients with either icteric hepatitis or elevated transaminase determinations have had HBAg identifiable hepatitis as measured by radioimmunoassay despite the observation that approximately one-quarter of the patients with abnormal laboratory tests were transfused with blood that was HBAg positive when tested by radioimmunoassay; all blood was HBAg negative when tested by counterimmunoelectrophoresis. It is believed that measurements of HBAb in many of these patients at periodic intervals to as long as 1 year after surgery will document that many of these patients had hepatitis B. However, it is evident that a significant number of patients who develop hepatitis following transfusion are probably infected with a different viral agent. A 90% followup of 3 months is being achieved largely by mail and telephone; noncompliance is largely limited to preschool children undergoing cardiac surgery. Surveillance of the study is being maintained by the National Heart and Lung Institute through the Hepatitis Advisory Committee. They review the data in an uncoded manner three times yearly to insure safety and to insure that significant differences have not already been achieved between the three coded groups. It is currently believed that at least one additional year of study will be needed for completion.

Biological specimens obtained in volunteer studies of hepatitis A in collaboration with Dr. Joseph D. Boggs of Northwestern University have been distributed to investigators. During the last year these specimens have been useful in the development of an immunofluorescent electron microscopic method for the identification of an antigen-antibody system associ-

ated with hepatitis A. These studies by Purcell et al at the NIH have stimulated widespread requests for fecal and serum specimens by investigators working in this field of research. Attempts are being made to develop culture systems, purify the antigen and antibody, and study the potentially infectious particles in animals. Larger sources of the fecal antigen will be needed in order to keep up with investigator requests.

Conclusion: A study is in progress to evaluate the efficacy of high titer HBAb gamma globulin in the prevention of transfusional hepatitis. The additional time of approximately one and one-half year will be required for completion of the study and analysis of the data.

New sources of feces from patients in the pre-icteric stages of hepatitis A should be sought for studies attempting to isolate the etiologic agent of this disease.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 126 Infectious hepatitis

Literature Cited.

Publications:

1. Ginsberg, A. L., Conrad, M. E., Bancroft, W. H., Ling, C. M., and Overby, L.R.: Antibody to Australia antigen: Detection with a simple radioimmune assay, incidence in military populations, and role in the prevention of hepatitis B with gamma globulin. J. Lab. Clin. Med. 82: 317-325, 1973.

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	74 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
73 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 114(f)			

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 74	3	70
FISCAL YEAR CURRENT 75	3	118

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
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ASSOCIATE INVESTIGATORS
NAME: Bardill, LTC D. R.
NAME: Rothberg, J. M. Ph.D.

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Adjustment; (U) Psychiatric Illness; (U) Stress Performance; 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) 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) 73 07 - 74 06 The study of the relationship between the trait of hypnotizability and the response to brief psychotherapy has been completed. The military family and adolescent dysfunctioning study describes the families of problem adolescents who seek help at an Army and outpatient psychiatric service. Data collected from the Racial Perceptions Inventory on the influence of social variables on communications between soldiers of different races has been utilized in planning the Race Relations Training Program at WRAMC. Project Home is a study of 173 Viet Nam veterans of which 70 have been followed for a year. Data analysis is in progress. The Career Outcome Study is a prospective study of the military and medical careers of urine positive and negative soldiers. The study population was selected from three basic training posts and will be followed for 14 months. A methodology for easily locating and following soldiers by their personnel records when their assignments are frequently changed is being developed. This technology is essential to establish more accurate incidence rates of various disorders. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*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 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, Project HOME, the Career Outcome Study, and the Demographic Research Methodology 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 collected from this project may contribute to the development of new primary and secondary preventive programs within Army psychiatry.

c. The study has been divided into three sections: the Family Activity Section, the Social Network Section, and the Problem Section.

(1) The Family Activity Section describes how each member of a family unit allocates his time. A questionnaire has been developed which elicits each individual's activities for three days prior to the interview session. Family members are also requested to draw a sketch of their home and to complete a diary of their activities for the week following the interview session. The amount of time spent in certain dyads, triads, and/or alone, the topics discussed and the activities shared, represent the data from which a description of the internal family workings will be developed.

(2) The purpose of the Social Network Section is to describe the extrafamilial relationships of family members. A questionnaire is utilized to determine the people the respondent associates with by choice outside the nuclear family unit (social network). Information such as the density of social networks, amount of duplication in the mother-father networks, the closeness described to persons mentioned, the amount of time spent with persons mentioned, and topics discussed will be obtained by questionnaire. This model has been adapted from Bott.[5]

(3) The Problem Section

(a) This section classifies the presenting problems of the families studied from a behaviorally descriptive framework. The data are gathered by means of a semi-structured interview given individually to each family member. The interview focuses on past and present problems, behavioral goals of the family, and parental influences on the children.

(b) Presenting problems will be classified by looking at such variables as: the number of problems; where problem behaviors take place; the amount of agreement or disagreement among family

members about the stated problems; the relationship between the reasons for seeking help and the desired behavioral changes within the family; and how each family member is affected by the problem behaviors.

(c) A psychometrically oriented research effort within the Problem Section is concerned with quantifying the stimulus value different family members present to each other during role enactments. A semantic differential technique is being used wherein each family member is being requested to rate their past role relations and that of every other family member. The rating for each family member is obtained on a series of scales anchored by descriptive antonyms (i.e., rewarding-punishing, sharing-withholding). The results will be factor analyzed to determine the commonalities employed in describing role relations with other family members. A subsequent analysis will be conducted to determine the behavioral correlates of individual and situational stimulus contributions as assessed by semantic differential. Other instruments being employed in the individual psychometric evaluation include the Rotter Internal-External Locus of Control Scale, the Marlowe-Crown Social Desirability Scale, the Taylor Manifest Anxiety Scale, the California F Scale, the Dogmatism Scale, and a study-specific behavior checklist. Correlations between specific behavioral classes, (i.e., eating, friendship) and each of the psychometric scale scores will be obtained.

(d) The pilot phase of the study has been completed and data collection will proceed over the next 12 months.

2. Project HOME

a. Project HOME is a study of 173 Viet Nam veterans of which 70 have been followed for a year. Drug use and certain other parameters of life style were followed. All subjects cooperated in a rather lengthy initial interview. Those subjects who were remaining in the Army (about half) were re-interviewed every two months. Those subjects who had separated from the Army were contacted by means of mailed questionnaires, about one year after the initial contact. The study contains approximately an equal number of respondents in each of the following four categories: (1) urine negative non-drug users, (2) urine negative recreational drug users, (3) urine positive recreational drug users, and (4) urine positive drug dependent users. Urine test results refer to the DEROS screen from Viet Nam.

b. Data collection is complete and data analysis is presently in progress. Preliminary findings from interviews and questionnaires suggest that 23% of urine positive subjects could be described as recreational users. The recreational user had less overall drug use, used drugs only during non-duty hours, was more than twice as likely to be black, smoked marijuana socially rather than in isolation, and was more than three times as likely to have been drafted.

c. Fifty-nine percent of urine negative subjects were non-users. Thirty-eight percent of urine negatives were recreational users. Non-users reported choosing as friends people who used less marijuana and more beer. The non-user was of higher rank (E-5) and much more often expressed a positive attitude towards his commanding officer.

3. The Career Outcome Study

a. Urine screening for illicit drugs of abuse at time of induction has defined a population of considerable interest to military psychiatry. Since behavior tends generally to be persistent and since persistent use of illicit drugs often has negative sequelae, a prospective study of the military and medical careers of urine positive and negative cohorts is in progress.

b. The cohorts consist of all of the urine positives and a fixed percentage random sample of the urine negatives processed through three reception stations over the period of April 1972 through June 1973. Of the five-thousand individuals so defined, approximately 90% have been located in the personnel system and their records are presently being acquired and analyzed. Characterizations of the initial demographic composition of the cohorts (by post and urine results) as well as measures of the military careers of these individuals have been selected and adequate computing programs have been produced and documented. Analysis of the error rates associated with locating individuals after different numbers of transcriptions of names and social security numbers is in process. Preliminary results suggest that comparatively more members of the urine positive cohort leave the service within the first year.

4. Demographic Research Methodology Study

Questions of the incidence of neuropsychiatric problems including drug and alcohol abuse in changing populations have resulted in the development of a system to characterize the temporal variability of the demographic composition of military populations. Sampling strategies have been investigated and sampling procedures have been developed based on the statistical properties of the social security numbers used for personnel identification. Analysis of the data in previously reported research has led to the development of new statistical criteria for the interpretation of stepwise linear discriminant procedures. These results are being prepared for publication under the title of "An Application of Stepwise Discriminant Analysis to the Characterization of Military Heroin Dependents, Illicit Drug Users, and Psychiatric Patients." These methodological techniques may contribute to future research programs which attempt to characterize specific military populations.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6456	74 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
73 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 114(f)			

11. TITLE (Precede with Security Classification Code): (U) Military performance and Stress; Factors Leading to Decrements of Performance and 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: NA
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	3	100
FISCAL YEAR CURRENT 75	3.5	148

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21. GENERAL USE
Foreign Intelligence not Considered

22. KEYWORDS (Precede 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) 73 07 - 74 06 Work on the times series analysis of psychophysiological performance decrements as a function of environmental loading factors has been suspended during the development and installation of controlled environmental research facilities. Pending completion of physical facilities, pilot investigations of the interactions between stressors and information processing and decision making performances has been ongoing. Particular emphasis has been placed on the tradeoff between speed response and accuracy of decision as it is affected by environmental loading factors. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 73 - 30 JUN 74.

* 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 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 to both 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, and temporal disorientation. 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 analogues 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-Offs in Speeded Classification

Reaction time (RT) experiments have traditionally been used to investigate a wide range of human performance characteristics, from the detection of isolated stimuli through the comparison of a stimulus word's meaning to that of words stored in memory. These experiments typically require subjects to respond as rapidly as possible without making errors, with mean RT constituting the primary dependent variable. A major difficulty with such an approach is that it assumes that changes in RT are uncontaminated by changes in subjects' criteria for generating a response. Previous experiments have demonstrated that subjects are able to achieve decreases in RT by sacrifices in response accuracy (cf. Schouten & Bekker, 1967; Pachella & Fisher, 1972; Pew, 1969). Thus, it is impossible to determine whether a given change in RT reflects true changes

in the time required for stimulus processing and response generation, or whether it reflects a change in subjects' response criteria. Recent experiments have suggested that a potential solution to this difficulty may be obtained by determining the complete trade-off function between response speed and accuracy (Lappin & Disch, 1972a,b; 1973). Speeded classification experiments are currently under way to determine optimal experimental procedures for generating reliable speed-accuracy trade-off functions for subsequent use in sustained performance and alcohol experiments.

2. A Normative Model for the Integration of Redundant Sources of Information in Speeded Classification

A general normative model has been developed to evaluate the integration of redundant sources of information in speeded classification tasks. Previous experiments have demonstrated that the addition of a completely redundant source of information to an existing stimulus dimension can significantly facilitate performance in speeded classification. That is, RT for classification of two redundant dimensions is shorter than RT for classification of either single dimension alone (Biederman & Checkosky, 1970; Morton, 1969; Garner, 1969; Wood, in press, a). Such "redundancy gains" have also been obtained in absolute judgment experiments in which accuracy rather than speed is the primary dependent variable (Eriksen & Hake, 1955; Lockhead, 1966, 1970). Implemented in equation form, the normative model enables RT distributions for the classification of redundant dimensions to be predicted from the empirical RT distributions obtained separately for the single stimulus dimensions. Thus, obtained redundancy gains under a given set of stress conditions can be compared with the theoretical maximum established by the model. The degree to which an obtained redundancy gain falls short of the predicted by the normative model constitutes a precise index of the degree to which performance is less than optimal. This procedure appears to be an extremely sensitive way to assess the effects of stress on speeded classification performance. A description of the application of this model to speeded classification data will be published in the coming fiscal year (Wood, in press, b).

3. Effects of Alcohol and Multiple Task Demands on Attention and Memory Performance

The widespread use of alcohol in the U.S. Army has recently been documented by Cahalan et al. (1972). In the present experiment the effects of moderate-to-heavy doses of alcohol on attention and memory performance are examined. Alcohol doses in this range have previously been shown to produce deficits in various cognitive processes (cf., Carpenter, 1962; Ryback, 1971), although the precise nature of the deficits remains to be determined. The interaction between alcohol and demands for multiple task performance is also investigated. Subjects are required to perform a verbal learning task and a probe RT task concurrently. Probe stimuli are presented systematically at different temporal intervals following the verbal stimuli in order to examine the time course of the memorizing sequence. Finally, a

perceptual salience manipulation is employed to determine whether recall deficits following alcohol intake are related primarily to deficits in attention, rehearsal, or retrieval. Preliminary to using RT as a dependent variable in this experiment, pilot experiments are establishing dose-response relationships between alcohol and RT using the speed-accuracy trade-off procedure described above.

4. Autonomic Correlates of Information Processing

Autonomic nervous system reactions have been considered primarily as indices of stress. Evidence is emerging that suggests, however, that autonomic reactions are involved in relatively nonstressful psychological acts, such as attending to the environment and problem-solving (Lacey & Lacey, 1974; Hahn, 1973). Since stress exerts marked influence on autonomic reactions, autonomic disturbances may be an integral part of the syndrome of stress-related performance decrements.

Previous work (Jemmings, 1974) has demonstrated the sensitivity of one aspect of autonomic activity, the cardiac inter-beat-interval, to differences in information processing. Information processing and its autonomic correlates were found further to interact with body weight. Specific relations having to do with attention and memory were suggested in the results. Thus, the results established the relevance of autonomic activity to characteristics of performance and to characteristics of the performing individual.

Current work has been designed to follow up the suggested specific relationship between different types of attentional processes and autonomic activity. Current work employs a combination memory and reaction time task. The reaction time task serves as a probe into the information processing required by the primary task, i.e. the memory task. Variations in the items memorized provide a manipulation of attentional salience. Variations in body weight within the set of randomly selected subjects will be related to the autonomic and performance results.

5. Measurement Technology for the Assessment of Sustained Performance

Studies of bio-rhythms and sustained performance are by nature time consuming, and are subject to certain practical problems that tend to make the most scientifically desirable data the most difficult to obtain. Physiological recordings from humans have generally required the subjects to be bedridden, restrained by wires and cables, or encumbered by bulky equipment that interferes with normal activities. The problems have been such that most of the data obtained to date has been drawn from a small number of motivated investigators willing to serve as their own subjects. Future progress will require normative data from large subject populations, over extended periods of time, during periods of sleep and waking activities. For this to become practical with volunteer subjects, biomedical recorders must become considerably smaller and cheaper than those heretofore available.

This Department has been investigating the feasibility of an all solid-state subminiature recorder using large scale integrated circuits. The goal is a recorder that is small enough to be taped to the body under the clothes, and that can store from 24 hours of data sampled at 15 minute intervals (worst case) up to seven days of data sampled every six minutes (best case).

Initial work showed the design to be technically and economically feasible, with an added gain in increased reliability, better resistance to mechanical shock, and higher time-compression rates than offered by mechanical recorders. However, the power consumption and multiple voltage requirements of existing circuits require several moderately large batteries, resulting in a recorder no smaller than existing mechanical ones. Hence, the recorder has been redesigned to use ultra low power components that can operate from a single voltage supply. All but two of the recorder components have been obtained in this low power version; one has yet to be released commercially, the other is on order.

A field trial of a miniature magnetic tape recorder capable of storing 24 hours of electrophysiological data has been completed. Five men wore the recorder continuously for three days while engaging in normal duty activities. The subjects reported no objectionable discomfort or impediment to their normal functioning. The signal monitored was the chest ECG. Foam disposable electrodes were generally reliable but still provide the major source of monitoring difficulty. Preliminary analysis of magnetic tapes indicates that signal to noise ratios are acceptable for reliable signal extraction.

6. Statistical Problems Associated with Biological Measures

Two aspects of work in this area have been published. The statistical distribution characteristics of cardiac inter-beat-interval were found superior to those of heart rate (Jennings, Stringfellow, & Graham, 1974). An initial description of the application of factor analysis to cardiac inter-beat-interval has been presented (Jennings, 1974), although the ultimate utility of this technique has not been established.

7. Longitudinal Study of Endocrine Reactions and Stress in Officer Candidate School

This work has been completed and results previously described. Publications will appear during the next fiscal year.

Project 3A762758A823 MILITARY PSYCHIATRY

Task ØØ Military Psychiatry

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

Literature Cited.

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2. Jennings, J. R.: Psychophysiological correlates of memory and attention in the obese and underweight. Psychophysiol. 11: 238, 1974 (A).

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6. Wood, C. C.: Parallel processing of auditory and phonetic information in speech perception. J. Acoust. Soc. Amer. 55: 435, 1974 (A).

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PROJECT 3A762758A824
RADIATION INJURY AND PROTECTION

Task 01
Radiation Injury and Protection

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6471	74 06 30	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	9. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 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	62758A	3A762758A824	01	055
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Synthesis of Anti-Irradiation Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

014000 Radio and Radiation

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
59 05	74 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 73	6	150
FISCAL YEAR CURRENT 74	1.85	138

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

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NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Activity; (U) Chemical; (U) Compound; (U) Dose, (U) Protection; (U) Radiation Injury; (U) Human Volunteers

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 research is to develop a militarily useful pill to protect personnel against lethal effects of ionizing radiation. Such a drug would reduce the initial effect of nuclear radiation as well as provide a margin of safety for personnel operating in a contaminated terrain. An efficient antiradiation compound would also be useful to the Army from a clinical standpoint.

24. (U) Approach to the objectives is through accepted drug development protocols. Synthesis and testing of potential agents is being carried out. Test results are analyzed for structure activity relationships and fed back into the synthesis program. Promising compounds are carried forward to testing in large animals and the pharmacology of these compounds investigated. In adcition, chronic toxicity studies, dose reduction factor studies and drug antagonism studies are being carried out.

25. (U) 73 07 - 74 06 The last of the chemical synthesis work, a preparations laboratory, has been phased out. The final phase of the work was devoted to the synthesis of some desired disulfides. A survey of the synthesis program is now being written. No further work is planned. This work unit isbeing terminated because of a change in mission emphasis by higher headquarters.

*Available to contractors upon originator's approval

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6484	74 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
73 07 01	D. CHG	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	3A762758A824	01	057
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Biological Effects & Hazards of Microwave Radiation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology 016208 Stres. 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. 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	5	250
FISCAL YEAR CURRENT 75	5	270

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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NAME: Hawkins, T.D. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Microwave Hazards, (U) Nonionizing Radiation, (U) Dosimetry, (U) Behavioral Effects, (U) Neurophysiology, (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 to delimit human operations in an electromagnetic environment to support maximum combat effectiveness at a minimum personnel risk. Delineate the interaction of radiofrequency and microwave radiation (100 MHz to 100 GHz) with biological systems. Survey and evaluate all known methods and techniques of microwave dosimetry and develop improved techniques and instrumentation where appropriate and necessary.

24. (U) Investigate each major organ system and biological process where there is reason to believe microwave effects may occur at reasonable power intensities. Where indicated, determine the military significance of the effects and the measures necessary to obviate them. A data bank of the world literature on the biological effects and hazards of electromagnetic radiation is to be maintained. Initial scientific efforts will use evaluative methods from experimental psychology, electrophysiology and ophthalmology. Exposure parameters will be chosen for relevance to Army radiating equipment and operational requirements.

25. (U) 73 07 - 74 06. During the reporting period, effort concentrated on the assessment of lethality of power levels up to 150 mW/cm^2 at various wavelengths; lethality studies in rodents have been completed; the interaction of circadian rhythms with microwave exposure has been demonstrated in mouse cornea. Also, the orientation of the biological specimen, with respect to both the E and H vectors, interacts strongly with the wavelength of the radiation. A survey for cataracts in military personnel exposed to microwaves has been completed; to date, no differences between non-exposed and exposed personnel have been found. The proceedings of a major conference dealing with the biological effects of microwaves were published. For technical report see Walter Reed Army Institute of Research Annual Report, 1 July 73 - 30 June 74.

*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 3A762758A824 RADIATION INJURY AND PROTECTION

Task 01 Radiation Injury and Protection

Work Unit 057 Biological effects and hazards of microwave radiation

Investigators.
Principal: Joseph C. Sharp, Ph.D.
Associate: H. Mark Grove, M.Sc.; Sandra H. Githens, B.S.; T. Daryl Hawkins, M.A.; MAJ Stuart E. Hirsch, MC; John H. Jacobi, M.Sc.; John F. Schrot, M.A.; Peter Brown, B.S.

Our efforts to systematize the behavioral and biological effects of microwave irradiation produced considerable gains in FY 74. Earlier observations of frequency-dependent behavioral effects were corroborated and expanded. Using indices of lethality and seizure, with several types of animal subjects, we have greatly extended the biological generality and implications of microwave exposure effects. The order of presentation below is roughly chronological.

Lethal effects of 2.45 GHz as a function of power density level in mice.

Our earlier work with rats showed that indices of lethality [determined by video monitoring, such as time elapsed to convulsion and time elapsed to final movement] could be varied systematically by manipulating power level. This work was extended to mice. Groups of eleven mice were exposed to one of five different power levels, 100, 150, 200, 300 or 400 mW/cm^2. Curves relating time to convulsion and time to final movement showed similar decreasing trends as a function of increasing power level, with average times to final movement being slightly longer than average times to convulsion. [Since all of our work indicates that these two measures show similar trends, statements of differential lethality in this report will be based on only the time to convulsion index to avoid unnecessary redundancies.] At all power levels the mice were consistently more vulnerable than the rats examined in FY 73, strongly suggesting that mice are generally more sensitive to 2.45 GHz than rats.

Differential lethal effects of 1.7 GHz and 2.45 GHz.

Using a power level of 100 mW/cm^2, twelve 150 g rats were exposed to 1.7 GHz and twelve were exposed to 2.45 GHz. The data showed an unequivocal frequency difference. Median time to convulsion at 2.45 GHz was 1260 sec, while the median time to convulsion at 1.7 GHz was 801 sec. In addition, there was no overlap in the range of scores

obtained for each frequency, providing a strong indication that 1.7 GHz is more lethal than 2.45 GHz in 150 g rats. These data provided the first indication that the frequency-dependent performance decrements observed in prior years with rats were a reflection of a general frequency-dependent biological phenomenon, thus mitigating our concern that the performance effects observed earlier were due to some uncontrolled artifact.

Lethality as a function of body orientation relative to E and H vectors.

The lethal effects of E ∥ a and H ∥ a (when a represents the major or long axis of the body) were compared at 1.7 GHz with a power level of 150 mW/cm^2 using rats (100-120 g). For one group of rats, the E vector was parallel to a and for another H was parallel to a. A small but significant difference indicated greater lethality with E ∥ a.

Microwave absorption data collected by Dr. Om P. Gandhi during a three-month visit to our laboratory suggested the possibility that larger lethality differences due to E-field orientation might be obtained with rats at frequencies lower than 1.7 GHz. In fact, his data provide the first known opportunity to scale from animals to man the resonant frequencies for maximum absorption ($\sim$ 40 to $\sim$ 55 MHz). There appeared to be larger orientation differences in power which Gandhi had made with mice at lower frequencies. Lethality data obtained with rats at 0.985 GHz confirmed this prediction. As in the 1.7 GHz study, rats (100-120 g) convulsed sooner with E ∥ a than H ∥ a; the difference in time to convulsion, produced by changing the E-field at 0.985 GHz, was quite large. Average time to convulsion with E ∥ a was only 269 sec, or about one-third the average time to convulsion with the H ∥ a (865 sec).

Lethality in mice (25-30 g) and rats (100-120 g) was compared at 1.7 GHz using a power level of 150 mW/cm^2. Consistently, the mice convulsed earlier than the rats. The median times were 331 sec and 498 sec, respectively. These observations in conjunction with similar differences found at 2.45 GHz suggested that mice are generally more vulnerable than rats to microwave exposure, at least in the range of 1.7 to 2.45 GHz.

Systematic interrelationships of microwave frequency E-field orientation and size of experimental subject as codeterminants of microwave lethality.

The cumulative process achieved throughout FY 74 on both the biological and engineering levels led us to conclude that there were a number of important microwave variables which combine in a complex

but systematic manner to produce the biological effects observed. The primary variables are presently considered to be the microwave frequency, the orientation of E-field, and the dimensions of the experimental subject. The combined and interacting effects of these variables are currently being explored in a major lethality study which is nearing completion. Three microwave frequencies have been examined: 0.985, 1.7, and 2.45 GHz. Work with 3.0 GHz is in progress. Work at 0.70 GHz is projected as the last stage. At each completed frequency, three types of animals were used: mice (25-30 g), small rats (100-120 g), and large rats (380-400 g). For each type of animal there were two groups: one group exposed with $H \parallel \underline{a}$ and another group exposed with $E \parallel \underline{a}$. Each subject was exposed to 150 mW/cm^2 and the time to convulsion and time to final movement were recorded. Data collected from this study have corroborated our earlier lethality findings and extended their generality.

Within the range of frequencies we have explored in the current lethality study, coupled with the absorption data collected by Gandhi, a number of tentative generalizations can be made:

(1) General Effect of Microwave Frequency.

For a particular animal, there is a frequency (or frequency range) to which the animal is more sensitive than it is to higher or lower frequencies. Given the general bitonic nature of the frequency sensitivity curve, the frequency at which maximum sensitivity obtains for a particular animal is dependent on both the dimensions of the animal and the E vector orientation. Larger animals show peak sensitivity at lower frequencies than smaller animals. This size/frequency relationship holds generally for both H and E parallel to $\underline{a}$. However, for a given size animal, the maximally effective frequency with the $E \parallel \underline{a}$ is lower than the maximally effective frequency with $H \parallel \underline{a}$.

(2) General Difference Between E vector orientations.

The magnitude of the differential effect produced by exposures with $H \parallel \underline{a}$ as opposed to exposures with $E \parallel \underline{a}$ is a function of the microwave frequency employed. At higher frequencies there is very little differential effect. At progressively lower frequencies, the difference increases with $E \parallel \underline{a}$ being consistently more effective than $H \parallel \underline{a}$.

(3) General Effect of Size.

As frequencies increase, e.g., up to 2.45 and 3.0 GHz, the data suggest that the larger the animal, the less is the vulnerability to the adverse effects of the radiation. This may be a simple function of the total mass of the subject. At lower frequencies, the effects are less regular, but current data are consistent with the statement that larger animals (100 and 400 g rats) are more vulnerable than

very small ones (mice). This relation is probably due to frequency dependent power absorption resulting from the interaction of differential penetration and the specific dimensions of the animal, and coupling with both E and H vectors of the electromagnetic energy.

Modulated microwave signals.

An initial series of studies designed to better understand the so-called hearing effect produced by extremely low average power levels have been completed. Two observations have led to a temporary cessation of this work. These were (a) the observation that non-biologic material could serve as a transducer of the energy to acoustic signals which can be used as a model without exposing biological material and (b) the report that signals similar to those used in the auditory study may cause changes in the blood brain barrier of experimental animals. This latter observation has been investigated during the latter half of the reporting period and, to date, not been replicated. However, the implications of these observations are of such great importance that further work is in progress.

Determination of dielectric properties of tissue.

To better understand and confirm earlier work, a series of measurements on the dielectric properties of various tissues was completed. This work took fat, brain, muscle, liver and lung from different species, including man, and, utilizing unique equipment in the laboratory, determined the dielectric constants at physiological temperatures. The findings resolved some ambiguities in the literature and, more importantly, showed that tissues from different species were similar. On this variable, human tissues are not significantly different from animal tissues which will now allow for detailed determinations in model systems.

Microwave bioeffects current-awareness digest.

During FY 74, a multiple-agency sponsored project to produce and publish a quarterly digest of the literature on biological effects of nonionizing radiation was initiated. This effort will be carried out by the Franklin Institute Research Laboratories under ARO Grant No. DAHCO4-74-G-0132. Funding has been provided by the Army, Air Force, Navy and FDA/BRH. The EPA is also cooperating in the activity. The first issue was readied for publication in June 1974. The quarterly publication will be distributed, without charge, to scientists and other workers in this discipline as a means of bringing together citations and abstracts of the diverse literature and is providing a method of informal communication.

Ocular effects of microwaves.

During FY 74 microwave ocular effects surveys continue in cooperation with the Army environmental hygiene agency. Populations within Army installations whose missions allow for the potential of exposure to microwave irradiation were examined. A detailed opthalmic examination including visual acuity, fundus examination and detailed slit lamp examination were done with particular emphasis on the lens. Photography was performed where indicated. Non-exposed personnel served as controls. Preliminary results have not shown a higher incidence of lenticular changes in the group with potential for microwave exposure.

The exposure of albino rabbits to microwave irradiation continue. Utilizing a focusing dish antenna, rabbits were exposed to varying power densities from low level to sub-lethal higher levels for periods of 15 minutes daily for 30 consecutive days. Both CW and pulsed duty cycles were used. Cataracts were produced only at power levels of 400 mW or above. The animals have now been followed for one year and to date have exhibited no lenticular changes or cataracts at power levels below 300 mW/cm^2.

Histology and electromicroscopy of rabbit lenses of exposed animals has progressed. A histologic correlated between posterior subcapsular irridescence, a slit lamp sign, and its electron microscopic counterpart has been demonstrated.

Project 3A762758A824 RADIATION INJURY AND PROTECTION

Task 01 Radiation Injury and Protection

Work Unit 057 Biological effects and hazards of microwave radiation

Literature Cited.

Reference:

1. Schrot, J., and Hawkins, T.D.: Lethal effects of 3000 MHz radiation on the rat. Radiation Research. (In press)

Publications:

1. Sharp, J.C., Grove, H.M., and Gandhi, O.P.: Generation of acoustic signals of pulsed microwaves. IEEE Transactions on Microwave Theory and Techniques. May 1974, 583-584.

2. Gandhi, O.P., Sharp, J.C., and Grove, H.M.: Strong dependence of whole animal absorption on polarization and frequency of RF energy. Proc. NYAS. Feb 1974.

3. Gandhi, O.P.: Polarization and frequency effects on whole animal absorption of RF energy. Proc. of IEEE. 62, Aug 1974.

4. Lenox, R.H., Gandhi, O.P., Meyerhoff, J.L., and Brown, P.V.: Modifications of in-vivo rapid microwave inactivation of enzymes in the central nervous system. Proc. International Microwave Power Inst. May 1974.

5. Larsen, L.E., Moore, R.A., and Acevedo, J.: A microwave decoupled brain-temperature transducer. IEEE Transactions on Microwave Theory and Techniques. April 1974, 438-444.

6. Lin, J.C., Grove, H.M., and Sharp, J.C.: Comparative measurement of dielectric properties of fresh mammalian tissues. Proc. CPEM. July 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6533	74 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
NIS	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	62758A	3A762758A824	01	058
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Biological Evaluation of Anti-irradiation Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

014100 Radiobiology 003200 Chemical, Biological and Radiological Warfare

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
59 05	74 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 73	1.65	136
FISCAL YEAR CURRENT 74	1.65	109

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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ASSOCIATE INVESTIGATORS
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NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Activity; (U) Chemical; (U) Compound; (U) Protection; (U) Radiation

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

23. (U) The objective of this research is to develop a militarily useful pill to protect personnel against lethal effects of ionizing radiation. Such a drug would reduce the initial effect of nuclear radiation as well as provide a margin of safety for personnel operating in a contaminated terrain. An efficient antiradiation compound would also be useful to the Army from a clinical standpoint.

24. (U) Approach to the objectives is through accepted drug development protocols. Test drugs are evaluated for ability to protect mice against lethal doses of ionizing radiation. Promising compounds are further evaluated in larger animals.

25. (U) 73 07 - 74 06 Testing of 300 drugs synthesized specifically to exploit known structure-activity relationships identified 100 drugs which protected 50% or more of mice exposed to lethal doses of ionizing radiation. Of the 100 active compounds, 40 gave protection when administered orally. The identification of classes of chemicals producing levels of protection as high as 100% in mice following oral administration is considered a major breakthrough. Of even greater significance was the first successful demonstration of protection of rhesus monkeys from lethal irradiation by an orally administered drug. Work is being terminated due to emphasis being shifted to other problems of Military significance. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 73 - 30 June 74.

*Available to contractors upon originator's approval

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Project 3A762758A824 RADIATION INJURY AND PROTECTION

Task 01 Radiation Injury and Protection

Work Unit 058 Biological evaluation of anti-radiation drugs

Investigators.
Principal: LTC Kenneth E. Kinnamon, VC
Associates: LTC Peter S. Loizeaux, VC; CPT Lyle L. Ketterling, MSC; Ms. Marie Grenan

During FY74, the phase-out year of Work Unit 058, emphasis was placed upon the oral administration of radioprotective drugs. Initial evaluation of drugs for their ability to protect against lethal doses of ionizing radiation was performed in mice, utilizing both oral and parenteral routes. Promising compounds were further evaluated in monkeys, but testing was limited to the oral route.

Radioprotection in mice

More than 300 drugs were evaluated for their ability to protect mice against lethal doses of cesium-137 irradiation. When administered intraperitoneally approximately 100 drugs protected at least 50% of the mice. When given orally 40 drugs were identified as providing at least 50% protection. The most interesting compounds were amidines with disulfide covering functions. Few compounds remain to be evaluated since termination of the synthesis effort.

Radioprotection in large animals

The highly radioprotective phosphorothioate WR 3689 was prepared in enteric coated tablets in an effort to carry the drug through the acid conditions of the stomach without breakdown. The number of tablets required to reach calculated radioprotective levels in monkeys was prohibitive and dogs consistently vomited the drug. The phosphorothioate class of radioprotectants contain a number of outstanding drugs, compounds which provide 100% protection to mice whether given orally or parenterally. They also provide 100% protection to monkeys if given parenterally; however the class is ineffective if administered orally to either monkeys or dogs. A variety of techniques were employed to enhance absorption of the drugs, but without success.

The second drug to offer protection to monkeys following oral administration was identified. Six M. mulatta received 85 mg/kg of WR 33,763, an amidine disulfide, by gastric intubation. Although only 2 of 6 monkeys survived, the four which died were the result of drug toxicity, not radiation. The other oral radioprotectant in monkeys, reported in the FY73 Annual Report is also an amidine disulfide. The two drugs represent a highly significant breakthrough, demonstrating

for the first time the feasibility of a pill to provide radioprotection in nuclear warfare.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6534	74 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
NIS	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	62758A	3A762758A824	01	059
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procode with Security Classification Code)

(U) Determination of Pharmacological Effects of Anti-irradiation Drugs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

014100 Radiobiology 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
73 05	74 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 73	1.0	68
FISCAL YEAR CURRENT 74	1.0	110

19. RESPONSIBLE DOD ORGANIZATION

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NAME:
NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procode EACH with Security Classification Code) (U) Radioprotectants; (U) Cardiovascular: (U) Autonomic Nervous System; (U) Human Volunteer

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

23. (U) The objective of this research is to determine the pharmacological and toxicological effects of potential radioprotectant drugs. These studies are to provide a basis for predicting human response and to fulfill requirements for submission of IND for clinical trials. Such drugs would provide a margin of safety for troops operating in contaminated areas as well as be useful from a clinical standpoint.

24. (U) Animal toxicity, animal cardiovascular and autonomic pharmacology and human tolerance (Phase I) studies are carried out.

25. (U) 73 07 - 74 06 Progress was previously reported under 3A062110A824 as work unit 055. Technical management continued for 2 contracts in pharmacology. One new IND application and 4 supplements were written. A Phase I human tolerance test has been completed on WR 2823. WR 2721 exhibited the properties of a ganglionic blocking agent in cat preparations. WR 2721 per se had little effect on blood pressure in either dogs or cats. WR 149,024 reduced endotoxin-mediated mortality in mice. Work is being curtailed due to termination of the Army's Radiation program. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 73 - 30 June 74.

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Project 3A762758A824 RADIATION INJURY AND PROTECTION

Task 01 Radiation Injury and Protection

Work Unit 059 Determination of pharmacological effects of anti-irradiation drugs

Investigators.
Principal: Melvin H. Heiffer, Ph.D.
Associate: Dr. A. Einheber

1. Introduction.

The main thrust of the anti-irradiation drug development program has been the study of compounds of substituted thioethylamines. The pharmacological areas emphasized here are those involving the cardiovascular and autonomic effects as well as protection against injury that may trigger an adverse effect upon those systems.

2. Acute cardiovascular and autonomic effects of WR 2721.

a. Background:

S-2-(3-aminopropylamino) ethyl phosphorothioic acid (WR 2721) is a chemical which has had considerable success as a protective agent in animals exposed to ionizing radiation. Our study was initiated to observe and characterize the acute cardiovascular and pulmonary effects of WR 2721 in dogs and cats and compare these effects with those of other members of the phosphorothioate series.

b. Experimental procedure:

WR 2721 AL and its congeners were made up in saline to a concentration of 100 mg/ml. The drugs were injected intravenously over a 2 min period in discrete doses of 25 to 200 mg/kg. Intra-arterial injections were made as a bolus in volumes no larger than 0.5 ml.

Adult beagle and mongrel dogs weighing between 9.5 and 13 kg were anesthetized with sodium pentobarbital, 30 mg/kg. Polyethylene catheters were inserted through both a femoral artery and vein into the abdominal aorta and vena cava respectively. This allowed for the measurement of aortic blood pressure and intravenous administration of drugs. Precordial EKG signals were taken with heart rate determined from the R wave to R wave interval. Respiratory rate and depth were measured by a pneumotachometer inserted on the end of an oral endotracheal tube.

In 4 intact dogs, WR 2721 was given in cumulative doses of 50, 100, 200, 400 and 600 mg/kg.

In 3 bilaterally vagotomized dogs the responses to norepinephrine, acetylcholine, angiotensin, histamine and isoproterenol were observed before and 30 min after the I.V. injection of WR 2721, 100 mg/kg, to determine whether or not WR 2721 potentiates or inhibits the action of these test substances. The sequence and doses of agonists were randomized. Also observed before and after WR 2721 administration was the response to clamping of the carotid arteries proximal to the carotid sinus (60 seconds) to determine the integrity of the carotid sinus baroreceptor pressor reflex. In 2 of these dogs, the blood pressure responses to the ganglionic stimulant, 1,1 dimethyl-4-phenylpiperazinium iodide (DMPP), in doses of 30 and 60 µg/kg, were observed before and after I.V. administration of WR 2721, 100 mg/kg. Additionally in these 2 dogs, the effect of WR 2721 was determined on the bradycardia produced by either electrical stimulation of the right vagus nerve or I.V. injection of acetylcholine.

Other dogs prepared as described above were also given the other thiophosphates listed in Table 1 to determine the effects of these substances on arterial blood pressure, heart rate and carotid artery occlusion reflex (3 dogs for each compound).

In 4 dogs, blood flow in the left femoral was measured continuously by means of an electromagnetic flowmeter. The hind limb was acutely denervated by the division of the femoral and sciatic nerves to eliminate possible reflex effects of flow. Circulation to the paw was occluded. The remaining perfused tissue was largely skeletal muscle, devoid of most of the arteriovenous shunts of the limbs. WR 2721 and WR 2823 were administered into the artery in doses ranging from 0.5 to 16 mg and flushed at a constant rate with saline (0.7 ml/min).

In 3 anesthetized cats the pre- and postganglionic fibers of the superior cervical ganglion were isolated. After attaching the ipsilateral nictitating membrane to a force displacement transducer and stretching to 3 gm basal tension, voltage response curves were obtained (single stimulus of 0.5, 1.0, 1.5, 2.0, or 3 volts at 60 Hz, 2 msec duration). Stimulation of both the pre- or postganglionic fibers was done with an insulated bipolar electrical probe.

c. Results:

WR 2721 given as a bolus injection in a dose of 100 mg/kg had only a small depressive affect on the blood pressure and heart rate of anesthetized dogs (Table 1). In fact, cumulative doses of up to

600 mg/kg delivered over a 2 hr period had little effect on these parameters. A slight gradual rise in blood pressure (30 to 40 mmHg) was noticed, however, over a 4 hr period following any cumulative dose over 100 mg/kg. At 50 mg/kg all dogs exhibited a slight mydriasis which increased with increasing doses. After this dose, carotid artery occlusion no longer elicited a pressor response. A watery type salivation was also noted in all doses above 100 mg/kg. WR 2822, WR 2823, and WR 2824 all produced more marked decreases in blood pressure and heart rate at a lower dose (Table 1). Salivation and mydriasis were not observed in any animal given these compounds.

Dose-blood pressure and heart rate response curves for norepinephrine, acetylcholine, angiotensin, histamine and isoproterenol were constructed before and 60 min after the introduction of WR 2721 (100 mg/kg) and were not different.

Figure 1 gives the typical blood pressure response in the dog to bilateral carotid artery occlusion, DMPP and norepinephrine before and 30 min after WR 2721, 100 mg/kg. The pressor responses to DMPP are vastly decreased following WR 2721 administration. The reduction of DMPP responses became even more profound after larger doses of WR 2721. Norepinephrine responses, however, were not affected. It is noteworthy that the basal arterial blood pressure is higher one hr following WR 2721 administration. Also observed in these dogs was that WR 2721 blocked the bradycardia previously observed with electrical stimulation of the right vagus nerve. However, the bradycardia produced by acetylcholine was not affected.

Figure 2 gives the responses of the cat nictitating membrane to pre- and postganglionic nerve stimulation. In the normal anesthetized cat both pre- and post- superior cervical ganglionic stimulation elicited contraction of the nictitating membrane. But following the administration of WR 2721, 100 mg/kg, there was a complete absence of contractions with preganglionic nerve stimulation. This persisted for over 4 hr. Postganglionic nerve stimulation, however, was capable at all times of producing contraction of the cat nictitating membrane. Dilated pupils and intense salivation were observed for up to 4 hr following WR 2721, but no change in blood pressure was seen. Similar results were seen in two other cats.

WR 2721 injected in doses of 1 to 16 mg into the femoral artery produced only meager increases in femoral bed blood flow (Figure 3). WR 2823 on the other hand produced marked increases in doses of 8 and 16 mg. All observed increases in flow were transient, returning to control values within 4 min. Arterial blood pressure (the perfusion pressure of the femoral bed) remained constant with all intraarterial injections, indicating femoral bed vasodilation with increased flow.

d. Discussion:

WR 2721 appears to produce only small acute effects on blood pressure, heart rate and respiration in doses up to 600 mg/kg. This is surprising since a congener of this compound, WR 2823 (Table 1), has very profound effects on these parameters at much lower doses as do the other thiophosphates tested. The inability of WR 2721 to reduce the vasopressor responses to norepinephrine and epinephrine is also striking since WR 2823 is a proven alpha adrenergic blocking agent. It was observed that WR 2823 had a much greater ability than WR 2721 to cause vasodilation in the femoral vascular bed.

The ability of WR 2721 to reduce the carotid artery occlusion pressor response was surprising. Our experiments using DMPP did demonstrate that WR 2721 was capable of blocking the effects of an extrinsic ganglionic stimulant. Furthermore, the ability of WR 2721 to differentially block the effects of preganglionic nerve stimulation and not postganglionic stimulation gives us confirming evidence that WR 2721 is a ganglionic blocking agent.

There are, however, several observations which are not consistent with classical ganglionic blockade. The profuse production of saliva and the lack of a blood pressure decrease in these animals following WR 2721 administration is surprising. Indeed, the gradual increase in blood pressure seen in dogs given WR 2721 is especially intriguing. This pressure increase was not blocked by prior treatment of the dog with the alpha adrenergic blocking agent, phentolamine, assuring us that this phenomenon was not due to norepinephrine-induced vasoconstriction. Determination of the other possible mechanisms which may be involved in the vasopression have not been made.

With the data now accumulated, it is obvious that the acute cardiovascular effects of WR 2721 are scant. However, the possibility that some variety of ganglionic blockade may be produced in individuals given this drug should be considered.

3. The protective effects of WR 149,024 in mice.

a. Background:

The active form of WR 2823 is believed to be the dephosphorylated disulfide dimer, WR 149,024. Since WR 2823 has been shown to prevent mortality and some of the cardiovascular effects of endotoxin in dogs, it was of interest to test WR 149,024 on endotoxic effect in mice and therefore this study was conducted.

b. Experimental procedure:

Female CDI mice, 7 to 8 weeks old, were used as experimental animals and maintained under usual laboratory conditions during the experiment. Endotoxin (Difco, E. coli) was suspended in saline and administered i.p. in a dosage of 600 µg per mouse. WR 149,024 was dissolved in water and given i.p. at a dose of 25 mg/kg of body weight.

c. Results:

Table 2 shows that 25 mg/kg of the drug given either 15 min before or 15 min after 600 µg of E. coli endotoxin effectively decreased the mortality due to the endotoxin.

Table 3 demonstrates that the drug is capable of preventing mortality from endotoxin when administered as long as one hr before the endotoxin.

4. Development of new anti-irradiation 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.

b. Investigational New Drug submissions:

One new IND application was written, for WR 149,024. Four supplements to IND submissions were written. They were supplements 4 and 5 for WR 2721 and supplements 2 and 3 for WR 2823.

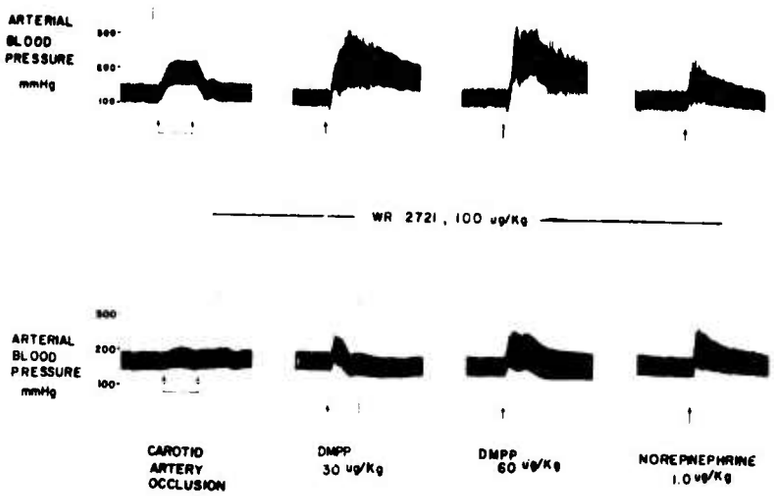

Figure 1. Arterial blood pressure response to bilateral carotid occlusion, I.V. injection of DMPP, 30 and 60 µg/kg, and norepinephrine, 1 µg/kg, before (upper panel) and 60 minutes after I.V. administration of WR 2721, 100 mg/kg (lower panel).

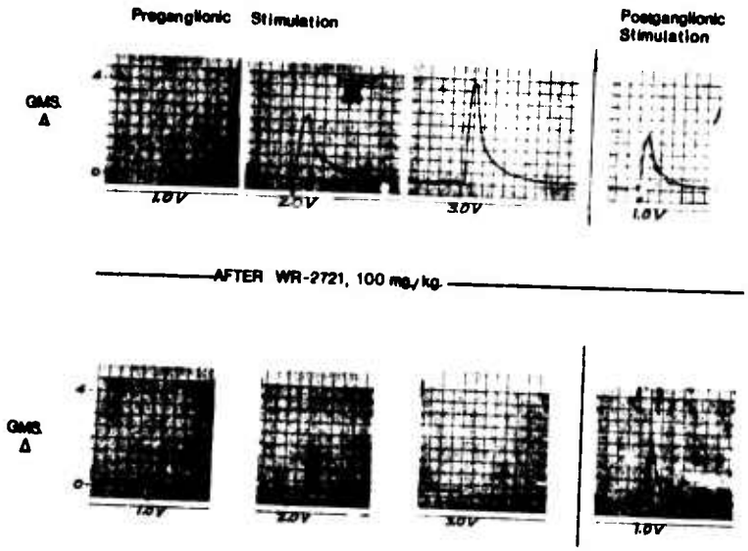

Figure 2. Change in tension of the cat nictitating membrane with electrical stimulation of pre- and postganglionic fibers of the superior cervical ganglia. Tracings were obtained before (upper panel) and 60 minutes after WR 2721, 100 mg/kg (lower panel). Contraction is produced by single stimulus of 1, 2 or 3 volts at 60 Hz and 2 msec duration.

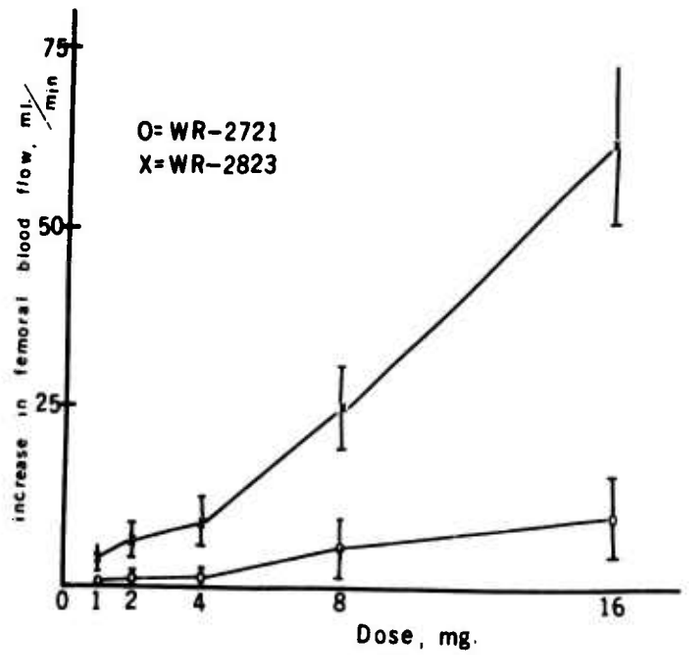

Figure 3. Increase in femoral blood flow in 3 dogs with femoral artery injection of WR 2721 or WR 2823. Brackets indicate S.E.M.

Table 1

Structure and Responses to Various Thiophosphates

Compound # Structure	I.V. Dose mg/kg	Decrease in Blood Pressure[1] (mmHg)[a]	Decrease in Heart Rate[1] (bpm)[a]	Duration of Initial Effects	Depression of Carotid Artery Occlusion Reflex?[b]
WR 2721 $H_2N-(CH_2)_3NH(CH_2)_2SPO_3H_2$	100 (3)[c]	5 to 15	15 to 30	5 min	Yes
WR 2822 $H_2N-(CH_2)_4NH(CH_2)_2SPO_3H_2$	50 (3)	25 to 65	60 to 85	30 min	No
WR 2823 $H_2N-(CH_2)_5NH(CH_2)_2SPO_3H_2$	50 (3)	55 to 90	55 to 75	15 min	No
WR 2824 $H_2N-(CH_2)_6NH(CH_2)_2SPO_3H_2$	50 (3)	30 to 75	40 to 65	Incomplete Recovery	No

[a]Range of initial peak change in these parameters following rapid I.V. administrations.
[b]Response observed 30 minutes after administration of given compound.
[c]Number in parentheses indicates number of dogs observed.

Table 2

Comparison of the Effects of WR 149,024 Prophylaxis and Therapy on the Lethal Effects of E. coli Endotoxin in Mice[a]

Group	Treatment				% Cumulative Mortality Hours After Endotoxin							
	First Rx		Second Rx									
	Agent i.p.	Time (min)	Agent i.p.	Time (min)	3	6	24	30	48	54	72	96
A	Water 1% BW	-15	Endotoxin[b]	"0"	0	0	40	63	80	80	83	83
B	Drug 25 mg/kg	-15	Endotoxin[b]	"0"	0	0	3	10	23	23	27	30
C	Endotoxin[b]	"0"	Water 1% BW	+15	0	0	43	60	87	87	93	93
D	Endotoxin[b]	"0"	Drug 25 mg/kg	+15	0	0	27	43	53	60	60	60
group means compared:					P value							
B vs A					-	-	<0.005		<0.001		<0.001	<0.001
D vs C					-	-	n.s.		<0.025		<0.01	<0.01

[a]Thirty mice per group.

[b]Endotoxin in sterile pyrogen-free 0.9% saline, 2 mg/ml; each mouse received 0.3 ml equal to 600 µg.

Table 3

Duration of Protective Effect of WR 149,024 in Mice Against Lethality of *E. coli* Endotoxin[e]

Group	Pretreatment 1st Rx i.p.	2nd Rx	Interval Between Injections (min)	% Cumulative Mortality Hours After Endotoxin 6	24	30	48	54	72	96
A, C, & E	Water, 1% BW	Endotoxin[d]	15, 30, or 60	0	41	64	94	94	96	97
B	Drug 25 mL/kg	Endotoxin[d]	15	0	7[b]	17[c]	30[c]	33[c]	43[c]	43[c]
D	Drug 25 mg/kg	Endotoxin[d]	30		17[a]	27[c]	47[c]	47[c]	47[c]	47[c]
F	Drug 25 mg/kg	Endotoxin[d]	60		17[a]	30[c]	50[c]	50[c]	53[c]	53[c]

[a,b,c]Difference from water-control group (A, C, & E) is statistically significant: [a]$p=<0.05$; [b]$p=<0.005$; and [c]$p=<0.001$.

[d]Endotoxin in sterile pyrogen-free saline, 2 mg/ml; each mouse received 0.3 ml equal to 600 µg.

[e]Thirty mice per group.

Project 3A762758A824 RADIATION INJURY AND PROTECTION

Task 01 Radiation Injury and Protection

Work Unit 059 Determination of pharmacological effects of anti-irradiation drugs

Literature Cited.

Publications:

1. Caldwell, R. W. and Heiffer, M. H.: Acute cardiovascular and autonomic effects of S-2-(3-amino-propylamino)ethyl phosphorothioic acid. Rad. Res. 55:548, 1973.

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	74 07 01	DD-DR&E(AR)636

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

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6275	3A762752A829	00	308
b. CONTRIBUTING				
c. [illegible]	CARDS			

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
66 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 74	2.95	212
	CURRENT 75	2.95	250

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

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) 73 07 - 74 06 Testing of compounds is accomplished by employing approximately 20 test systems at eight different laboratories. Testing includes screening for both prophylactic and suppressive antimalarial activity. The primary suppressive screen utilizes a mouse - P. berghei model. During the period 1 Jul 73 thru 31 May 74 11,035 compounds have been tested. Of these 345 were found to have antimalarial activity. Questions of drug resistance, activity by different routes of compound administration and other various secondary evaluations received regular attention employing several rodent systems, two in vitro methods and two primate models. The primary prophylactic test system included the use of chicks and P. gallinaceum. During the reporting period 2645 compounds were tested; 219 were found to be active. Secondary testing of compounds for prophylactic activity was conducted using rodent and simian models. For technical report see Walter Reed Army Institute of Research Annual Progress, 1 Jul 73 - 30 Jun. 74.

* Available to contractors upon originator's approval

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 308 Biological evaluation of anti-malarial drugs

Investigators.
Principal: LTC Kenneth E. Kinnamon, VC
Associate: CPT Lyle L. Ketterling, MSC

Introduction

Testing of compounds is accomplished by employing approximately 20 test systems at eight different laboratories. Testing includes screening for both prophylactic and suppressive antimalarial activity.

Progress

a. Primary Screens

A primary screen is designed to show if a compound has activity against malaria parasites. One may choose to examine few compounds in such a way that there is little chance of missing even minimal activity or one may wish to look at many compounds at the expense of a lesser degree of sensitivity. In the Army Malaria Research Program the latter approach was adopted for the following reasons: (1) it is recognized that no compound is known that is equally effective against all developmental stages of a particular species of Plasmodium or that is equally active against the same stage of different species, thus, no screen employing a single Plasmodium species in a single host is capable of detecting all active compounds, irrespective of the technique and (2) new classes of compounds worthy of more detailed investigation should be sufficiently active to be detected by the less sensitive "high volume" testing. Therefore, the high volume screen should provide the greatest yield when evaluated in terms of discovering clinically safe and effective compounds in the least possible time at a reasonable cost.

1. Suppressive Testing

The primary test employed was developed by Dr. Leo Rane (University of Miami). The endpoint of the system is extension of survival time of ICR/HA Swiss mice that are given a standard inoculum of Plasmodium berghei (KBG 173) on day zero and a single subcutaneous injection of the test agent in peanut oil on day 3, i.e. 72 hours later. Untreated animals die within 6 to 8 days with a mean survival time of 6.2 days. An increase of 100% in mean survival time is considered the minimum effective response for a candidate compound. Treated animals are kept under observation for 60 days. Survivors at the end of this period are considered to be cured. Compounds are

routinely tested at three drug levels using five animals per group. Both positive and negative controls are run simultaneously. Compounds found to be active are confirmed in subsequent tests and other testing done to obtain data for active compounds at a minimum of seven drug levels. During this report period 11,949 compounds were tested. Of these, 462 were found to be active.

2. Prophylactic Testing

White Leghorn cockerels weighing 50-57 grams are parasitized by intrajugular injection of 0.5 ml of a suspension of Plasmodium gallinaceum sporozoites. The sporozoite preparation is a filtered suspension of ground Aedes aegypti mosquitoes infected 9-11 days earlier by feeding on infected donor chicks. Test drugs are suspended in peanut oil and administered subcutaneously on the day of infection in a single dose. Every drug is routinely tested in 5 chicks at each of three dose levels. As in the mouse test in treated groups of chicks, a drug is considered active if the mean survival time is at least twice as long as in the corresponding control group. During the reporting period 2672 compounds were tested; 232 were found to be active.

b. Secondary Rodent Testing

1. Suppressive

Compounds sufficiently active for further testing were evaluated in one or more secondary tests employing mice as the experimental animal. Approximately two percent of the compounds evaluated in the primary screen are tested in these secondary murine systems. Evaluation of any given compound included efficacy relative to quinine, activity against various drug resistant strains of murine malaria, duration of active, activity via various routes of administration, paraaminobenzoic acid (PABA) antagonism, synergism and/or antagonism in combination with one or more other compounds and approximation of the therapeutic index.

2. Prophylactic

Secondary testing is conducted at two laboratories. As test animal one employs young rats and mice (Most Laboratory), the other uses mice only (Peters Laboratory). The test used by Dr. Most is designed to demonstrate direct evidence of drug action on exoerythrocytic (EE) stages of Plasmodium berghei in young rats. CFN female rats 3-4 weeks of age when given 125,000 to 250,000 sporozoites intravenously develop EE forms in the liver within 40-45 hours. These can be readily demonstrated and quantitated after histological preparation. Compounds with causal prophylactic activity can eliminate or reduce the EE forms. The throughput of this test has been essentially doubled during the reporting period by introducing a "preliminary parasitemia screening" procedure.

The test used by Professor Peters is designed to differentiate between prophylactic activity and residual suppressive activity of test compounds in mouse malaria infections. The test is based on the inverse linear relationship between the logarithm of the sporozoite inoculum and the mean time taken for the resulting erythrocytic infection rate in groups of mice to reach 2 percent. Differences in the pre-2% patency period between control and treated sporozoite-inoculated animals can reflect a drug action on EE stages, erythrocytic forms or both. Cross-inoculation in parallel series of groups with infected red cells allows the residual drug action on erythrocytic forms to be assessed, leaving a value proportional to the action on the EE stages alone.

c. In vitro Testing

Approximately 100 compounds evaluated in the secondary murine systems were sent to be tested for their antifolate properties and/or their ability to inhibit drug resistant strains of P. falciparum in an in vitro system.

1. Antifolic Acid Assay

The antifolic acid test is designed to assay compounds for interference with the metabolism of folic acid. Three bacterial species which require the addition of a form of folic acid for growth are used as test organisms. These are Streptococcus faecium var. durans, ATCC 8043, which can use the simpler pteroic acid as well as folic and folinic acids; Lactobacillus casei, ATCC 7469, which cannot use pteroic acid but can use folic acid, 5-methyltetrahydrofolic acid and folinic acid, and Pediococcus cerevisiae, ATCC 8081, which requires the reduced form of folinic acid. By the use of these three organisms valuable information can be obtained not only in terms of general activity against this metabolic pathway but also of the site of such action.

2. Erythrocytic Schizont Inhibition

The in vitro test employed requires incubation of the candidate compound with human blood parasitized with P. falciparum. When the parasitized blood is incubated trophozoites progress to mature schizonts after 24 hours. The addition of drugs with antimalarial activity inhibits the maturation of trophozoites to normal schizonts. This procedure can be used to evaluate the blood schizonticidal activity of many antimalarial agents with both sensitive and drug-resistant P. falciparum. Five strains of parasites are used. The Ethiopian (Tamenie) and Uganda I are the chloroquine sensitive strains utilized. The Malayan (Camp), Vietnam (Marks) and Cambodian (Buchanan) are the resistant strains employed.

d. Subhuman Primate Testing

1. Suppressive

(a) Simian Malaria; Simian Host

Eighteen compounds were tested for suppressive activity in rhesus monkeys during this reporting period. Compound tolerance and/or efficacy studies employing Plasmodium cynomolgi strain B were done. In the determination of the drug tolerance, primary emphasis is placed on the close clinical observation of each test animal during the time increasing doses of the test drug are given. In addition, the clinical data may be supplemented by the use of hematologic and pathologic findings that will serve as additional indicators of toxic effects. Therapeutic effectiveness of the candidate drugs is determined by testing the capability of various dosages of each compound to reduce or eliminate parasitemia in monkeys that have been infected with the simian parasites. All monkeys used in the test for therapeutic effectiveness are subject to continuous clinical observation as well as hematologic and pathologic examinations. For efficacy each test compound initially is evaluated at its maximum tolerated dose or at a dose that is otherwise specified on the basis of previous experimental findings. The evaluation of a compound is concluded when the drug is given either at the maximum tolerated dose or one of the lower dose levels that fails to show evidence of therapeutic effectiveness. The test for therapeutic effectiveness consists of administering the test compound once daily for seven consecutive days beginning four days after the intravenous inoculation of each animal with 5×10^8 P. cynomolgi parasitized erythrocytes. The course of the parasitemia in each animal is determined daily for 14 days. Thereafter blood parasite determinations are made every other day to day 30. Monkeys free of parasitemia, ascertained by blood smears, by day 30 are then splenectomized and the blood monitored for parasites every other day to day 60. Monkeys surviving to this point are killed and necropsied.

(b) Human Malaria; Simian Host

This test system was designed to assess the in vivo response of an infection with human malaria organisms without using human subjects. This was made possible by establishing the "human infection" in the owl monkey, Aotus trivirgatus. Nine strains of P. falciparum and two strains of P. vivax have been successfully established by Dr. Leon Schmidt. All infections appear to respond to drugs in much the same manner as the corresponding infection in man, with the possible exception of species variation in the bioavailability of the drugs. To test compounds, 2 to 3 animals per group and two controls are treated for seven consecutive days beginning on the day the parasitized erythrocytes reach 0.2 to 1%. Five hundred thousand parasitized cells are administered intravenously to establish the infection. Blood films are made daily continuing for three days after the end of treatment in animals that become parasite free, then twice weekly for two weeks and then once weekly up to 90 days after

the last treatment. When the parasitemia cannot be detected by microscopic determination the monkeys are scored as "clear." Animals at the end of 90 days are considered cured. During this reporting period work on several combinations of antimalarials as well as on individual compounds was done.

2. Prophylactic

This test was designed to determine not only the causal prophylactic and radical curative properties but also the blood schizonticidal activity of test compounds. Rhesus monkeys with simian malaria are used. Animals are given an i.v. injection of 100,000 to 500,000 sporozoites prepared from a homogenate of A. freeborni mosquito salivary glands infected with the Bastianelli strain of P. cynomolgi. The test compound is administered for nine consecutive days beginning on the day before sporozoite inoculation. Blood examinations are made on the last day of drug administration and are repeated until parasitemia is found. If patency is not observed within sixty days after challenge the compound used will be scored as having prophylactic activity. Untreated control monkeys are consistently patent on the eight day after inoculation. Compounds may be further tested for radical curative and/or blood schizonticidal activity. During this fiscal year approximately 100 selected compounds were tested. The compounds with the best efficacy-toxicity ratios were found to be the Lepidines. In this class WR 181,023 [8-(5-amino-2-pentyl)amino-6-methoxylepidine] was found to be about twice as active as primaquine and about one half as toxic thus affording a fourfold advantage over primaquine.

Summary

Biological evaluation of potential anti-malarial drugs continued during the reporting period. In the Primary Suppressive Screen 11,949 compounds were tested, 462 were active. In the Primary Prophylactic Screen 2672 compounds were tested, 232 were active. Secondary testing to evaluate leads found in the primary screens continued in several in vivo animal models and two in vitro systems.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6536	74 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	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
NIS	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. [redacted]	CARDS 114F			

11. TITLE (Procede 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 74	4.5	292
FISCAL YEAR CURRENT 75	4.5	300

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 (Procede 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. Procede 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) 73 07-74 06 Progress was previously reported under 3A663713D879 as work unit 134. Technical management continued for 13 contracts in pharmacology. Five new IND applications and 14 supplements were written. Pharmacokinetic investigations using radioactively labelled drugs in healthy mice were carried out for WR 142,490, WR 171,669 and WR 184,806. Pharmacodynamic evaluations in anesthetized dogs were compared for WR 2975 and WR 181,023. The mouse oral efficacy and toxicity and the effects of microsomal modifiers on these were studied for WR 2975, WR 5990, WR 159,412, WR 172,435, WR 181,023, WR 182,232 and WR 182,284. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*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

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1. Description.

The efforts of the department can be divided into two broad areas of pharmacological study. First is the effect of the body or system on the drug -- absorption, distribution, biotransformation and excretion. Second, and more familiar, is the effect of the drug on the body or system. Of necessity a pronounced overlap exists between the two areas.

2. Absorption, distribution and excretion in mice.

The absorption, distribution and excretion of three new antimalarials were studied in mice after oral administration using radioactive compounds.

a. WR 142,490-^{14}C:

Studies were continued on this very important new antimalarial. Two areas were investigated.

Blood levels of radioactivity in mice after oral dosing indicated that peak levels were not reached until approximately 24 hr, with a slow disappearance thereafter. However, this did not correspond to the excretory pattern of metabolites versus parent drug. Therefore, the following experiment was carried out.

Thirty-six female ICR mice from the Walter Reed colony, all weighing 25 $\pm$ 1 gms, were dosed orally, 8.4 mg/kg, with a suspension of drug. Blood from groups of 6 mice each were pooled at 4, 8, 24, 48, 72 and 96 hr, and plasma and red cells separated. The drug was extracted from both plasma and red cells at pH 7.4 with ethyl acetate. The ethyl acetate extract was washed at pH 11 and the drug and remaining metabolites separated by thin layer chromatography. Figures 1 and 2 show the difference in actual WR 142,490 values from those assumed from gross radioactivity alone. In plasma the drug level peaked at or before 4 hr, while the metabolite levels peaked around 24 hr. In

red cells the drug level peaked at 4 to 8 hr, while the metabolite levels peaked at around 24 hr. The T 1/2's in plasma and red cells respectively were 12 hr and 26 hr for drug, and 33 hr and 52 hr for metabolites.

Table 1 lists the ratio of concentration in the RBC's to the concentration in the plasma. It can be seen that the ratios stabilized by about 8 hr. The drug concentrates strongly in the RBC fraction while the metabolites tend to concentrate in the plasma.

The second question investigated was that of the ratio of parent drug to metabolites in those mouse organs that strongly concentrated radioactivity after oral administration of WR 142,490-^{14}C. Accordingly, 8 mice were dosed orally as above, except at 10 mg/kg instead of 8.4 mg/kg. One group of 4 was killed at 24 hr and the remainder at 48 hr. The corresponding organs or tissues from the 4 mice for a given time period were pooled and homogenized in methanol. The methanol extracts were chromatographed using thin layer chromatography.

The results are presented in Table 2. The parent drug represented the majority of radioactivity present in salivary gland, lung, liver, spleen, kidney, stomach (including contents) and small intestine (including contents). However, the percentages of radioactivity representing metabolites predominated in heart, gall bladder (including bile) cecum and large intestine. The material in the remaining carcasses was primarily parent drug.

b. WR 171,669-^{14}C:

To determine the nature of the drug-related radioactivity, mice were dosed orally, 20 mg/kg, and killed 6 hr later. The gastrointestinal tracts (including lumen contents), the remaining carcasses and the excreta were homogenized separately in methanol. The extracts were chromatographed using thin layer chromatography. Analysis of the data indicated that virtually all of the radioactivity present in the carcasses was the parent drug. In contrast, about 75% of the radiolabel present in the gastrointestinal tract and 65% of that present in the feces was the parent drug. This indicates relatively slow metabolism coupled with rapid excretion of the metabolites back into the gut.

c. WR 184,806-^{14}C:

WR 184,806·H_3PO_4-^{14}C was administered to mice orally by gavage at 10 mg/kg. The excretory pattern was studied by analysis of urine, feces and expired air for total radioactivity. This data is presented in Table 3.

The data in Table 3 indicate that the major route of excretion of WR 184,806·H_3PO_4-^{14}C and its metabolites was via the feces. Expired air failed to show an appreciable amount of radioactivity (< 0.1%) and is not considered an important route of excretion for this compound.

The urine samples from above were treated with a saturated solution of NH_4Cl (pH 9.5), passed through Amberlite XAD-2 resin columns and washed with water. The columns were eluted with methanol. The resulting methanol eluates were concentrated and chromatographed on silica gel plates, yielding the information summarized in Table 4.

The data indicate that WR 184,806·H_3PO_4-^{14}C was readily absorbed from the gastrointestinal tract after oral administration. It also shows that the parent drug was metabolized as seen in Table 4. The proportionate amount of total radioactivity excreted in the urine tentatively identified as parent (Compound IV) decreases steadily while Compound V, a presumed metabolite, increases rapidly.

An additional study was undertaken to determine the levels of total radioactivity in the plasma and red blood cells of mice after 10 mg/kg orally of WR 184,806·H_3PO_4-^{14}C. The data from this experiment are summarized in Table 5.

Peak plasma and red blood cell levels occured at 2 hr post dose and again at 8 hr post dose. The estimated primary half-life of the first rise was 15.7 hr in plasma with an elimination rate constant of 0.044 hr^{-1}. The secondary rise gave an estimated secondary half-life of 9.8 hr with an elimination rate constant of 0.071 hr^{-1}. These data indicated an initial rise of parent drug followed by a distribution into the blood of metabolites giving the secondary peak. This view is supported by the rapidly changing RBC/plasma ratio.

The foregoing data indicate that WR 184,806·H_3PO_4-^{14}C is well absorbed from the gastrointestinal tract and is metabolized readily Work is in progress to amplify on these findings.

3. Cardiovascular and pulmonary pharmacology in dogs.

a. Background:

Although primaquine is used in the radical cure of relapsing forms of malaria, its usefulness is limited by a fairly high incidence of side effects such as gastrointestinal distress, hemolysis and methemoglobinemia. The 4-methyl analog of primaquine, WR 181,023, is an 8-aminoquinoline which is being developed as a more potent and less toxic drug than primaquine itself to cure P. vivax malaria. In an effort to develop improved therapeutic agents with which to treat and prevent malaria, comparative studies were carried out on the

pharmacology of WR 181,023 and of primaquine. In this series of investigations, the acute cardiovascular and pulmonary responses of the dog to intravenous administration of primaquine (WR 2975) and 4-methyl primaquine (WR 181,023) were determined and compared.

b. Methods:

Fifteen adult beagle dogs weighing between 10.0 and 15.5 kg were anesthetized with sodium pentobarbital, 30 mg/kg of body weight. Both femoral veins were cannulated with polyethylene catheters, one for administering drugs and the other for maintenance doses of the anesthetic. For the latter purpose a Harvard infusion pump was employed 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. Temperature was continuously monitored rectally with the thermistor probe of a tele-thermometer 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 first phase of the study involved 5 dogs, 3 of which received WR 2975 intravenously in doses ranging from 2.5 to 10 mg/kg, the other 2 dogs receiving WR 181,023 in doses ranging from 2.5 to 20 mg/kg of body weight. Both drugs were administered as the phosphate salt but the dosage was calculated as the free base. The drugs were dissolved in 0.9% sodium chloride solution and administered over a 5 min period. Dose response curves to epinephrine, norepinephrine and isoproterenol were obtained before and after each intravenous administration.

The second series of experiments involved 6 dogs. Three received WR 2975 at doses ranging between 2.5 and 12.5 mg/kg of body weight and the other 3 received WR 181,023 in doses of 2.5 to 20 mg/kg of body weight. In each experiment the drugs were given in a series of increasing doses at one hr intervals so that there was a cumulative effect. After each dose of the drug, responses were monitored and recorded continuously until they returned to control levels or until death ensued.

To test the integrity of the carotid sinus pressor reflex, the common carotid arteries were clamped bilaterally in 3 dogs from the first series of experiments and in 3 dogs from the second series of experiments. This test was conducted just prior to and 2 min after the administration of the drug.

c. Results and discussion:

The effects of drug treatment on arterial blood pressure, heart rate and respiratory rate are shown in Table 6. The peak effect on blood pressure occurred within a 5 to 10 min period. Both drugs caused a slight initial rise in arterial pressure at the low doses of 2.5 and 5.0 mg/kg. This was followed by marked hypotension at the higher, toxic dosage levels of 10 mg/kg for primaquine and 20 mg/kg for 4-methyl primaquine. At the highest dosage level, 4 of 7 primaquine-treated animals survived and 4 of 5 of the 4-methyl primaquine-treated animals survived. The blood pressure of the survivors returned to near control values after 30 to 45 min. The diastolic pressure responses paralleled the systolic changes and thus there was no significant change in the pulse pressure. All of the blood pressure alterations appeared to be secondary to disturbances of the normal electrical innervation of the heart and the accompanying cardiac arrythmias.

The first indication of an effect on the heart rate occurred at 7.5 mg/kg for primaquine and 10.0 mg/kg for 4-methyl primaquine. At the highest doses, a marked bradycardia developed, with slowing to about 50% of control levels. The presence of arrhythmias at the high doses made accurate determinations of the heart rate difficult.

The effect of WR 2975 or WR 181,023 on the duration of the P-R interval and the Q-T interval is shown in Table 7. Each drug produced a dose-related increase in the duration of both of these parameters. The lengthened P-R interval reflects an increased conduction time from the auricles to the ventricles and may partially explain the pattern of heart block with subsequent fibrillation observed at the toxic dosage levels. The drug-related effect on the Q-T interval appears to indicate an increase in the refractory period of the ventricular myocardium. This in turn may contribute to the pattern of heart block and fibrillation observed after drug treatment.

The effects of the drug on the carotid sinus baroreceptor response is shown in Figure 3. Both drugs produced a dose-related depression of the pressor response with complete abolition at a dose of 10 mg/kg of primaquine and 20 mg/kg of 4-methyl primaquine. These results are in good agreement with similar studies by others (Moe et al, 1949) and are consistent with the hypothesis that these 8-aminoquinolines impair central vasomotor regulatory mechanisms.

Neither WR 2975 nor WR 181,023 produced a significant change in the arterial blood pressure responses to test doses of the catecholamines, with the exception of isoproterenol. At doses of 7.5 mg/kg of WR 2975 or 20 mg/kg of WR 181,023, the typical cardiovascular response to 0.5 µg/kg test doses of isoproterenol was significantly altered. As shown in Figure 4 the expected decrease in arterial

pressure was abolished at the highest doses tested. Furthermore, the tachycardia normally produced by isoproterenol did not occur after administration of these drugs. Other investigators (Bass et al, 1972) have presented evidence that primaquine depresses the cardiac response to isoproterenol and suggested that it blocks beta-adrenergic receptors. Our results confirm these findings and are consistent with beta-adrenergic blockade. However, before any conclusions can be reached concerning the mechanism of action, further testing will be necessary to show specificity of these responses to primaquine and related 8-aminoquinoline compounds.

In summary, our results are generally in good agreement with the work of others reported in the literature. From this study we have data which show that WR 181,023 (4-methyl primaquine) produces changes in the cardiovascular system which are qualitatively similar to those produced by WR 2975 (primaquine). However, since the dose required to produce quantitatively similar responses is 2 fold greater, it can be concluded that 4-methyl primaquine is only one-half as toxic as primaquine on the cardiovascular system of the anesthetized dog.

4. Oral efficacy and toxicity of candidate antimalarials and the effects of modification of host drug-metabolizing capacity on these parameters.

a. Background:

Comparison of antimalarial efficacy was carried out in mice by determining either the relative curative or parasitemia-suppressing potency of the various agents against the drug-sensitive KBG 173 strain of P. berghei. Mice were inoculated with 5×10^5 parasitized RBCs on Day 0. On Day 3, they received, by gavage, the antimalarial suspended or dissolved in MCT (0.5% Tween 80 and 0.2% methyl cellulose in 0.9% saline); control mice received MCT only. Parasitemia suppression was assessed by means of Giemsa-stained thin blood films taken at various intervals after treatment, as described last year. All control mice died. Those treated mice alive on Day 28 were exsanguinated and each donor's blood was inoculated i.p. into a previously normal mouse. Recipients were monitored by weekly smears for 2½ days, and if malaria did not develop the donors were considered to have been cured.

Methods to determine the effect of host drug-metabolizing enzyme induction (phenobarbital pretreatment) and inhibition (SKF 525-A pretreatment) on antimalarial efficacy in P. berghei-infected mice were described last year. The effect of these pretreatment regimens on the toxicity of the antimalarials was assessed in uninfected mice. Mice received, by gavage, a single oral dose of the drug and were observed for 7 days. During this time, gross

appearance, overt toxic manifestations and cumulative mortality were noted daily. At the end of 7 days a final mortality count was taken and the surviving mice were killed and autopsied. Mice dying during the 7-day period were also necropsied when feasible.

b. Comparison of the oral curative potency of unmicronized vs micronized WR 172,435:

Equivalent single oral doses of micronized (lot AD) and unmicronized (lot AC) WR 172,435, a pyridine methanol, were tested for their comparative curative potency. The average particle size of the micronized lot was < 2μ and that of the unmicronized lot 15μ. The 5 mg/kg doses of lot AC or AD provided no cures. At the 10 mg/kg dose, both lots produces cures. The pooled results of two experiments indicated that the cure rate was significantly greater ($p < 0.05$) after the micronized (9/24) than after the unmicronized (?/24) WR 172,435.

c. Effect of phenobarbital or SKF 525-A pretreatment on the acute oral toxicity of WR 159,412 AD:

We reported last year that the induction of host drug-metabolizing enzymes by pretreatment with phenobarbital caused a significant decrease in the parasitemia-suppressing potency of WR 159,412, a thioquinazoline. Conversely, inhibition of host drug-metabolizing enzymes by pretreatment with SKF 525-A resulted in a significant enhancement of the antimalarial efficacy of this compound.

Further studies were then undertaken in uninfected mice to determine the effect of phenobarbital or SKF 525-A pretreatment on the toxicity of oral WR 159,412 given once at an approximate LD_{50} (1,500 mg base/kg). The results are shown in Table 8. Mice receiving WR 159,412 after pretreatment with vehicle only may exhibit overt toxic signs within several hours, characterized by muscle weakness with listing to one side and with resp[illegible] followed by episodes of prostration and convulsive-type [illegible], and ultimately death. Pretreatment of mice with phenobarbital not only reduced or prevented the acute toxic manifestations but also promoted survival. However, pretreatment with SKF 525-A significantly increased overall toxicity of WR 159,412. The same qualitative effect was demonstrated when mice were pretreated with piperonyl butoxide (50 mg/kg i.p., 1 hr before challenge), which inhibits the same microsomal oxidative enzymatic system as SKF 525-A but allegedly at a different step. These results, together with those obtained in the efficacy study, suggest that the parent chemical, not a metabolite, is responsible both for acute toxicity and for antimalarial efficacy.

d. Comparison of the oral parasitemia suppressing potency of primaquine (WR 2975) and related 8-aminoquinolines:

Primaquine and 4 other 8-aminoquinolines were compared by means of their parasitemia-suppressing potency when given in single oral doses (equivalent to 11.4 mg base/kg). The increasing order of parasitemia-suppressing potency was as follows: WR 182,232 AB < WR 2975 AG ≦ WR 5990 AC ≦ WR 182,234 AB < WR 181,023 AC.

On the basis of these findings more definitive studies were undertaken to compare further the efficacy and toxicity of WR 181,023 (4-methyl primaquine) with that of primaquine. The role of host drug-metabolizing enzymes in affecting these parameters was assessed through the use of pretreatment with phenobarbital or SKF 525-A.

e. Comparison of the oral efficacy and toxicity of WR 181,023 and primaquine (WR 2975) and the effect of phenobarbital or SKF 525-A pretreatment on these parameters:

Table 9 shows the results of two experiments comparing the parasitemia-suppressing potency of WR 181,023 AC and WR 2975 AG at two different subcurative dose levels. While both agents, Groups B and C, suppressed parasitemia (in comparison with Group A) it is apparent that WR 181,023 was more efficacious at both doses tested. Subsequent experiments also revealed that, whereas cures could be obtained with non-toxic doses of WR 181,023, doses of primaquine in the lethal range were required to effect cures.

Table 10 shows the effect of enzyme induction (phenobarbital pretreatment) and enzyme inhibition (SKF 525-A pretreatment) on the parasitemia-suppressing potency of WR 181,023 and primaquine. Neither of these pretreatment regimens in itself significantly affects the course of the P. berghei infection. The antimalarial dose, which differed for each agent, was chosen to be most suitable to allow us to view any effect of the phenobarbital or SKF 525-A pretreatment on the antimalarial action of the test compound.

It can be seen from Table 10 that enhancement of host drug metabolism by phenobarbital pretreatment decreased the efficacy of primaquine while suppression of host drug metabolism by SKF 525-A increased its efficacy.

The results with WR 181,023 revealed that neither pretreatment regimen altered the antimalarial efficacy of WR 181,023. This finding suggests that WR 181,023 is not metabolized by those hepatic drug-metabolizing enzymes affected by phenobarbital and SKF 525-A.

Toxicity studies revealed that WR 181,023 was about half as toxic as primaquine, based on 7 day LD_{50} determinations. The acute LD_{50} of WR 181,023 given in mg of base/kg was 412 (366-451) while that of primaquine was 194 (153-239). Consistent with the results of the efficacy study, pretreatment regimens of phenobarbital and SKF 525-A had no demonstrable effect on the LD_{50} of WR 181,023 (Table 11). However, although SKF 525-A pretreatment was without effect on the LD_{50} of primaquine, phenobarbital pretreatment resulted in complete protection against its lethal effect. These results, together with those of the efficacy study, point out that the mouse handles WR 181,023 differently from primaquine. The change in toxicity of primaquine after phenobarbital pretreatment and the change in efficacy observed after both phenobarbital and SKF 525-A pretreatments are taken as presumptive evidence of the participation of host drug-metabolizing enzymes. The lack of effect of these pretreatments on either the efficacy or toxicity of WR 181,023 suggests that this drug is probably not handled by the microsomal drug-metabolizing system in the mouse.

As stated earlier, cures against P. berghei with primaquine were attainable only with doses in the lethal range. However, the ability of phenobarbital pretreatment to completely protect against a routine LD_{50} of primaquine afforded us an opportunity to attempt to achieve cures with a formerly LD_{50} of primaquine. Thus we were able to compare the curative rate of primaquine with that of WR 181,023 given at a dose equivalent in terms of base. It was also of interest to determine how much antimalarial activity would remain after an LD_{50} of primaquine in mice pretreated with phenobarbital since phenobarbital, in addition to reducing the toxicity of primaquine, also reduced its efficacy when primaquine was given in low, subcurative doses.

A comparison of the malaria-curative activity of an acute LD_{50} of primaquine after phenobarbital pretreatment and of an equivalent dose of WR 181,023 is given in Table 12. Protection by phenobarbital from the toxic effects of an LD_{50} of primaquine resulted in a significant number of cures of the blood-induced infection. However, the cure rate was no better than that seen with the same dose of WR 181,023 given alone.

Studies in the mouse with WR 181,023 have demonstrated that: the compound does not appear to be metabolized by hepatic drug-metabolizing enzymes affected by phenobarbital or SKF 525-A; it is half as toxic as primaquine; and it is more efficacious as a blood schizontocidal agent than primaquine and, in fact, can effect cures against P. berghei in non-toxic doses.

If the blood schizontocidal action of this agent, as seen in the rodent, also applies to primate malaria (P. cynomolgi or P. vivax), then its use as a radical curative agent (tissue schizontocide) may have the added advantage that the dosage or duration of treatment with any blood schizontocide (e.g., chloroquine) used in combination with WR 181,023 may be minimized.

5. 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:

Five new IND applications were written. They were 2 single drugs and 3 combinations. The individual drugs were WR 159,412 and WR 203,659. The combinations were WR 448/WR 2978, WR 2978/WR 4873 and WR 158,122/WR 7557.

Fourteen supplements to IND submissions were written. They were for 7 single drugs and 3 combinations.

c. Technical monitoring of contracts necessary for data generation:

Thirteen 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.

Among the quinazoline groups, an antibacterial assay for WR 158,122 plasma levels was developed for both humans and non-human primates; levels as low as 10-20 ng/ml can be measured. Absorption of formulated drug seemed poor, so a dosage form increasing bioavailability by utilizing an oil carrier is being developed.

Among the phenanthrenemethanols, the absorption, distribution and excretion of WR 171,669 has been extensively examined in both rat and rhesus monkey. Development of assay for blood level measurement of WR 33,063 and WR 122,455 have proceeded apace.

Among the quinolinemethanols, a high pressure liquid chromatographic method has been developed for assaying blood levels of WR 30,090. This drug is incompletely absorbed and a form with greatly enhanced bioavailability has been produced. A well absorbed form of WR 184,806 has been developed, which is undergoing extensive toxicological evaluation. A soluble form of WR 142,490 has been derived and is also being intensively studied toxicologically prior to IND submission. Chronic toxicological testing has also been carried out on the less soluble form of WR 142,490 which is currently being evaluated clinically.

Table 1

Distribution of Drug and Metabolites in Mouse Blood
after 8.4 mg/kg of WR 142,490-^{14}C Orally

Hours Postdose	WR 142,490			Metabolites		
	RBC (µgm/gm)	Plasma (µgm/ml)	RBC/Plasma	RBC (µgm/gm)	Plasma (µgm/ml)	RBC/Plasma
4	0.981	0.287	3.4	0.708	0.677	1.04
8	0.998	0.190	5.2	0.718	0.979	0.73
24	0.699	0.120	5.8	0.894	1.429	0.62
48	0.208	0.038	5.5	0.574	0.915	0.62
72	0.104	0.017	6.1	0.361	0.550	0.66
96	trace	trace		0.317	0.475	0.67

Table 2

Distribution of Drug and Metabolites in Selected Mouse Tissues after 10 mg/kg of WR 142,490-^{14}C Orally

Organ or Pool	Time of Sacrifice (hours postdose)	Proportion of Parent Drug Present (%)
Heart	24	47
	48	28
Lung	24	68
	48	72
Salivary Gland	24	73
	48	66
Spleen	24	59
	48	84
Kidney	24	71
	48	56
Liver	24	72
	48	62
Gall Bladder (Including Bile)	24	19
	48	12
Stomach[a]	24	65
	48	63
Small Intestine[a]	24	71
	48	54
Cecum[a]	24	29
	48	25
Large Intestine[a]	24	38
	48	30
Carcass	24	79
	48	64
Feces[b]	0-24 hr sample	47
	24-48 hr sample	30

[a]Organs include lumen contents.
[b]From 48 hr sacrifice mice only.

Table 3

Recovery of Radioactivity from Mice after Oral Administration of WR 184,806·H_3PO_4-^{14}C[a]

Hours Postdose	Urine (%)		Feces (%)		Expired Air (%)		Carcass[b] (%)
	Sample	Total[c]	Sample	Total[c]	Sample	Total[c]	
0-8	4.8	4.8	2.4	2.4	0.003	0.003	
8-24	5.4	10.2	10.7	13.1	0.008	0.011	
24-48	9.9	20.1	18.1	31.2	0.014	0.025	
48-72	4.7	24.8	10.6	41.8	0.017	0.042	
72-96	1.6	26.4	4.5	46.3	0.017	0.059	
TOTAL		26.4		46.3		0.059	4.0

[a]Samples were counted in a Packard Tri-Carb Model 3320 Liquid Scintillation Spectrometer.

[b]All carcass samples were determined 96 hrs post dose when the animals were sacrificed by cervical dislocation.

[c]Cumulative total.

Table 4

The Radioactive Urinary Components from Mice after Oral Administration of WR 184,806·H_3PO_4-^{14}C[a,b]

Compound	Rf	% ^{14}C in Sample			
		8 hr	24 hr	48 hr	72 hr
WR 184,806·H_3PO_4	0.79	-	-	-	-
I	0.54	9.14	3.80	3.85	3.19
II	0.61	0.00	3.40	3.64	2.98
III	0.73	11.83	11.40	19.84	3.83
IV	0.79	25.00	15.60	13.36	7.23
V	0.86	9.41	31.20	37.25	55.32
VI	0.95	44.62	34.60	22.06	27.45

[a]All chromatograms were developed 10 cm past the origin on Brinkman Silica Gel F_{254} (0.25 mm thickness) 20 x 20 cm glass plates using a n-BuOH:HOAc:H_2O (66:17:17 v/v/v)solvent system.

[b]Radioactive areas on the plates were measured by scanning with a Varian 6000 Radioscanner. The peaks were integrated using a Varian LB242K integral measuring system.

Table 5

Mean Levels of Total Radioactivity in Plasma and Red Blood Cells of Mice Dosed Orally with 10 mg/kg (Calculated as Weight of WR 184,806·H_3PO_4-^{14}C).[a,b]

Hours Post Dose	Hematocrit %	RBC Level (µg/ml)	Plasma Level (µg/ml)	RBC/Plasma
1	49	1.52	0.92	1.67
2	48	2.33	1.84	1.26
4	45	1.91	1.68	1.13
6	45	2.52	1.72	1.47
8	43	3.38	2.55	1.32
10	47	2.36	2.08	1.13
12	47	1.77	2.00	0.89
24	45	1.21	1.36	0.89

[a]All values are the average of four animals per group.

[b]All samples were counted in a Packard Tri-Carb Model 3320 Liquid Scintillation Spectrometer.

Table 6

Effects of Intravenous Administration of WR 2975 or WR 181,023 on Blood Pressure, Heart Rate and Respiratory Rate in the Dog[a]

		Dose (mg base/kg body weight)					
		Control	2.5	5.0	7.5	10.0	20.0
Arterial Blood Pressure (mmHg)	b	175±13 / 115±5	173±13 / 117±8	181±10 / 120±5	165±30 / 107±14	110±0 / 55±7	e / e
Systolic / Diastolic	c	185±23 / 120±13	188±21 / 125±9	200±0 / 133±3	212±18 / 143±15	180±7 / 117±4	42±37 / 23±21
Heart Rate (bpm)	b	168±24	166±23	164±20	124±0[d]	84±0[d]	e
	c	175±32	176±27	170±17	170±7	162±14	arrythmia
Respiratory Rate (rpm)	b	3±3	9±3	12±6	16±9	11±2	e
	c	7±1	8±2	9±3	13±4	15±4	22±11

[a] Each value is taken at time of peak effect and is the mean ± S.E.M. for 3 experimental animals.

[b] WR 2975 (Primaquine).

[c] WR 181,023 (4-methyl Primaquine).

[d] one animal.

[e] No dose given.

Table 7

The Effect of WR 2975 or WR 181,023 on the Duration of the P-R Interval and on the Q-T Interval

		Dose (mg base/kg body weight)[a]				
		Control	2.5	5.0	7.5	10.0
Duration of P-R Interval (msec)	b	2.0±0	2.4±0.2	3.0±0.4	3.3±0.4	5.0±0.5
	c	1.9±0.1	2.0±0	2.1±0.1	2.5±0.4	2.7±0.3
Duration of Q-T Interval (msec)	b	4.8±0.4	5.2±0.8	5.4±0.2	6.6±0.6	6.7±0.7
	c	5.0±0.3	5.0±0.3	5.2±0.2	5.3±0.3	5.6±0.4

[a]Each animal received a series of increasing doses.

[b]WR 2975 (Primaquine).

[c]WR 181,023 (4-methyl Primaquine).

Table 8

Effect of Phenobarbital or SKF 525-A Pretreatment on an Approximate Oral LD_{50} (1300 mg base/kg) of WR 159,412 in Uninjected Mice

Pretreatment	Number of Mice Used	Cumulative Mortality (%)			
		Time After Treatment with WR 159,412			
		3 hrs	6 hrs	1 Day	7 Days
Control	30	17%	23%	23%	47%
Phenobarbital	28	0%	0%[b]	0%[b]	0%[b]
SKF 525-A	30	40%	77%[b]	93%[b]	93%[b]

[a]Pretreatment regimens consisted of a) a daily i.p. injection of phenobarbital sodium, 100 mg/kg in water given for 3 days, the last dose given 24 hrs prior to oral dosing; or b) a single i.p. injection of 50 mg/kg of SKF 525-A in 0.9% saline one hour before oral dosing.

[b]Significantly different from control using chi-square analysis.

Table 9

Comparison of the Oral Antimalarial Efficacy of Primaquine (WR 2975) and 4-Methyl Primaquine (WR 181,023) in *P. berghei*-infected (Day 0) Mice

Group	Agent	Dose, mg/kg as base	Median % Parasitemia, Day of Infection				
			Before oral Rx	After oral Rx			
			3	6	8	10	14
			Experiment 1				
A	Vehicle (Control)	(1% BW)	4.0 (12)	49.0 (11)	50.4 (3)	-- (0)	-- (0)
B	WR 2975	5.7	4.2 (12)	10.6 (12)	17.0 (12)	38.4 (12)	47.2 (7)
C	WR 181,023	5.7	4.8 (12)	2.4[a] (12)	16.4 (12)	25.2 (11)	43.0 (10)
			Experiment 2				
A	Vehicle (Control)	(1% BW)	2.4 (10)	51.2 (10)	44.8 (5)	-- (1)	-- (0)
B	WR 2975	11.4	3.2 (10)	0.6 (10)	9.4 (10)	22.2 (4)	36.6 (4)
C	WR 181,023	11.4	2.4 (10)	0.0 (10)	1.8[a] (10)	9.0 (10)	26.8 (8)

[a]Difference from WR 2975 (Group B) is statistically significant (p0.05 or less by Mann-Whitney Rank Test).

Table 10

Effect of Phenobarbital or SKF 525-A Pretreatment on the Oral Antimalarial Activity of Primaquine (WR 2975) and 4-Methyl Primaquine (WR 181,023) in *P. berghei*-infected (Day 0) Mice

		Median % Parasitemia				
		Day of Infection				
		Before oral Rx	After oral Rx			
Group	Pretreatment[a]	4	6	8	10	14
	I. WR 2975, 11.4 mg of base/kg on Day 3					
A	Vehicle (Control)	.8 (24)	1.6 (23)	8.8 (23)	23.0 (14)	36.8 (7)
B	Phenobarbital	2.6 (24)	12.6[b] (24)	12.8[b] (23)	30.0[b] (23)	54.0[b] (20)
C	SKF 525-A	2.0 (24)	1.2[b] (24)	2.0[b] (24)	21.4 (24)	30.4 (11)
	II. WR 181,023, 5.7 mg of base/kg on Day 3					
D	Vehicle (Control)	4.2 (20)	1.6 (20)	7.0 (20)	26.4 (17)	44.8 (12)
E	Phenobarbital	4.4 (20)	2.2 (20)	13.6 (20)	22.2 (15)	44.0 (11)
F	SKF 525-A	3.6 (20)	1.6 (20)	6.2 (20)	26.0 (7)	45.6 (7)

[a] Pretreatment regimens consisted of: a) a daily i.p. injection of phenobarbital sodium, 100 mg/kg in water (Groups B and E) for 3 days (Days 0, 1, 2) before oral dosing on Day 3; or b) a single i.p. injection of 50 mg/kg of SKF 525-A in 0.9% saline (Groups C and F) on Day 3, one hour before oral dosing.

[b] Difference from control (Group A) is statistically significant ($p \leq 0.05$ by Mann-Whitney Rank Test).

Table 11

Effect of Phenobarbital or SKF 525-A Pretreatment on the Lethality of an Otherwise Acute Oral LD_{50} of Primaquine (WR 2975) or of 4-Methyl Primaquine (WR 181,023) in Uninfected Mice

Agent	Usual LD_{50} mg/kg as base	7 Day Cumulative Mortality		
		Pretreatment[a] Regimen		
		H_2O	Phenobarbital	SKF 525-A
WR 2975	194	10/20	0/20	11/20
WR 181,023	412	30/60	41/60	30/60

[a]See Table 8 for description of pretreatment regimens.

Table 12

Comparison of the Malaria-Curative Activity of an Acute Oral LD_{50} of Primaquine (WR 2975) Following Pretreatment with Phenobarbital and of an Equivalent Dose of 4-Methyl Primaquine (WR 181,023)

Group	Pre-treatment[a]	Agent	Dose mg/kg as base	Cumulative Mortality (No. dead/No. used) Day of Infection Before Oral Rx	After Oral Rx 3	5	7	14	21	28	% Malaria Cures (No. cured/ No. used)
A	Vehicle	Vehicle	1% BW	0/24	0/24	19/24	24/24	24/24	24/24 (100%)		0 (0/24)
B′	Pheno-barbital	WR 2975	194	0/20	0/20	0/20	0/20	8/20	8/20 (40%)		60 (12/20)
C	None	WR 181,023	194	0/21	0/21	0/21	0/21	2/21	2/21 (10%)		76 (16/21)

[a]See Table 10 for description of pretreatment regimen with phenobarbital.

Distribution of Radioactivity in Mouse Plasma Following Oral Dosing (8.4 Mg/Kg) with WR 142,490-^{14}C

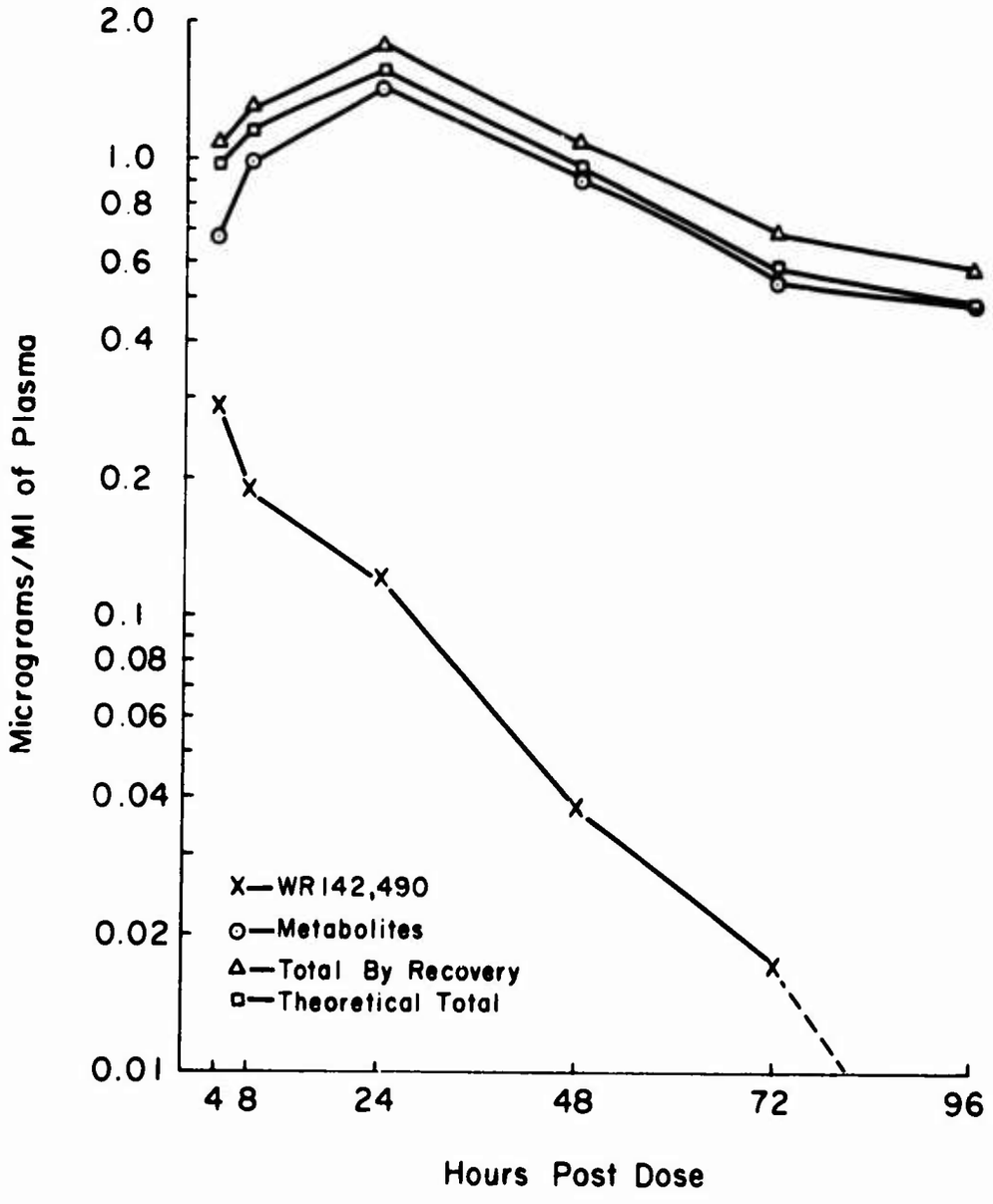

Figure 1

Distribution of Radioactivity in Mouse Red Blood Cells Following Oral Dosing (8.4 Mg/Kg) with WR 142,490-^{14}C

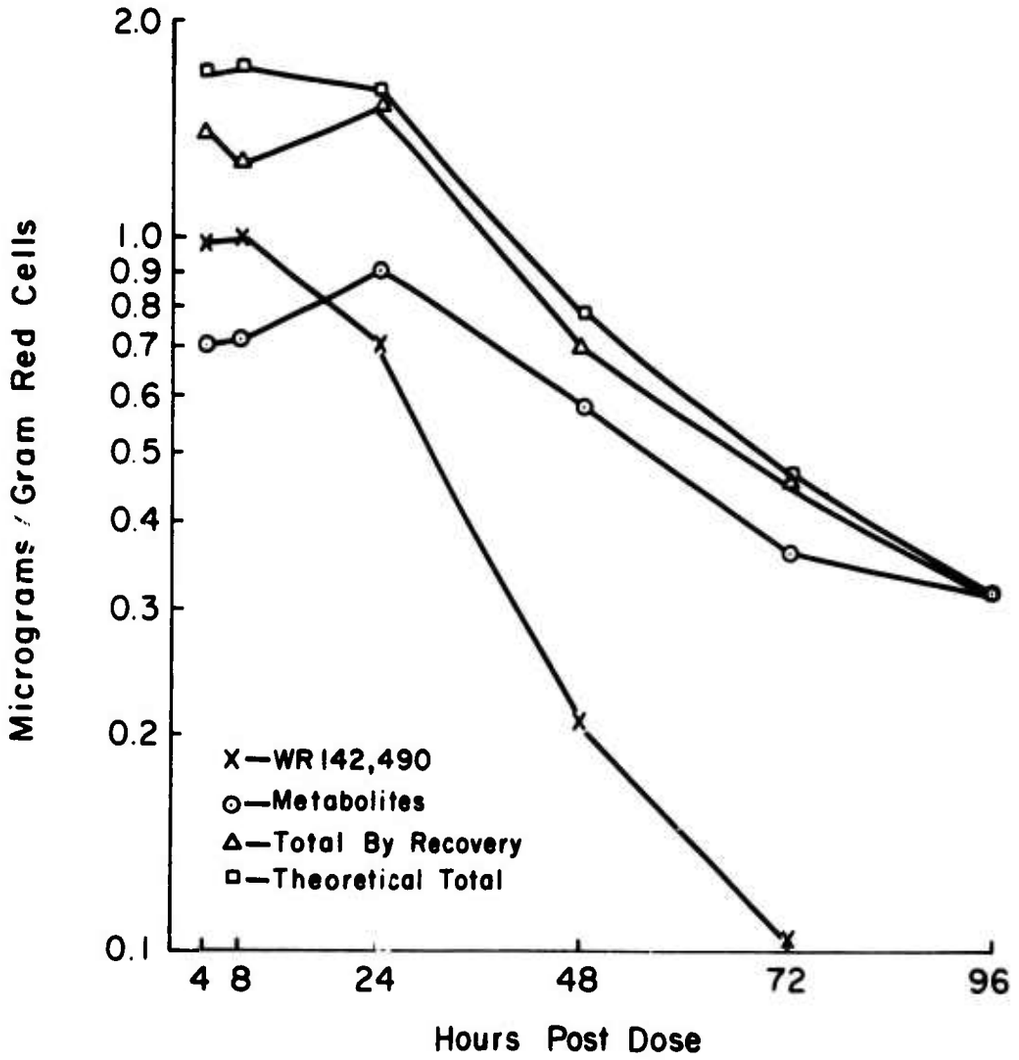

Figure 2

The Effect of WR-2975 or WR-181,023 On Blood Pressure Response to Bilateral Carotid Artery Occlusion (45 sec.)

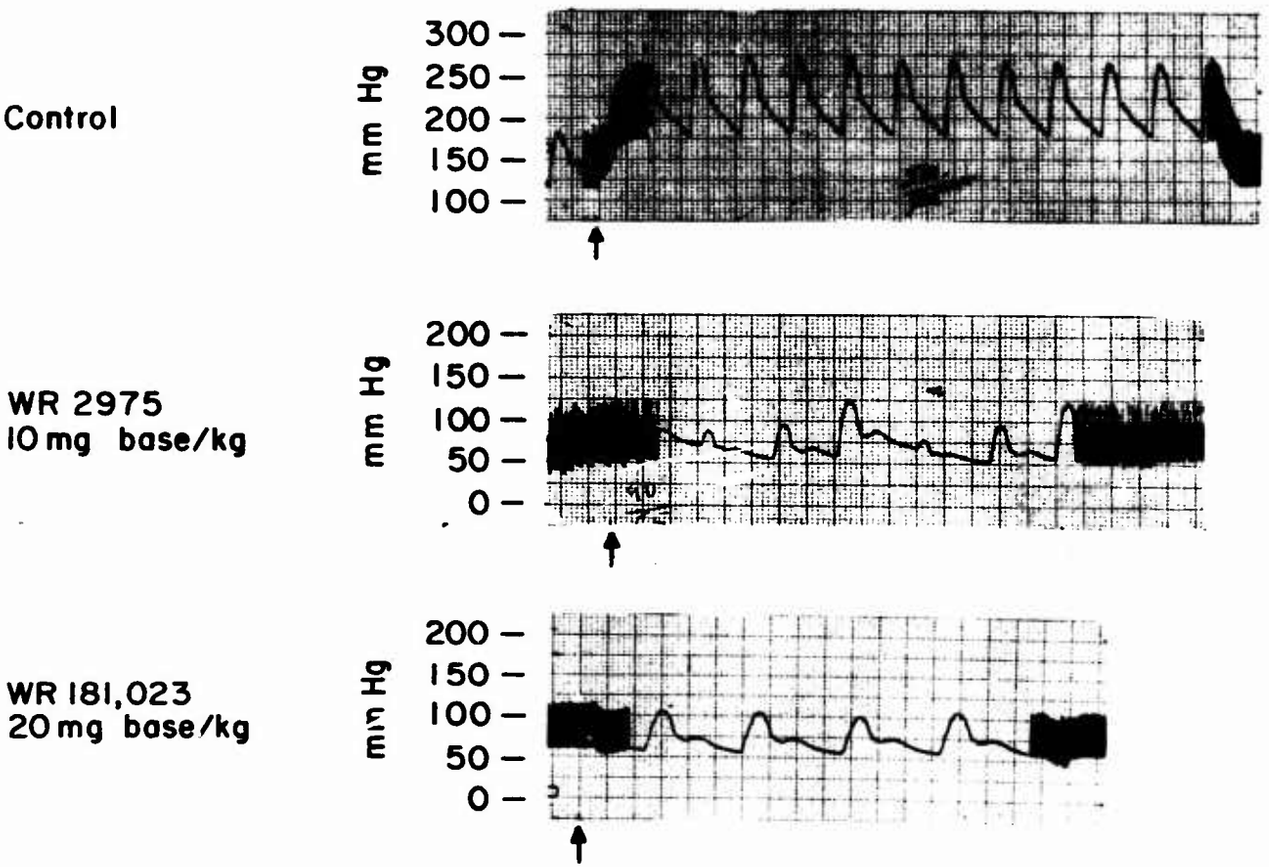

Figure 3

The Effect of WR-2975 or WR-181,023 on Blood Pressure Response to 0.5μg/kg Isoproterenol

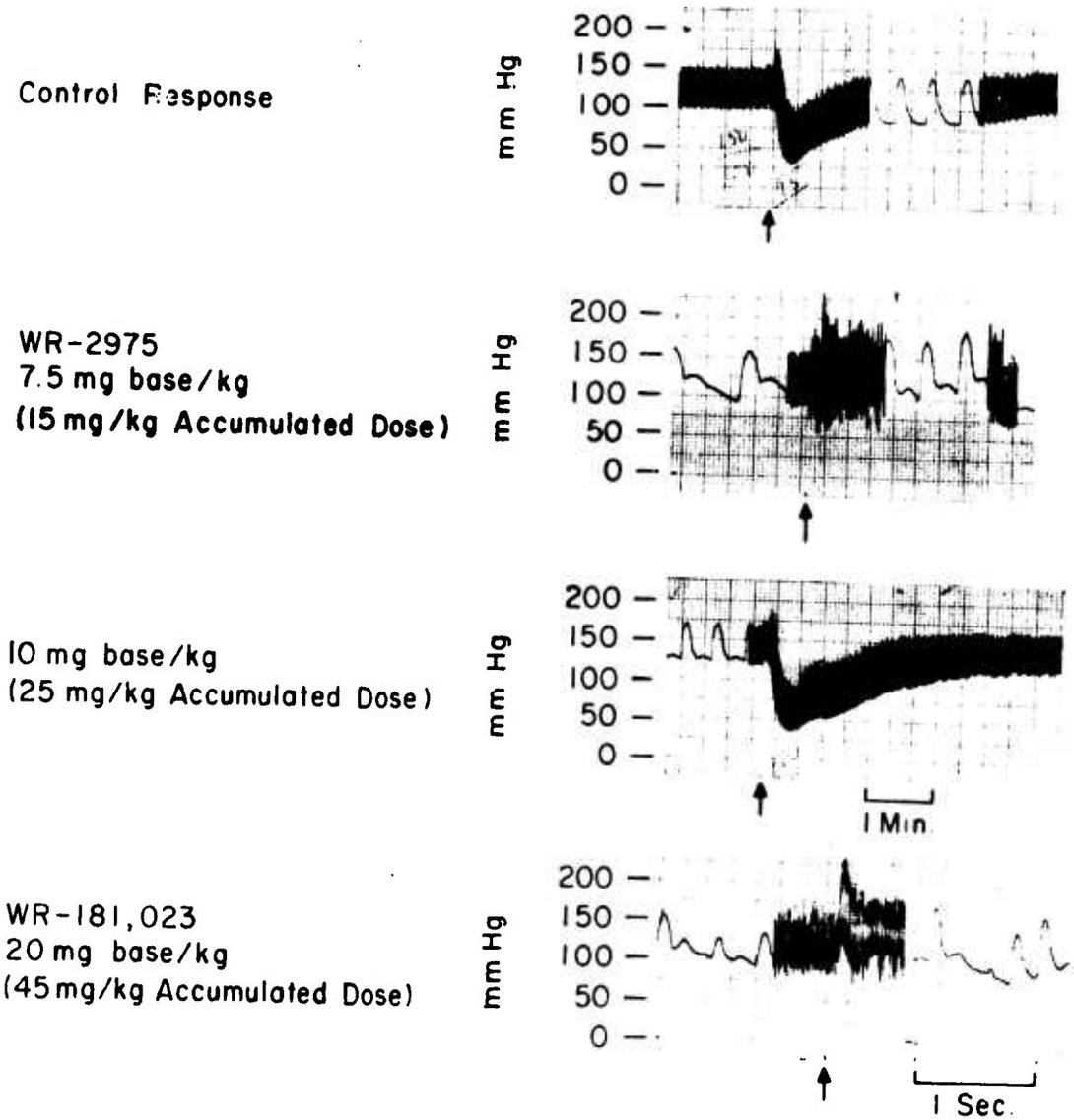

Figure 4

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 309 Determination of pharmacological effects of antimalarial drugs

Literature Cited

References:

1. Moe, G. K., Peralta, B. and Seevers, M. H.: Central impairment of sympathetic reflexes by 8-aminoquinolines. J. Pharmacol. Exp. Ther. 95:407-414, 1949.

2. Bass, S. W., Ramirez, M. A. and Aviado, D. M.: Cardiopulmonary effects of antimalarial drugs. VI. Adenosine, quinacrine and primaquine. Toxicol. App. Pharmacol. 21:464-481, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6506	74 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
73 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				CH FR 108
[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 74	20	463
FISCAL YEAR CURRENT 75	3	162

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 Analysis: (U) Plasmodium; (U) Antimalarial; (U) Gas Chromatography: (U) RIA

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 develop methods for the analysis of antimalarial compounds in blood from human users in Army-sponsored research.

24. (U) Investigation of instrumental techniques such as gas chromatography, high performance liquid chromatography, ESR and mass spectrometry and immunoassay techniques such as radioimmunoassay (RIA) and hemagglutination inhibition (HI) for use as reliable and effective methodologies.

25. (U) 73 07 - 74 06 A method using electron capture gas chromatography has been developed for analyzing WR 30090 in serum. A problem of column degradation with fresh specimens prevents unrestricted utilization at present. Investigations are underway to define and correct for this interference. Antibody formation to a variety of antimalarials in rabbits is being investigated for use in developing radioimmunoassays for detection and quantitation of antimalarial drugs in biological fluids. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*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 315 Blood level assays for anti-malarial drugs

Investigators.
Principal: LTC Douglas J. Beach, MSC
Associate: PFC Kenneth P. Arnold, B.S.; Billy G. Bass, M.S.; Ann R. Berman, B.S.; Betty J. Boone, Ph.D.; LTC Gale E. Demaree, MSC; Leo Kazyak, B.S.; Elvio A. Levri, M.S.; H. Kenneth Sleeman, Ph.D.

The objective of this work unit is to develop and establish methods and analytical techniques for the quantitation of anti-malarial compounds in the blood of human subjects. The development of these methods is expected to permit a definition of toxicity, mechanism(s) of action and pharmacokinetics of the anti-malarial drugs. Efforts have been focused on the following areas:

1. Radioimmunoassay for anti-malarial drugs.

2. Gas chromatographic analysis of WR 030090.

1. Radioimmunoassay for the anti-malarial drugs.

Radioimmunoassay (RIA) is a sensitive and, relatively specific method for the determination of submicrogram quantities of drugs in biological fluids. RIA potentially is able to detect a substance of analytical interest with a minimum amount of extraction and isolation-costly, time-consuming and error prone steps. RIA requires the production, purification and characterization of specific antibodies or binding proteins.

Two anti-malarial compounds have been tested for direct antigenicity. The drugs, di-n-butylaminoethyl)-2(3',4'-dichlorophenyl)-6,8-dichloro-4-quinoline methanol hydrochloride (WR 030090) and 6-bromo-alpha-(di-n-heptyl aminomethyl)-9-phenanthrene methanol hydrochloride (WR 033063), have been injected into New Zealand rabbits. Sera from these animals were examined for antibody production. Preliminary screening for antibodies to the drugs using ^{14}C-labelled compounds have been discouraging. Further studies are underway to determine if any specificity of binding exists. Rabbit serum albumin (RSA) coupled primaquine has been prepared for injection and will be investigated for antigenicity. Coupling of WR 030090 and WR 033063 to RSA is in the initial stages.

2. Gas chromatographic analysis of WR 030090.

Work continued in the refinement of a gas chromatographic method for analyzing WR 030090 in serum. This method involves a chloroform extraction and derivatization with BSA (N,-O-bis-(tri-methylsilyl)-acetamide) and chromatography on an OV-1 column in a gas chromatograph equipped with an electron capture detector. The GC response to the drug was linear for a concentration range of 25 to 200 nanograms on column.

Recovery of WR 030090 from aqueous solution was virtually complete. When the drug was added to pooled serum or plasma in a concentration of 0.5 µg/ml or less, the recovery dropped to about 40% of total. With higher concentrations of the drug, recovery improved; at a level of 2.0 µg/ml, 70% of the WR 030090 was recovered. Problems were experienced with what is believed to be a binding of WR 030090 to protein. Further study of this protein binding effect is planned.

A group of 28 patients infected with P. falciparum were treated with WR 030090. Blood levels of the drug in these individuals were determined. Specimens were drawn four hours after the third dose and four hours after the 18th dose. In 11 patients the concentrations increased from the third dose to the 18th dose; in 6 patients the concentrations decreased from the third dose to the 18th dose; in 8 patients no amount of the compound was detectable; in 3 patients specimens were available for only one sampling period but the drug was detected. The results indicate that the drug is detectable in serum but the data are inadequate for elucidating the pharmacokinetics of the drug in humans.

Investigations are in progress for assessing the practicality of using thin layer chromatography as a detection/separation technique followed by quantitative GLC analysis on the scraped and extracted TLC spots.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6514	74 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
73 07 01	D.CHG	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. xxxxxxxxx	CARDS 114 (f)			CH FR 124

11. TITLE (Procede 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 74	6	193
	CURRENT 75	6	193

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Schneider, Dr. I. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Anopheles; (U) Mosquitoes; (U) Plasmodium; (U) Susceptibility; (U) Falciparum malaria; (U) Aotus; (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) Development of physiological means of interrupting malaria transmission through an understanding of factors influencing susceptibility of anopheline vectors to malaria and of factors determining transmission efficiency of vectors. Test systems are developed for the evaluation of antimalarial drugs and the study of sporozoite immunity for the eventual prevention and control of malaria in military troops.

24. (U) Studies are conducted on the infectivity of human and closely related simian malarial parasites to various mosquito vectors of SE Asian areas. Attempts to transmit falciparum malaria to lower primates are made. Feedings of anophelines on gametocytemic hosts are conducted, followed by dissections of samples of the mosquitoes at intervals thereafter to determine level and progress of infections.

25. (U) 73 07 - 74 06. Anopheles stephensi mosquitoes were successfully grown axenically using a modification of a medium designed by Rosales-Ronquillo. Two attempts were made to immunize Rhesus monkeys against Plasmodium cynomolgi. In the first experiment, 9 million sporozoites were injected into 2 monkeys at 14 day intervals for 42 days. In the second experiment 30 million sporozoites were injected. Four times over an 83 day period. No protection against sporozoite challenge was provided either time. A new technique is being tested for the mass isolation of intact salivary glands from infected mosquitoes. New insect cell lines were developed from Anopheles stephensi, Glossina austeni, and G. morsitans. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*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 318 Biological studies of insect infection and disease transmission

Investigators

Principal: Ronald A. Ward, Ph.D.; Imogene Schneider, Ph.D.

Associate: LTC Bruce F. Eldridge, MSC; CPT Anthony Bosworth, MSC; SP4 David M. Spooner; SP4 John Brandt; SP4 Lawrence Macken; PVT Amy Goodmann; PVT Ralph W. Martin

Description

Studies are conducted on the relationship of human and non-human malarial parasites to their mosquito vectors. Included are a consideration of the role of genetic and environmental factors in mosquito susceptibility, the attempted cyclic transmission of malarial parasites in unusual hosts, the effect of mosquito pathogens on mosquito biology, the design of insect culture systems which will support the growth and development of the insect phase of malarial parasites, the use of density gradients to isolate sporozoites from mosquitoes for immunization purposes and the development of primary and established insect cell lines from selected dipteran species.

Progress

1. Experimental vivax and falciparum malaria in Aotus monkeys

Blood induced infections of human plasmodia can be established in certain primate hosts with appropriate treatment. It is more difficult to infect the natural mosquito vector and cyclically transmit the parasite to the unusual host due to changes in the infectiousness of the malarial gametocyte in the new host. These studies were undertaken in an attempt to analyze mosquito infectivity and the production of the complete sporogonous cycle under such modified conditions.

A New World monkey, Aotus trivirgatus, which in nature is completely refractory to human malaria, was subjected to a variety of experimental procedures which suppressed the normal immune response towards infections with various human plasmodia. Infection of Aotus with Plasmodium vivax and P. falciparum isolates was facilitated by splenectomy of the host prior to infection and often after treatment with an immunosuppressant agent, azathioprine (Imuran). Under these circumstances, malarial gametocytes comparable to those observed in the normal human host would be produced and mosquito infection achieved if some or all of the following conditions were fulfilled: (1) the parasite strain used to initially infect the monkey was one either recently isolated from an endemic area, with few intervening serial blood transfers in man, or if it had been extensively passaged in the laboratory in man, it had a good history of cyclical transmission; (2) extremely virulent strains of falciparum

could not be used; (3) the mosquito vector for infection with falciparum was an anopheline from the same geographical region as that of the parasite; and (4) to maintain mosquito infectivity, the parasite had to be transferred by mosquito passage rather than by consecutive blood transfer or by use of stabilate material, i.e., a quantity of early passage parasites preserved at low temperature for reference purposes.

2. Cultivation of malaria parasites and mosquito tissues

Within the past year, Rosales-Ronquillo (1) has reported the successful development of Plasmodium berghei ookinetes in vitro following the introduction of heavily parasitized red blood cells into both primary cultures and Schneider's cell line of Anopheles stephensi. The culturing period never exceeded 50 hours with most ookinetes being formed within 24 hours. Her methods differed from the earlier attempts of Chao and Ball and of Schneider in two respects: (1) the cultures were first "conditioned" 2-24 hours before the infected blood was added by removing the old medium and replacing it with serum-free medium and (2) the gametocytes comprised a minimum of 3.5% of the infected cells before being placed in culture.

Both conditions were met in four replicate runs employing both P. berghei and P. cynomolgi infected cells. Each replicate consisted of 3 primary, 3 established and 3 control cultures. After 26-48 hours, each set of cultures was fixed in methanol and stained with Giemsa. Although an estimated 10^5 gametocytes were routinely placed in each culture, ookinetes were very sparse (10-40/slide) in the cell cultures and not always detected in the control slides. However, those ookinetes formed in the established cell line were invariably much healthier in appearance than those from either the primary or control cultures.

Preliminary attempts were made to obtain greater numbers of ookinetes by modifying the method of Chen and Schneider (2,3) for isolating the sporozoite stage. Mosquitoes infected with P. cynomolgi were ground up and subjected to an enzymatic treatment in an attempt to dissociate the midguts and free the ookinetes. After the larger debris was removed by a series of filtration and centrifugation steps the remaining material was placed on a linear density gradient of bovine serum albumin (1.070 to 1.110 g/cm^3). Although some ookinetes were found, the final preparation was heavily contaminated with bacteria and yeast, and hence, unsuitable for incorporation into any cell culture system.

A. stephensi mosquitoes were successfully grown axenically using a medium designed by Rosales-Ronquillo (4) but modified to the extent of having autoclaved hay infusion broth substitute for the diluent. The interval between hatching of the larvae and the emergence of the adults averaged 12 days for the anophelines raised axenically compared to 9 days for the controls. Unfortunately, the mortality rate is usually quite high, often 60% or more, which effectively precludes raising the mosquitoes in large numbers, at least for the time being. The numbers are sufficient, however, for initiating primary cultures from the midgut cells of the axenic adults.

3. Attempts to immunize Rhesus monkeys against P. cynomolgi malaria

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 (5). To ascertain whether comparable protection could be obtained with a primate malaria, two attempts were made to immunize Rhesus monkeys (Macaca mulatta) against P. cynomolgi. Both control and infected A. stephensi mosquitoes were irradiated with 9 kilorads in a "Gammacell 220" cobalt source. The sporozoites, largely freed from mosquito debris, bacteria and the like, by density gradient centrifugation, were innoculated IV into the monkeys.

In the first experiment, two monkeys were given 8-9 x 10^6 attenuated sporozoites at 14 day intervals during a 42 day period. The control monkey received extracts made from uninfected, irradiated mosquitoes (Table 1). The challenge with infected mosquitoes took place 14 and 15 days after the last inoculation. The prepatent periods for both experimental and control mosquitoes were 10-11 days. The subsequent parasitemia for all monkeys appeared normal with no observed differences in maximum numbers of parasites or gametocyte counts (Table 3, Exp. 1).

In the second experiment, approximately 30 million sporozoites were used to inoculate one monkey (Table 2). Four inoculations were made in 83 days at intervals of 5, 30 and 48 days. The experimental and control monkeys were challenged with infected mosquitoes 13 days after the last inoculation. The experimental monkey's parasitemia was slightly lower than that of the control but the infection was well within the normal range.

The peak parasitemia and gametocytemia of both control and experimental monkeys was not markedly different in either experiment (Table 3). Oocyst counts from mosquitoes fed during the course of the infections differed as much between controls as between controls and experimentals.

Investigators using the P. berghei model have relied upon the circumsporozoite precipitation reaction (CSP) to demonstrate the presence of anti-sporozoite antibodies in the blood serum of immunized animals. In the present study, attempts to use the CSP reaction to document the course of an immune response were not successful, i.e., the presence of detectable CSP antibodies appears to have little, if any, relationship to protective immunity.

4. Mass isolation of intact salivary glands from infected mosquitoes

The mass isolation of sporozoites from infected mosquitoes by the density gradient centrifugation technique is efficient, i.e., an estimated 70% recovery rate, only if the great majority of the sporozoites are within the process of breaking out of the oocysts and thus within the hemocoel proper. Once having penetrated the salivary glands, the

sporozoites are technically much more difficult to extract and the recovery rate falls to perhaps 1/10th of that given above. This means, for example, that the optimum interval for recovery of P. berghei sporozoites from A. stephensi mosquitoes lies between 12 and 14 days post feeding, some 2 to 3 days before the majority of the sporozoites reach the glands. However, Vanderberg (6), has recently shown that immature, irradiaged P. berghei sporozoites, i.e., those still within the oocysts, failed to confer immunity on mice while older sporozoites, some of which may have already reached the glands, did so to a limited extent. Full immunity was elicited only when the sporozoites were fully mature and within the glands. Hence, it was deemed necessary to develop a technique which would effectively isolate and concentrate only such sporozoites.

Infected female mosquitoes, housed in pint-sized cardboard containers, were placed in a Revco freezer (-70^{o}) for 3 minutes, then removed and shaken quite roughly for an additional minute or so. Heads, legs, wings and, in some cases abdomens, detached from the thoraces and were easily removed by filtering through a 1 mm plastic screen. The thoraces (and some abdomens) were placed on a flat glass plate and crushed by rolling a steel bar over feeler guage steel of a specific thickness. A tolerance of 0.381 mm between bar and plate provided enough pressure to pop the paired salivary glands from the neck of the decapitated body. A second roll, using a tolerance of 0.254 mm, effectively freed approximately 85% of the glands from the thoraces. After extraction, the glands plus debris were washed with saline into an enamel pan and much of the debris removed by a series of filtration steps. The glands were then concentrated by centrifugation.

At present, the recovery rate (see Table 4) varies considerably depending upon such factors as numbers of mosquitoes used, the numbers and extensiveness of the washing and filtration steps and the "stickiness" of the glands. The latter characteristic is particularly troublesome as the glands, once detached, may not only adhere to mosquito debris but also to glass surfaces, such as the walls of glass pipets or of centrifuge tubes.

5. Establishment of insect cell lines from various dipteran species.

A cell line of Anopheles stephensi established in this laboratory in 1969 was lost due to fungal contamination. The line had been passaged more than 200 times and some 26 subcultures had been sent upon request to various investigators in this and other countries during the past five years. In view of the usefulness of that particular cell line a new line was initiated in December 1973 and has been successfully subcultured a total of four times in the past five months. Once fully established the cells will be used in this laboratory for malaria research as well as being made available to interested individuals elsewhere.

Primary cultures, varying from 5 to 8 months in age, were initiated from the early larval stages of Glossina austeni and G. morsitans. None,

as yet, are capable of sustained multiplication in subcultures. It is anticipated that if successfully established, the cells will be used to study the metabolism of the crithidial and metacyclic forms of African trypanosomes.

Conclusions and recommendations

1. Splenectomized Aotus trivirgatus monkeys, particularly if treated with immunosuppressant drugs, lose their refractoriness to both P. vivax and P. falciparum and undergo an infection similar in course to that in humans. Transmission to mosquito can also occur under certain conditions. Further studies should be undertaken to increase the transmission efficiency of the vector as well as to determine which factors preclude and which promote the establishment of an exoerythrocytic cycle in an abnormal host.

2. The isolation of ookinetes by enzymatic treatment of mosquito midguts followed by concentration on a density gradient appears quite promising. Ookinetes so isolated appear to be healthy and retain their motility. Elimination of the bacterial contaminants may be possible through the use of another gradient medium, such as colloidal silica, reputed to give finer separation than the bovine serum albumin currently used. Until this can be accomplished the preparations are not suitable for incorporation into in vitro systems.

3. A. stephensi mosquitoes have been successfully raised axenically and cell cultures are being initiated from midgut cells of the adults. Cells of such origin may prove more adequate for the development of the insect cycle of the malarial parasites than are cells of undefined embryonic origin which have been used almost exclusively in the past. Further studies on the nutritional and physical requirements of anophelines raised under axenic conditions are needed to reduce the mortality to a more acceptable level.

4. Two attempts to immunize monkeys against P. cynomolgi malaria were unsuccessful. Reliance on a single phenomen, the so-called circumsporozoite precipitation reaction, to monitor the build-up of immunity was inadvisable as the presence of CSP antibodies apparently has little relationship to protective immunity. A reliable method, consisting of one or more serological assays, for documenting the course of the immune response against the sporozoite stage is obviously needed. The use of such assays should initially be tested and evaluated with the P. berghei model for both practical and monetary reasons. If promising results are obtained the assays can subsequently be extended to a primate malaria model.

TABLE 1

Inoculation and challenge schedule for initial attempt to immunize monkeys against P. cynomolgi malaria

Inoculation	Day	Estimated No. of sporozoites ($x10^5$) Monkey 1	Monkey 2	Control*
1	0	2.8	2.8	0
2	14	26.1	29.8	0
3	28	65.1	46.3	0
4	42	1.4	1.8	0
	Totals	96.0	80.7	

Challenged on day 56/57 with 6 infected mosquitoes

* Control consisted of a similar number of noninfected mosquitoes, irradiated, subject to the same procedures as the infected mosquitoes and the material from the peak gradient fractions injected IV.

TABLE 2

Inoculation and challenge schedule for second attempt to immunize a monkey against P. cynomolgi malaria

Inoculation	Day	Estimated No. of sporozoites ($x10^6$) Monkey 1	Control*
1	0	11.7	0
2	5	16.0	0
3	30	1.7	0
4	48	0.2	0
	Total	29.6	

Challenged on day 61 with 2/3 infected mosquitoes.

*Control subjected to same treatment as in initial immunization attempt

TABLE 3

The course of infection in experimental and control monkeys and resulting insect infections after challenge with P. cynomolgi sporozoites

Group (Treatment	Animal	Prepatent period (days)	Day peak parasitemia	Max.# parasites (gametocytes)/ 10,000 RBC	Peak (average) oocyst counts/ midgut from resulting feeds
Exp. 1 Irradiated mosquitoes (None)	Control	11	18	868 (14)	76 (42)
Irradiated mosquitoes (with sporozoites)	Monkey #1	10	17	800 (23)	89 (56)
	Monkey #2	11	15	753 (17)	70 (34)
Exp. 2 Irradiated mosquitoes (none)	Control	10	18	772 (19)	90 (50)
Irradiated mosquitoes (with sporozoites)	Monkey #1	11	18	457 (10)	68 (28)

TABLE 4

Recovery of intact salivary glands from infected mosquitoes

Tolerence	No. of mosquitoes	No. of glands recovered	% Recovery	Hrs needed for extraction & collection
.254	87	83	48	2
.381	720	125	9	2
.203	110	30	14	1
.381/.254	290	291	50	4
.381/.254	56	69	62	2
.330	200	90	23	1
.254	290	116	20	2

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 318 Biological studies of insect infection and disease transmission

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6520	74 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
73 07 01	D.CHG	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. [redacted]	CARDS 114(f)			CH FR 129

11. TITLE (Precede 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	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	4	153
FISCAL YEAR CURRENT 75	4	153

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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. Comparison of various serological tests for reliability in routine malaria diagnosis.

25(U) 73 07 - 74 06 The studies in Aotus support the hypothesis that deep vascular schizogony occurs and that periodicity is similar to that in humans. Both cellular and humoral roles are involved in malaria immunity, and partial protection can be artificially transferred with cells or serum. Tolerance can be induced by use of malaria antigens as with other antigens that have been better defined. Refinement and standardization of serological tests have led to more reproducible results. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*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 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 324 Host responses to malaria

Investigators.
Principal: COL J.C. Burke, MSC
Associate: W.L. Bowie; W.H. Hildreth; CPT F.A. Hines, VC; MAJ L.K. Martin, MSC; CPT R.A. Wells, MSC

1. Protective immunity in rodent malaria.

Studies have been made on protective immunity to rodent malaria, to further describe the relative roles of cellular and humoral immunity, as evidenced by passive protection to malarial challenge following the transfer of cells or serum from immune donors.

A cooperative study by the Departments of Medical Zoology and Neurophysiology has attempted to determine whether immune response to malaria is controlled or influenced by one or more areas of the hypothalamus, (anterior, dorsomedial or posterior nuclei), and that an ablation of protective immunity is possible by electrolytic lesioning of the specific hypothalmic areas.

I. Protective Passive Immunity from Cells and Serum

A. CRF Rats. Lymphoid cells from spleens or lymph nodes and peritoneal exudate macrophages and serum were transferred from immune donor CRF rats into nonimmune rats of the same inbred strain. Comparisons were made of parasitemia levels in the recipients following a malaria challenge.

In each of four experiments donor rats were immunized with IP injections of 10^9 γ-irradiated parasitized erythrocytes (γ-PRBC). The inocula were irradiated at 20,000 rad, and administered in 5 or 6 weekly injections. Control groups included nonimmunized and active-infection-recovered immune rats. Cell transfer was generally on a 1:1 donor-recipient basis. Spleen and node cells were collected from the excised organs by maceration and passage through a 40-ga steel mesh screen, in Hank's Buffered Salt Solution (HBS) and under slight vacuum. Counts of lymphoid cells were made by hemocytometer and viability was measured by trypan blue dye exclusion cell counts. Macrophages were harvested 24 hours after injection of donor rats with sterile mineral oil (20 ml, IP). They were suspended in HBS and counted. All cell suspensions were triple washed with centrifugation before counting and aliquoting for injection into recipients. Heart puncture blood was collected from each donor, for serum transfer studies.

Cell suspensions were injected into recipients on day (-1) and malaria challenge with 10^7 PRBC was made on day 0. Serum injections were

made on days 1 and 3. All injections were IP. Giemsa-stained tail-blood smears were made each Monday, Wednesday and Friday, and from each smear a percentage parasitemia was calculated.

In rats receiving cells (either lymphoid and/or macrophage) or serum from immune-recovered donors, there was an almost total suppression of parasitemia. There was only a partial reduction (10%-50%) of parasitemia in rats that had received lymphoid cells or serum from γ-PRBC immunized donors.

B. Walter Reed Rats. Two experiments were performed on non-inbred Walter Reed rats, in which the recipients were pre-exposed to whole-body irradiation.

On day (-7) the recipient rats were exposed to 500 rad whole-body γ-irradiation. Spleen cells from recovered-immune donors were collected and processed as described above, and the recipient rats were challenged with 10^7 PRBC.

Cell transfers in the non-inbred rats were successful in suppressing parasitemias (median % PRBC <2% throughout experiment, as compared to % PRBC of up to 22% in controls).

C. C57/BL 6J Mice. Cell transfer studies with P. berghei in laboratory mice are difficult, due to the generally fulminating course of infection which precludes the development of an active acquired immunity. However, mice that can be kept alive for a relatively long period of infection are able to develop such an immunity. Hence, several methods have been explored for the prolongation of infection and eventual recovery of mice which are immune to challenge and thus are suitable as cell donors.

1. Immunization by repeated challenge and chemotherapy. One experiment has been performed in which experimental mice were immunized by long-term active infection (8 parasite injections and drug treatments over a period of 13 months) alternating challenge and chemotherapy to maintain parasitemia levels below 10%. There were 41 to 78 mice surviving, and these were used as donors in a cell transfer experiment. However, in the cell recipients any protective effect from transferred immune cells were masked by an increased parasitemia caused by the transfer of infected erythrocytes which had been sequestered in the spleen of donors which had overcome patent parasitemia. In present studies this problem of inadvertent transfer of parasites is being obviated by the treatment of cell preparations with a solution to lyse the erythrocytes.

2. Immunization by challenge followed by periodic transfusions of normal homologous erythrocytes (NRBC). P. berghei-infected mice were divided into four groups, viz., mice which had received (1) an IP injection of NRBC in a 1:1 transfer each time a parasitemia of 10% PRBC was reached (2) an IP injection of NRBC in a 4:1 transfer whenever a

level of 10% was reached (3) an IP injection of NRBC in a 1:1 transfer whenever a level of 60% was reached or (4) an IP injection of NRBC in a 4:1 transfer whenever a level of 60% was reached. Survival was comparable for these donor groups (30%) except in group 3 and in an untreated infected control group, where there were no survivors. The 12 surviving mice are presently serving as cell donors in an experiment involving incubation of cells in anti- θ antiserum to lyse the T-cell component of the transferred inoculum. The anti- θ serum was prepared by periodic injections (over a 12-month period) of AKR/J inbred mice with thymocytes which had been removed from C3H/He J inbred mice (which have thymocytes that bear an identical θ locus).

3. Immunization by multiple injections of γ-irradiated PRBC. In none of the experiments with irradiated parasite injections has there been sufficient survivors to permit cell transfer experiments.

4. Pre-infection with P. berghei yoelii. Continuing studies involve further attempts to immunize donor mice by first infecting them with P. berghei yoelii, to which they are capable of developing a sterile immunity, and to challenge with P. berghei berghei. Initial experiments indicate that a cross-immunity does result from pre-infection with this less-virulent strain of parasite.

II. Ablation of Immune Responses to Malaria by Induction of Hypothalmic Lesions

Studies in Europe have indicated that by the electrolytic lesioning of specific areas of the hypothalamus, the immune response to certain antigens can be suppressed. In a cooperative study with the Department of Neurophysiology, a study was made to determine whether or not such an impairment of acquired immunity might result in rats subjected to hypothalmic lesioning and challenged with P. berghei.

Adult COBC rats were subjected to electrolytic lesions in the anterior, dorsomedial or posterior hypothalamus. Sham lesions were produced in control rats by the placement of the electrode within the hypothalamus, but without use of an electric current. After recovery from surgery, they were challenged with P. berghei and monitored throughout the course of infection by blood-smear parasite counts and by indirect hemagglutination titer.

There were no consistent detectable differences between groups of rats that had been lesioned, compared to those which had received "sham" lesions. Studies are continuing, to reevaluate our model with another antigen (viz., ovalbumin), and then to repeat the study with malaria, with variation in challenge dosages.

3. Active and serum mediated passive immunity to Plasmodium falciparum malaria.

Data indicates that Aotus monkeys develop an immunity to a second challenge by Plasmodium falciparum. Also, that some protection from a heterologous strain of P. falciparum is afforded Aotus monkeys by the administration of human antiserum. Three experiments reported herein are designed to further test for homologous active immunity and to determine the degree to which homologous antiserum can protect Aotus monkeys from P. falciparum.

The line of P. falciparum used in these experiments was derived from a Malaysian isolate, designated as the Camp strain, which has been serially passaged in Aotus monkeys. A history of the strain and the course of the disease it produces have been described.

Physical, hematological, and hemoparasitological examinations were performed on the Aotus monkeys and only apparently healthy animals were chosen for experimental use. The animals varied in weight from 660 to 1100 grams. Procedures for maintenance of the monkeys has been described.

Each 5 ml ampule of the antimalarial drug Aralen^{R} (Winthrop) contained 250 mg of Chloroquine hydrochloride (equivalent to 200 mg of Chloroquine base).

Normal Aotus serum was obtained by bleeding normal monkeys at various times, and the serum was stored at -70°C. Immune serum was obtained by challenging Aotus monkeys at designated times with 10^{8} parasitized red blood cells of P. falciparum. When the parasitemia reached 20% PRBC the animals were cured with Chloroquine hydrochloride. They were bled at specified times, and the serum stored at -70°C. Some of these immune animals were challenged two to four times. If no clinical evidence of parasites was observed two weeks after challenge, the animals were bled of 20 ml blood, and the serum stored as before. The amount of serum given to each recipient animal was 7.5 ml per Kg body weight.

Experiment I

The first experiment was designed to evaluate active immunity in Aotus monkeys. Six monkeys were each infected with 10^{8} parasitize red blood cells from a donor animal. When the parasitemia reached 20% the animals were cured with Chloroquin hydrochloride. Each monkey was later challenged with 10^{8} parasitized red blood cells at one month, three months, six months, seven months and eight months.

Five of the monkeys remained clinically free of the parasites; one monkey after the seventh month developed a parasitemia of one per cent. This data verifies that Aotus monkeys develop an acquired immunity to P. falciparum.

Experiment II

The second experiment was designed to evaluate the protective effect of homologous antiserum. Five groups of monkeys were used and the results are contained in the 5 groups that follows:

Group I - These animals received serum from animals challenged twice.

Monkey #	Prepatent Period	Day to Death
1893	11 days	survived
1390	41 days	survived
1745	30 days	Day 38
1760	21 days	Day 31

Group II - These animals received serum from animals challenged once.

Aotus #	Prepatent Period	Day to Death
1587	7 days	Day 14
1597	15 days	survived
1609	9 days	Day 23
1425	12 days	Day 22

Group III - These animals received serum from animals challenged more than two times.

Aotus #	Prepatent Period	Day to Death
1874	28 days	survived
1883	never patent	survived
1881	7 days	survived
1781*	never patent	-

*Never became patent, but died on day 32 as a result of a cage fight.

Group IV- These animals received serum from animals who received Chloroquin HCl.

Aotus #	Prepatent Period	Day to Death
1770	5 days	Day 15
1764	9 days	Day 16
1589	7 days	Day 10

Group V - These animals received normal serum

Aotus #	Prepatent Period	Day to Death
1608	5 days	Day 13
1437	3 days	Day 11

The results indicate that each animal was protected in direct proportion to the number of times the respective donor animal was challenged.

Experiment III

This experiment was designed to compare protection from a multiple dose regimen of antiserum and as single dose regimen. Three groups of four animals were divided as follows:

Group I - These animals received one dose of hyperimmune serum the first week and each week thereafter received normal serum for three more weeks.

Aotus #	Prepatent Period	Day to Death
2017	23 days	survived
2096	25 days	survived
2107	never patent	survived
2030*	never patent	-

*Died of peritonitis as a result of cage fight on day 43.

Group II - These animals received normal control serum every seven days.

Aotus #	Prepatent Period	Day to Death
1952	9 days	Day 16
1966	5	11
2025	4	10
2265	4	10

Group III - These animals received hyperimmune serum each week for four weeks.

Aotus #	Prepatent Period	Day to Death
2024	15 days	survived
2026	41	survived
2028	never patent	survived
2055	13	survived

The results of this experiment suggests that a single dose provided protection equal to that from a multiple dose regimen. A question arises whether or not antierythrocyte antibody plays any role in the protective effect of the antiserum. An experiment designed to explore that question is being performed.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 324 Host responses to malaria

Literature Cited

Publications:

1. Diggs, C.L. "Approaches to the study of acquired immunity against malaria" in IMMUNOLOGY METHODS AND TECHNIQUES, Ed. Zacharia and Breese. Marcel Dekker, Inc., New York, 1973, pp 169-196.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6525	74 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. DSEP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	C. 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	328
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			M FB 132

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	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 74	2	200
FISCAL YEAR CURRENT 75	2	180

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21. GENERAL USE

Foreign intelligence not considered

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, and assess various modes of antimalarial therapy with respect to clinical responses and radical cure. Document metabolic alterations of human and animal red blood cells when infected with malaria parasites and assess the effect of antimalarial drugs on these alterations in order to develop new drugs effective against resistant falciparum malaria. Malaria is an extremely important factor in military operations in the tropics.

24. (U) Document clinical features of acute disease, evaluate available therapeutic agents with respect 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 the effects of antimalarial drugs on morphologic growth and radiolabelled precursor incorporation into protein and nucleic acids during in vitro schizogony. Establish presence of metabolic pathways in the malaria parasite.

25. (U) 73 07 - 74 06 Consultations with many physicians treating patients for malaria from Vietnam continued on an informal basis. Several new drugs and new drug combinations were introduced into clinical test centers, and cooperative field trials with investigational compounds in Thailand were conducted. In vitro culture of P. knowlesi parasites has continued as a screening procedure for antimalarial activity and for demonstration of drug potentiation. Biosynthesis of methionine was observed during in vitro culture. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

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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 to assess the effect of antimalarial drugs on these pathways in order to develop new drugs effective against resistant falciparum malaria.

Progress.

Admissions to Walter Reed General Hospital 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 reported at two international meetings (1,2). This latter publication included a summarization of all human work to date with U.S. Army Investigational Antimalarial Drugs.

In vitro study of synergism between antimalarial drugs has continued, using the ^{14}C-orotic acid system with Plasmodium knowlesi (simian) malaria. Combinations of pyrimethamine and WR 158122 were found to be additive in effect in this system. The system was used to screen investigational compounds for antimalarial activity. Of 40 compounds studied, 19 were effective at the 80% inhibitory level. A majority of the effective compounds were analogs of folates and of purine nucleosides. A report of a previously completed in vitro screening study was accepted for publication (3).

The P. knowlesi parasite is being examined for presence of metabolic pathways involving metabolites of folic acid. Drug-susceptible processes sequential to dihydrofolate reductase may lead to potentiation of effect of antimalarial drugs which are inhibitors of that enzyme (e.g., pyrimethamine, methotrexate). The single-carbon transfer involving folates in which methionine is formed by transfer of the methyl group of serine to homocysteine has been demonstrated in vitro, using radio-isotopically labeled compounds.

In a collaborative study with Dynatech Corporation, antimalarial efficacy of implanted drug preparations is being studied. WR 158122 in a polymer vehicle (glycolic acid/lactic acid copolymer) which releases the drug over a four-week period in vitro, was implanted subcutaneously in mice and immediate and repetitious (weekly) challenges with P. berghei were commenced. At drug dosages of 80 mg/Kg and above, patency of parasitemia was delayed for at least 3 to 4 weeks. Dosages of 40 mg/Kg and lower were less effective (patent parasitemia at two weeks or earlier). Some rejections of implanted material were observed at high drug dosage (640, 320, 160 mg/Kg).

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malarial Investigations

Work Unit 328 Clinical studies of human malaria

Literature Cited.

References:

1. See "Publications" below.

2. Canfield, C. J., and Rozman, R. S.: Clinical testing of new antimalarial compounds. Bull. World Health Org. (In press).

3. 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. (In press).

Publications:

1. Canfield, C. J.: Progress in clinical testing of new antimalarial agents. Proc. 9th Inter. Cong. Trop. Med. & Mal.: 284-285, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6471	74 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
73 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. [redacted]	CARDS 114F			CH FR 112

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 74	5.6	135
FISCAL YEAR CURRENT 75	5.6	135

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Drug Resistance; (U) Chemotherapy; (U) Chemoprophylaxis; (U) Human; (U) Monkey; (U)Vectors; (U)Chloroquine;(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 in military personnel. 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 malaria patients.

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) 73 07 - 74 06 In a study of 207 patients with falciparum malaria both WR 33063 and WR 30090 were as effective as quinine but less toxic. Pyrimethamine with DFD (WR 6798) given as a single dose cured only 43 percent of 23 patients with falciparum malaria. Pyrimethamine with DFD (WR 6798) was administered weekly as a prophylactic in central Thailand; results of the study are not yet available. Eighteen new drugs were tested against P. cynomolgi in the rhesus monkey. Infrared aerial photography was introduced as a survey technique for locating dry-season breeding sites of mosquitoes. Differences in rate of infection between A. balabacensis and A. minimis could be at least partially explained by differences in blood-meal volumes. Coma and pulmonary edema in falciparum malaria are often caused by or exacerbated by excessive intravenous infusion of fluids.

For technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 73-30 Jun 74.

*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 3A762759A829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 336 Field studies on drug resistant malaria

Investigators.

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Associate: Sanong Kosakal, MD; Katchrinee Pavanand, MD; Joseph E. Scanlon, Ph. D.; Panya Sonkom, MD; Markpol Tingpalapong, DVM

A. CLINICAL STUDIES

1. Comparison of a 9-Phenanthrene Methanol (WR33063), a 4-Quinoline Methanol (WR30090), and Quinine for Treatment of Falciparum Malaria in Thailand

BACKGROUND: WR33063 and WR30090 are the code numbers for two antimalarial drugs developed by the U.S. Army Malaria Research Program.

WR33063 is a 9-phenanthrene methanol which in volunteers did not cause toxicity up to 4.6 grams daily for 10 days; and 1.6 grams daily for 6 days was highly effective against all the strains of P. falciparum that were tested. (1)

WR30090 is a 4-quinoline methanol which was non-toxic in volunteers who received 0.69 grams daily for 6 days. At this dose it radically cured all chloroquine-sensitive and most chloroquine-resistant infections with P. falciparum.(2)

Canfield et al (3) tested both drugs in U.S. soldiers with acute falciparum malaria in Vietnam. The radial cure rate for WR33063 was 92% (23/25) and for WR30090 was 88% (23/25). Other patients with recrudescent infections were all cured by one or the other of

these new drugs. Segal et al (4) studied indigenous patients in Northeast Thailand and found a cure rate of 92% (23/25) for WR33063 and of 94% (24/25) for quinine.

The present study extends the comparison of WR33063, WR30090 and quinine to Southeast Thailand, another area of known multi-drug parasite resistance.

DESCRIPTION: Colwell et al (5) reported on the first of several malaria studies sponsored jointly by the SEATO Medical Research Laboratory and the Trad Provincial Hospital in Southeast Thailand 400 km from Bangkok. Malaria is transmitted continuously throughout the year in Trad Province.

The present studies were begun on 11 January 1973. Patients reporting to the hospital with suspected malaria were referred to a special malaria clinic. Blood was obtained from a finger prick and a thin film, a circular thick film, and a measured 3 x 15 mm rectangular thick film containing 0.002 ml blood were made. The slides were stained with Giemsa at 1:5 dilution for 5 minutes. If examination of the blood films showed plasmodia, a direct parasite count was done on the measured film using the method of Earle and Perez (6) as described by Powell et al (7).

On patients admitted to a therapeutic study, the second and all subsequent slides, which included 2 rectangular films each using 0.05 ml blood, were stained with Giemsa at 1:50 dilution for 30 minutes. Parasite counts were performed twice daily on capillary blood specimens taken at 0700 and 1400 hours from day 0 to day 6, or until two negative blood films were obtained; thereafter, specimens were obtained once daily. Parasite counts were performed at other times when indicated. Follow-up smears were made on days 14, 21 and 28.

Determinations of hematocrit levels and leucocyte counts were made on admission and on days 3, 6 and 28. Sera were collected on days 0, 3, 6 and 28, and whenever the clinical response was unusual, for measurements of creatinine, glutamic-oxaloacetic transminase (SGOT), bilirubin and alkaline phosphatase. Urinalysis was performed on admission and on days 3, 5 and 28.

From the patients with falciparum malaria who required treatment in hospital, volunteers were selected for the study who met the following criteria:

1. Males at least 15 years old who agreed to the use of the drugs which were under investigation and who were willing to be in hospital for 6 days and to attend 3 follow-up visits on days 14, 21 and 28 (the day of admission being called day 0).

2. Asexual parasite counts of P. falciparum between 1,000 and 100,000 per cmm.

3. Evidence of active disease as shown by a rising parasite count, a high fever, or obvious symptoms (e.g. headache).

Patients with severe or complicated falciparum malaria were not studied nor were those with coincident vivax malaria.

The patients were attended by the SEATO physicians throughout the study, under the supervision of the Trad Medical staff. The nurses took oral temperatures routinely at 0600 and 1800. Additional readings were taken during febrile episodes. Ward rounds were made at least twice daily and at other times when clinically indicated. Palpation for splenomegaly was done at least once daily.

On the day of discharge (day 6 or later) the patients were handed a follow-up card and then taken home in a truck by the SEATO driver who drew a map of the route on returning to the clinic. If the patients failed to attend the follow-up clinic on days 14, 21 and 28, home visits were made.

A table of random numbers was used to assign the patients to one of the following drugs.

a). WR33063 was formulated in yellow and green gelatin capsules containing 200 mg of the drug. The dose was 600 mg (3 capsules) every 8 hours for 18 doses (6 days). The total dose was 10.8 grams.

b). WR30090 was formulated in red and white gelatin capsules containing 250 mg of the drug. The dose was 250 mg (1 capsule) every 8 hours for a total of 18 doses (6 days). The total dose was 4.5 grams.

c). The quinine was administered in brown sugar-coated tablets of quinine sulfate, USP, each containing 270 mg quinine base. The dose was 540 mg (2 tablets) every 8 hours for 18 doses (6 days). The total dose was 9.72 grams.

Throughout the study, the drugs were administered by the technicians or the doctors. We evolved a team concept whereby the doctor and the technician combined the ward rounds and medication administration at 0600, 1400 and 2200 hrs. The drug for each patient was kept in a bottle labelled with the patient's name, study number and bed number. The number of capsules or tablets in each bottle was double checked before treatment. The "Medication Ward Round" was conducted in the following sequence:

1). With the technician as interpreter, the patient was asked how he felt. Evidence of drug toxicity would result in a reduced or deleted dose.

2). The patient swallowed the drug followed by water.

3). The spleen was palpated while the patient breathed through his mouth. We could thus detect whether the drug had been swallowed or retained in the mouth.

4). The symptoms and signs were noted and the doctor recorded each dose on individual sheets for each patient.

5). The patients were then asked to stay in bed for 30 minutes and were observed by the study team during this period.

PROGRESS: Between January and August 1973, blood films were examined on 4213 people of whom 1705 had falciparum malaria (including a few mixed infections) and 284 had vivax malaria.

214 patients were admitted to the therapeutic study between 11 January and 2 August 1973. 207 of these patients completed

the 6 day treatment in hospital.

The similarity of the study groups is shown in Table 1. The average age was 27 years. There was no difference in the mean temperature or parasite count. WR33063 cleared parasitemia slower than quinine (77 vs 70 hours), but temperatures returned to normal more rapidly with WR33063 than with the other 2 drugs. The differences were not statistically significant.

In 204 patients the parasitemia was cleared during the hospital course. In the 3 other patients the parasitemia was not cleared by the initial therapy.

The drugs caused no detectable changes in hematocrit, WBC count, creatinine, SGOT, bilirubin, alkaline phosphatase or urinalysis.

Table 1. Comparison of WR33063, WR30090 and Quinine Initial Response

Drug	No. Patients	Mean Asexual Parasite Count (per cmm)	Mean* Parasite Clearance (Hours)	Mean** Highest Temperature (° C)	Mean*** Fever Clearance (Hours)
WR33063	69	27,894	77.2±2.6(68)	39.2	54.5±3.8(56)
WR30090	68	27,520	71.6±2.7(65)	39.4	58.4±3.7(61)
Quinine	70	27,745	69.8±2.5(69)	39.0	63.7±4.5(57)

* Mean ± Standard Error of Mean. The figures in parentheses refer to the number of patients observed.

** Highest temperature on day of admission.

*** Fever clearance times computed only if initial fever >38.0°C.

One patient (case No. 216) had heavy proteinuria (2.3 grams per cent) and a serum albumin of 1.13 grams per cent on the day of admission before therapy with WR33063. During treatment he

Table 2. Treatment of Falciparum Malaria in Southeast Thailand 1973

Drug*	Mean Parasite Count Per cmm	RIII**	RII	RI	S	Total	Cure Rate	No. Doses In Regimen
WR33063	27,000	1	0	4	57	62	92%	18
WR30090	28,000	0	1	8	54	63	86%	18
Quinine ***	28,000	1	0	9	55	65	85%	18
Fansidar	28,000	0	2	1	14	17	82%	1
Amodiaquine	20,000	0	2	6	5	13	38%	4
Maloprim***	24,000	0	0	17	4	21	19%	1
Chloroquine	15,000	2	8	1	0	11	0%	4
Pyrimethamine	3,000	0	3	0	0	3	0%	3

*Dosage

WR33063: 600 mg T.I.D. 6 days
WR30090: 250 mg T.I.D. 6 days
Quinine: 540 mg (base) T.I.D. 6 days
Fansidar: Sulformethoxine 1.0 g. } 1 dose
Pyrimethamine 50 mg }
Maloprim: DDS 200 mg } 1 dose
Pyrimethamine 25 mg }
Amodiaquine: 1.5 g in 3 days
Chloroquine: 1.5 g in 3 days
Pyrimethamine: 150 mg in 3 days

**RIII, no marked reduction of asexual parasitemia; RII, marked reduction of asexual parasitemia but no clearance; RI, clearance, followed by recrudescence; S, Clearance without recrudescence. World Health Organization (1967) Chemotherapy of malaria. WHO Tech Rep Ser No. 375, p 42.

***Study performed in Northeast Thailand by Segal et al (1974 b)

developed gross pitting edema of the abdomen and legs and the heavy proteinuria continued. The malaria was cured but the nephrotic syndrome persisted despite corticosteroid therapy.

Complete follow-up was achieved in 190 of the 207 patients who were admitted to the study (92%). Follow-up was 100% for local residents but only 63% (29/46) for patients who had recently migrated from other parts of Thailand.

21 patients developed a repeat attack, on or before day 28, diagnosed as a recrudescence. 12 patients had a repeat attack, between day 29 and day 147, diagnosed as a reinfection. This arbitrary classification followed established practice.

Patients whose infections were cleared and did not recur on or before day 28 were considered to be cured. The cure rates for the 3 study drugs compared with 5 other drugs we have studied is shown in Table 2. Chloroquine and pyrimethamine resistance were confirmed. WR33063 had the highest cure rate, although the differences between the 3 drugs were not statistically significant.

Patients who were not cured had significantly higher initial parasite counts and more prolonged parasitemia and fever (Table3).

Table 3. WR30090, WR33063, Quinine Comparison of Patients Cured and Not Cured

Characteristic	Cured (S)	Not Cured (RI-III)	Statistical Significance
Number of Patients	183	24	
Mean Initial Parasite Count (per cmm)	25,600	43,900	t=2.7, p<0.02
Mean Parasite Clearance (Hours)	71	88	t=2.9, p<0.01
Mean Initial Fever (^{o}C)	39.2	39.2	t=0, Not Significant
Mean Fever Clearance (Hours)	56	79	t=3.2, p<0.01

WR33063 was the least toxic drug and caused no specific side effects. In no patient did drug administration have to be stopped or the dose reduced.

Headache and backache were more common in the patients receiving WR30090. Urticaria, probably due to WR30090, occurred in two patients.

Quinine caused cinchonism in many patients and was the most toxic drug. Many patients were seen lying down at unusual times, presumably because of postural hypotension. The dose of quinine had to be reduced in 15 patients (20%) because of severe toxicity.

Drug fever was tentatively diagnosed in 9 patients on quinine, 3 patients on WR30090, and 1 patient on WR33063.

All 3 drugs were inconvenient to use because they had to be administered every 8 hours for 6 days. Most patients were otherwise fit for discharge on the third day, so that the treatments caused delay in discharge and overflow of the wards. Convenient therapy should be finished within 3 days, and, if possible, be comprised of only 1 or 2 doses daily.

DISCUSSION: WR33063 was a highly effective drug. In comparison with WR30090 and quinine, WR33063 had the highest cure rate, cleared fever the most rapidly and had the lowest toxicity. WR33063, however, was slower than quinine in clearing parasitemia. The main disadvantage of WR33063 was its bulky formulation (3 capsules for each dose).

WR30090 was also as effective as quinine, especially in clearing parasitemia. This drug appeared to cause headache, backache and urticaria but was not as toxic as quinine. In contrast, WR30090 showed no toxicity in the more limited number of cases reported by Martin et al (2) and Canfield et al (3).

Altogether, WR33063 appears to be the more promising of the 2 new drugs.

In this study, patients with a prolonged parasitemia or a prolonged fever were more liable to develop recrudescences. Similar results were obtained in U.S. troops by Hall (8). Thus, these 2 parameters

(parasite clearance time and fever clearance time) may be a useful guide in evaluating the radical curative potential of an antimalarial drug.

We have observed that intravenous quinine is more effective than oral quinine in bringing a malarial attack under control; therefore, it is wise to use oral quinine alone against mild infections only, and to precede oral therapy with intravenous quinine for more severe infections.

20% of the patients developed attacks of vivax malaria during the follow-up period and these caused many days lost from work. Routine administration of primaquine after discharge (e.g., 15 mg daily for up to 14 days) may be the answer.

In patient No. 216 the nephrotic syndrome was probably caused by the falciparum malaria. Such an entity has been proposed by Berger et al (9).

SUMMARY: Quinine was compared with a 9-phenanthrene methanol (WR33063) and a 4-quinoline methanol (WR30090) in the treatment of 207 patients with falciparum malaria in Southeast Thailand. Quinine eradicated parasitemia (average 70 hours) more rapidly than either WR30090 (72 hours) or WR33063 (77 hours); but WR33063 had a higher cure rate (92%) than WR30090 (86%) or quinine (85%).

WR33063 was the least toxic drug. WR30090 appeared to cause headache, backache and urticaria. Quinine was the most toxic drug. All 3 drugs were inconvenient in having to be administered every 8 hours for 6 days. One patient did not respond to oral quinine but did respond to an intravenous quinine infusion.

A "Medication Ward Round" was perfected during the study and comprised sequential history, drug administration, physical examination, dose notation and patient observation.

Falciparum nephrosis was diagnosed in one patient.

2. Amodiaquine Resistant Falciparum Malaria in Thailand

BACKGROUND: Interest in 4-aminoquinolines other than chloroquine was reawakened by Schmidt who found in owl monkeys that

two chloroquine-resistant strains of P. falciparum were more susceptible to amodiaquine than to chloroquine. Rieckmann also reported the superiority of amodiaquine both in vitro and in vivo although radical cures were not achieved with amodiaquine in volunteers. Fitch demonstrated that owl monkey erythrocytes infected with chloroquine-resistant P. falciparum had a deficiency of chloroquine-^{14}C uptake, but not a deficiency of amodiaquine-^{14}C uptake. Therefore, we compared the therapeutic efficacy of the two drugs in an area endemic for chloroquine-resistant falciparum malaria.

DESCRIPTION: The study was performed at Trad Hospital in Southeast Thailand between March and July 1973. Chloroquine, given either orally or parenterally, is used frequently to treat patients with the clinical diagnosis of malaria; amodiaquine is not used at all. Chemoprophylaxis is not practiced in the community. All patients were fully informed of the nature of the drug trial and consent was granted voluntarily. They all had mild or moderate falciparum malaria with asexual parasitemias between 1,000 and 100,000 per cmm. Alternate patients were assigned to chloroquine or amodiaquine. The dosage form of chloroquine used was a non-enteric coated tablet ("Nivaquine", May and Baker, 150 mg of chloroquine base). The dosage form of amodiaquine was a non-enteric coated tablet ("camoquine", Parke-Davis 200 mg of base). The initial regimen was 1.5 g of the appropriate drug, administered over 3 days, 600 mg initially, 300 mg 6 hours later, and 300 mg on each of the succeeding 2 days.

Because only few patients were cured by 1.5g of amodiaquine given for 3 days, a 2.0g course for 4 days was studied in 9 additional selected patients with low parasitemias. Most patients received 400 mg (2 tablets) initially followed by 400 mg 6 hours later on day 0, then 400 mg on the morning of days 1, 2 and 3.

CHLOROQUINE 1.5 g:

In hospital, chloroquine cleared parasitemia in only 3 of 13 patients (Table 1). One of these patients developed a recrudescence during the 1 month follow-up period; the other two patients could not be traced and the responses are recorded as "RS" (parasitemia was cleared; thus RII or RIII were ruled out, but follow-up examinations were not achieved and the final result had to be either RI or S). In many patients chloroquine had little or no effect on parasitemia - a potentially dangerous situation. No patients were cured

Table 1. Falciparum Malaria in Southeast Thailand Treated with Chloroquine 1.5g over 3 Days

Case Number	Age	Asexual Count *P. falciparum* (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance* (Hours)	Result**	*P. vivax* after Discharge
1	17	1820	6d	37.7	-	RS	
3	21	3240	N.C.***	37.7	-	RII	
5	17	12820	N.C.	39.5	N.C.	RIII	
7	26	1500	N.C.	38.8	74	RII	
9	57	6552	N.C.	39.0	51	RII	
11	20	18200	69	36.8	-	RS	
13	18	13160	N.C.	40.0	N.C.	RIII	
15	21	2340	N.C.	37.5	-	RII	
17	40	27391	N.C.	37.2	-	RII	
19	23	2710	41	39.0	63	RI	
21	25	21021	N.C.	38.8	52	RII	
23	43	49572	N.C.	38.5	N.C.	RII	
25	23	20637	N.C.	40.2	N.C.	RII	
Average	27	13920	N/A	38.5	60		

* Fever clearance was computed only when the initial fever was at least 38°C.

** RIII, no marked reduction of asexual parasitemia; RII, marked reduction of asexual parasitemia but no clearance; RI, clearance, followed by recrudescence; S, Clearance without recrudescence. World Health Organization (1967) Chemotherapy of Malaria. WHO Tech. Rep. Ser. No.375, p42.

We have added a suggested new symbol, namely; RS, clearance, but no follow-up examinations. (see text)

*** No clearance.

by chloroquine. Five of the treatment failures were next treated with amodiaquine 1.5g, and their subsequent responses were as follows: 1 patient was cured (S); 2 patients had recrudescence (RI); 1 patient responded only partly (RII) but did respond to a combination of sulfadoxine with pyrimethamine (S); and 1 patient could not be traced (RS response). The other 5 treatment failures were treated with other drugs but follow-up examinations were not obtained. Due to the obviously poor response to chloroquine, use of this drug was discontinued after 13 patients had been treated.

AMODIAQUINE 1.5 g:

Amodiaaqine was significantly ($p<0.01$) more effective in clearing parasitemia (15 out of 17 patients) than chloroquine (Table 2). Four patients could not be followed-up (RS response); radical cures were achieved in 5 (38%) of the remainder.

The 8 patients who were not cured were then further treated as follows:

1). Five patients were treated with 3 tablets of Fansidar (sulfadoxine with pyrimethamine). One of these patients developed another recrudescence and the other 4 responded satisfactorily but did not return after discharge (RS response).

2). Two of the eight received quinine with an RS response.

3). One patient was cured by WR33063 (a 9-phenanthrene methanol under investigation).

AMODIAQUINE 2.0 g:

The parasitemia was cleared in all 9 patients (Table 3). Two patients did not return for follow-up examination; 1 of these patients had no parasitemia on days 14 and 24 but did not return on day 28 (RS responses). Two other patients returned only once, one on day 26, and one on day 30. They did not have detectable parasites in peripheral blood films and were considered radically cured. Two patients recrudesced on days 14 and 15 respectively. Over-all, 5 of 7 patients were adjudged to be radically cured by the 2.0g course. Since only patients with mild disease (average parasitemia 8,000) were treated, the over-all response was not considered favorable.

Table 2. Falciparum Malaria in Thailand Treated with Amodiaquine 1.5g over 3 Days

Case Number	Age	Asexual Count P. falciparum (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)	Result	P. vivax after Discharge
2	44	4320	66	37.7	-	RS	
4	47	8730	90	37.5	-	RI	
6	27	12376	76	37.7	-	RI	
8	18	34830	60	39.5	46	S	
10	18	18425	N.C.	38.6	55	RII	
12	18	14256	93	39.5	19	RS	
14	30	7917	47	37.5	-	S	Day 51
16	34	9100	65	39.7	13	RS	
18	44	13190	117	39.0	79	RI	
20	37	21060	75	38.0	43	RS	
22	30	9100	48	37.6	-	S	Day 44
24	49	7735	105	39.4	55	RI	
26	15	35900	N.C.	37.9	-	RII	
27	29	54000	70	40.3	86	S	
28	17	20000	69	38.0	56	RI	Day 45
29	25	18428	70	37.3	-	RI	
30	18	18200	100	38.2	20	S	
Average	29	18092	76.7	38.4	47.2		

Table 3. Falciparum Malaria in Southeast Thailand Treated with Amodiaquine 2.0g over 4 Days

Case Number	Age	Asexual Count *P.falciparum* (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)	Result	*P.vivax* after Discharge
31	38	7392	94	37.7	-	S	
32	15	21140	75	39.8	43	RS	
33	41	2700	41	38.9	32	S	
34	17	3680	41	37.7	-	RS	Day 59
35	15	2916	67	40.0	42	S	
36	20	5265	43	37.2	-	S	
37	23	16562	115	39.4	18	RI	
38	29	9696	88	37.3	-	RI	
39	27	4050	63	39.6	14	S	
Average	25	8155	69.7	38.6	29.8		

Table 4. Comparison of Cure Rates

Drug	Mean Parasite Count (per cmm)	RIII	RII	RI	S	Total	Cure Rate
Chloroquine 1.5g	14000	2	8	1	0	11	0%
Amodiaquine 1.5g	18000	0	2	6	5	13	38%
Amodiaquine 2.0g	8000	0	0	2	5	7	70%

Urine specimens were obtained before treatment from 33 patients. Chromatography of these specimens showed spots corresponding to one or both of the 4- aminoquinolines in 29 of them. This indicated that a high proportion of the patients had received antimalarial drug therapy before admission. All 33 patients had evidence of 4-aminoquinolines in post-treatment urine specimens. The two drugs could not be reliably differentiated by the chromatography techniques employed.

Symptoms were frequently observed during chloroquine therapy and were at least partially attributable to the disease, which in most patients was not responding satisfactorily. Abdominal tightness, dizziness and other symptoms were fairly common in patients on amodiaquine therapy (although they were not more frequent than with chloroquine).

DISCUSSION: Falciparum malaria in Thailand responds poorly to chloroquine whether given as treatment or for suppressive prophylaxis. The logical deduction is that chloroquine should not be used for falciparum malaria in Thailand. In practice, chloroquine is frequently prescribed either orally or 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 resistance.

In accord with recent studies we found (Table 4) that amodiaquine was more effective (38% cure rate) than chloroquine (0%) in the treatment of chloroquine-resistant falciparum malaria. The predominant response was RI rather than RII; however, 38% is not a very impressive cure rate. The difference in cure rates may be partly explained by the fact that chloroquine is widely used in the community whereas amodiaquine is not used at all.

We do not recommend amodiaquine for the treatment of falciparum malaria in Thailand since both quinine and the combination of sulfadoxine and pyrimethamine (Fansidar) are more efficacious. Harinasuta (personal communication) recommends a short course of quinine (1-3 days) followed by a single dose of sulfadoxine with pyrimethamine as the preferred course of therapy.

SUMMARY: Amodiaquine cured 38% (5/13) of patients with falciparum malaria in Southeast Thailand. Chloroquine cured none (0/13). In

hospital, amodiaquine cleared parasitemia significantly more frequently than did chloroquine, but neither drug was effective enough to warrant further use for falciparum malaria in Thailand. Insidious toxicity was observed with both drugs.

The responses of patients whose parasitemia was cleared in hospital but who did not attend for follow-up examination were recorded as RS (i.e. RI or S). This notation is presented as a suggested new addition to the W.H.O. classification.

3. Clindamycin Toxicity in Falciparum Malaria

OBJECTIVE: To determine whether a 3 day course of quinine and clindamycin is effective and acceptable therapy for falciparum malaria in Thailand.

BACKGROUND: Workers at the National Institutes of Health (NIH) have claimed that 3 days therapy with quinine and clindamycin is effective and not toxic.

PROGRESS: Six patients with P. falciparum malaria (Table 1) were started on quinine 540 mg (base) every 8 hours together with clindamycin 450 mg every 8 hours, for a 3 day course. In 4 patients the clindamycin was stopped because of severe drug toxicity principally manifested as severe nausea and retching ("dry heaves"). After the symptoms of the malaria and drug toxicity had subsided, the clindamycin was resumed without any further problem; however, it was decided that full dose combination therapy with quinine and clindamycin was too toxic.

Eight patients with falciparum malaria (Table 2) received combination therapy with reduced doses of quinine and clindamycin (usually quinine 270 mg every 8 hours and clindamycin 150 mg every 8 hours). Five of these patients developed gastrointestinal symptoms, especially retching.

Quinine alone for 3 days (Table 3) was given to two of these patients which brought the disease under control in hospital. One of these patients developed a drug-induced fever. Clindamycin alone for 3 days (Table 3) brought the disease under control in 2 other patients. Follow-up in all these studies has not yet been completed.

Although the combination of quinine and clindamycin appeared to be efficacious, its toxicity was unacceptable. Clindamycin or quinine

Table 1. Full Dose Combination Therapy with Quinine and Clindamycin

Case No.	Parasite Count (per cmm)	Parasite Clerance (Hours)	Initial Fever (° C)	Fever Clerance (Hours)	Comment
1	101556	93	39.6	60	
2	55419	64	40.0	39	
3	146692	117	38.3	82	Drug Toxicity
4	8654	69	40.0	69	Retching
5	33943	85	40.6	60	Retching
6	5642	69	40.0	92	Retching
Average	58649	83	39.8	67	

Table 2. Half-dose Combination Therapy with Quinine and Clindamycin

Case No.	Initial Parasite Count (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clerance (Hours)	Comment
7	13312	92	38.9	32	Retching
8	236600	116	39.1	-	Fever
9	358830	119	37.5	-	Fever and Retching
10	214760	115	38.5	42	
11	2730	59	40.9	58	
12	4914	96	40.2	-	Abdominal Pain
13	61880	-	38.0	-	Toxicity
14	54432	67	39.9	18	
Average	104671	95	39.1		

Table 3. Quinine Therapy for 3 days

Case No.	Initial Parasite Count (per cmm)	Parasite Clerance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)
15	87804	84	37.9	-
16	21708	69	39.8	-

Table 4. Clindamycin Therapy for 3 days

Case No.	Initial Parasite Count (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)
17	820	61	39.2	-
18	18144	92	39.9	76

alone was effective in treating relapses.

4. Treatment of Falciparum Malaria with Pyrimethamine and DFD

OBJECTIVE: Pyrimethamine in combination with DFD has been developed and tested for the prophylaxis of malaria. We have tested a single dose for therapy.

BACKGROUND: DDS (Dapsone) is a sulfone with antimalarial activity, but its short half-life (17 hours) dictates daily administration for prophylaxis. DFD, a diformyl derivative of DDS, was developed as a longer acting compound (half-life 30 hours) suitable for weekly dosage. Clyde found that DFD with pyrimethamine, given weekly, was effective in the prophylactic suppression of both falciparum and vivax malaria.

DESCRIPTION: Patients with clinically mild or moderate falciparum malaria were treated with pyrimethamine 50 mg and DFD 800 mg (4 tablets) Initially a few patients received a 2 tablet dose.

PROGRESS: Three patients received a 2 tablet dose (Table 1). One patient was cured (S response) but in two others the parasitemia was not cleared in hospital (RII response).

Thirty patients were given a single dose of 4 tablets (Table 2). The results are given in Table 2. The over-all cure rate was 43% (10/23).

The cured cases included many mild infections. Generally the initial clinical response was slow. In cases No. 2 and 4 the patients remained ill from their malaria on day 2 with a high fever. In both patients the total parasite count was low, although schizonts were seen on the blood films. It is probable that DFD

has the unusual effect of clearing the parasites out of the blood into the deeper tissues.

SUMMARY: Single dose therapy with pyrimethamine 50 mg and DFD 800 mg was insufficient to cure a majority of patients infected with falciparum malaria.

Table 1. Falciparum Malaria Treated with 25 mg Pyrimethamine and 400 mg DFD

Case No.	Parasite Count (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)	Result*
1	57785	NC	39.0	NC	RII
2	3360	-	40.4	NC	RII
3	1539	41	37.8	-	S

*
RIII = parasitemia did not respond to treatment
RII = partial response of parasitemia
RI = clearance of parasitemia followed by recrudescence
S = cure
NC = not cleared

5. The Suppression of Plasmodium falciparum and Plasmodium vivax Parasitemias by a Diformyldiaminodiphenyl Sulfone-Pyrimethamine Combination

OBJECTIVE: To study the effectiveness of the combination of diformyldiaminodiphenyl sulfone (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.

Table 2. Falciparum Malaria Treated with 50 mg Pyrimethamine and 800 mg DFD.

Case No.	Parasite Count (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)	Result*
4	41040	NC	38.4	NC	RII
5	1560	45	38.7	37	RI
6	7209	44	37.1	-	S
7	20200	44	39.0	50	S
8	1820	61	39.6	12	RS
9	1420	61	38.8	36	S
10	5400	92	39.2	67	RI
11	12393	45	37.3	-	S
12	11220	NC	40.5	-	RIII
13	7290	66	37.7	-	RS
14	6885	43	37.8	-	S
15	12150	67	36.9	-	S
16	5670	40	37.0	-	S
17	13013	52	40.5	92	RS
18	2568	41	38.6	NC	RI
19	12312	70	40.4	93	RI
20	18382	117	37.8	NC	RI
21	4920	72	40.3	40	RI
22	11664	84	39.8	N/A	S
23	1040	61	38.7	-	RI
24	30294	69	39.1	69	S
25	1220	14	39.8	-	S
26	1120	-	40.0	-	unknown
27	9963	-	40.4	-	RIII
28	7220	60	39.3	59	RI
29	40708	-	40.0	-	RII
30	12960	62	38.3	12	RS
31	95580	93	39.0	84	RI
32	46137	43	39.2	42	unknown
33	63700	-	39.2	-	unknown
Average	16900	60	38.9	53	

DESCRIPTION: Six hundred and seventy-four 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.

PROGRESS: The twenty-six week course of medication was concluded on 9 February 1974. Currently the study subjects are being monitored monthly for evidence of malaria transmission in the absence of chemosuppressive agents. Until the termination of medication the average weekly study subject participation was approximately 88%. As the first slide reading has not been completed, no data reduction is possible at this time.

6. Treatment of Falciparum Malaria with Septrin:

Septrin, a 5:1 combination of sulfamethoxazole and trimethoprim, is commonly used in the treatment of bacterial infections. It has been moderately successful in antimalarial therapy. Three patients with P. falciparum infections were given Septrin. Results are summarized in Table 1.

Table 1. Falciparum Malaria Treated with Septrin

Case No.	Parasite Count (per cmm)	Parasite Clearance (Hours)	Initial Fever (°C)	Fever Clearance (Hours)	Comment
1	7280	51	39.0	44	
2	46980	51	38.6	20	
3	49248	48	37.5	-	Quinine Therapy added

In cases 1 and 2 a satisfactory clinical response occurred in hospital. In case 3, it was decided on the day of admission that, because of the patient's symptoms, quinine therapy was also indicated. Follow-up studies on these patients have not yet been completed.

7. Treatment of Falciparum Malaria with Proguanil

Proguanil, probably the least toxic antimalarial, was introduced in 1945. By 1950 falciparum malaria resistant to proguanil had been encountered and the drug fell into disfavor. Nonetheless, the Australian Army in Vietnam utilized proguanil with DDS, daily, as prophylaxis with apparent success.

One patient with P. falciparum infection at Trad Hospital was treated with proguanil. The patient was a 49 year old man with clinically mild disease. The parasite count was 48,924 per cmm, and oral temperature 38.2 °C. Proguanil 200 mg was given as a loading dose then 100 mg was given twice daily for a total of 12 doses. His fever responded slowly (clearance time 115 hours), as did his parasitemia (clearance time 98 hours). Follow-up has not been completed.

In the patient studied, proguanil, like many other partially effective drugs (e.g. Fansidar, Septrin, Amodiaquine), was slow to bring the clinical symptoms under control. These partially effective drugs should not be used for the primary treatment of the disease. Quinine is the drug of choice, although it is often toxic, and falciparum malaria in Thailand often shows partial resistance. Further studies with proguanil will be conducted.

8. Quinine Fever in Falciparum Malaria

In a recently completed study, quinine was compared with 2 investigational drugs (WR30090 and WR33063). Each of the 3 drugs was given to malaria patients for 6 days. The temperature charts and and clinical histories were reviewed for evidence of drug-induced fever.

The criteria for the diagnosis of quinine fever (QF) were a fever which persisted or developed after parasitemia had been cleared, and the absence of evidence of another disease. All cases in which the fever clearance time exceeded the parasite clearance time by at least 12 hours were reviewed.

The study involved 207 patients. The mean fever clearance times for WR33063, WR30090 and quinine were 55, 58 and 64 hours respectively; and the mean parasitemia clearance times were 77, 72 and 70 hours. Thus the differences between mean parasite clearance times and mean fever clearance times were 22, 14 and 6 hours for WR33063, WR30090 and quinine respectively. The magnitude of these differences appears to inversely reflect the potential of the drugs to cause a drug fever. QF was diagnosed in 10% (9/70) of the patients treated; WR30090 fever in 4% (3/68); and WR33063 fever in 1% (1/69). Details of the 9 QF patients are shown in Table 1; on average the fever clearance time exceeded the parasite clearance time by 40 hours.

Quinine fever is one, but certainly not the most serious, manifestation of quinine toxicity.

Table 1. Quinine Fever in Thailand

Case No.	Initial Fever (°C)	Parasite Clearance Time (Hours)	Fever Clearance Time (Hours)	Difference FCT-PCT (Hours)	Peak Fever During QF (°C)*
24	40.3	46	114		39.5
45	40.7	76	96	20	39.3
54	39.7	93	107	14	38.3
99	38.2	58	104	46	39.0
120	39.5	69	133	64	39.3
134	39.5	54	93		38.1
184	38.0	41	81		38.0
198	38.4	87	132		39.4
213	40.4	88	111		39.0
Average	39.4	68	108	-	38.9

* Oral temperature

9. Chloroquine-Responsive Vivax Malaria in Thailand

Although vivax malaria is not always radically cured by chloroquine and primaquine, the acute clinical attack does respond to chloroquine alone. Since falciparum malaria in Thailand is highly resistant to chloroquine, we wished to confirm the sensitivity of vivax malaria to this drug.

Patients with clinically severe vivax malaria and microscopic

confirmation of a pure infection were selected for study. Patients with mixed infections of P. falciparum and P. vivax were separately studied.

All of 10 patients with vivax malaria responded rapidly to chloroquine (Table 1). The mean fever clearance time was 44 hours compared to 64 hours for falciparum malaria treated with quinine at the same hospital. The mean parasite clearance time was 44 hours compared to 69 hours for falciparum malaria treated with quinine. The parasitemia remained negative in the patients who completed the 28 day follow-up.

The rapid action of chloroquine in acute attacks of vivax malaria was confirmed in an area where falciparum malaria is highly resistant to this drug.

Table 1. Vivax Malaria Treated with Chloroquine: Initial Clinical Response

Case No.	Asexual Count (per cmm)	Parasite Clearance Time(Hours)	Initial Fever (°C)	Fever Clearance Time (Hours)
1	11040	37	38.8	35
2	8100	47	37.2	-
3	24570	52	41.0	56
4	14023	47	39.8	-
5	4540	62	40.5	38
6	14580	44	40.6	-
7	17063	42	37.9	-
8	7614	44	40.0	43
9	7000	40	39.0	40
10	5265	24	39.6	28
Average	11379	44	39.4	40

10. Jaundice in Falciparum Malaria

Over a 2 month interval sera were taken from a group of outpatients with malaria at Trad Provincial Hospital. The Department of Biochemistry, SEATO Medical Research Laboratory kindly performed biochemical determinations. Sera of 235 patients with falciparum malaria were examined. 79% had serum total bilirubin below 2 mg% and did not have clinically visible jaundice (Table 1). 21% of the patients had increased serum bilirubin. 14% of all the patients had mild biochemical jaundice (bilirubin between 2 and 4 mg%); 5% had

moderate jaundice (bilirubin between 4 and 10 mg%); and 2% had severe clinical and biochemical jaundice (bilirubin over 10 mg%). There was a positive correlation between the parasite count and the bilirubin level (Table 2), a finding which we had expected. The fact that jaundice in malaria is related to the severity of the infection is confirmed by analysis of 11 patients recently studied (Table 3).

We alsc studied a group of 24 patients with . ix malaria. The mean total bilirubin level was only 0.99 mg%. The highest single value was 1.7 mg%.

Most falciparum patients with jaundice have a high parasitemia and require urgent intravenous antimalarial therapy. Quinine is the only effective drug available; however, the juandice may indicate liver damage. Quinine is metabolized by the liver and can be toxic to that organ. Quinine toxicity may be induced even with orthodox dosages. Over the first 24 hours optimum therapy is usually 20 gr quinine in 1000 ml saline given as 2 separate infusions of 500 ml. The infusion rate should not exceed 100 ml per hour. A similar regimen can be given on succeeding days. The quinine should be given orally when the patient has improved. Radical cure of these patients is difficult and oral therapy should be prolonged; however, the patients are usually fit for discharge before the jaundice has cleared.

Table 1. Incidence of Jaundice in 235 Patients with Falciparum Malaria

Total Bilirubin (mg percent)	Number of Patients	% of Total	Diagnosis
0 - 2	186	79	No Jaundice
2 - 4	33	14	Mild Jaundice
4 - 10	12	5	Moderate Jaundice
10+	4	2	Severe Jaundice

Table 2. Correlation of Parasite Counts with Bilirubin Levels in Falciparum Malaria

Range of Asexual Parasite Count*	Number of Patients	Mean Total Bilirubin mg% (± Standard error)
0 - 100	16	0.91 ± 0.15
100 - 1000	22	0.92 ± 0.10
1000 - 10,000	66	1.33 ± 0.11
10,000 - 100,000	100	1.62 ± 0.18
100,000+	31	3.36 ± 0.74

* Parasites per cmm

Table 3. Patients with Falciparum Malaria and Clinical Jaundice

Case No.	Asexual Parasite Count*	Total Bilirubin mg%
1	109382	32
2	53508	39
3	2106	11
4	16686	8
5	96288	28
6	7720	7
7	437710	8
8	131606	21
9	546000	11
10	46656	12
11	146692	9
Average	144,941	17

* Per cmm

11. Management of Anemia in Falciparum Malaria

In many patients with falciparum malaria, pulmonary edema and hemoglobinuria have followed blood transfusion. We have successfully managed several patients without blood transfusion and wish to record our findings.

In our patients with malaria, we define the anemia as severe if the hematocrit (packed cell volume) is below 15%. Anemias with hematocrits above this level seldom require special attention. We have recently conservatively managed 5 patients with hematocrits that fell below 15% (Table 1). In most patients the lowest hematocrit was recorded a few days after admission. None of the patients developed cardiac failure. They all received quinine therapy initially by the intravenous route. Infusions were given slowly. Slow but steady progress was observed in all cases. Radical cure was achieved in the 3 patients who were followed-up. None of the patients developed pulmonary edema or hemoglobinuria.

We have not yet treated an anemic falciparum malaria patient by blood transfusion. We consider that eradication of the parasitemia is of prime importance but that, because of the weakened state of the patients, the antimalarial therapy should be given cautiously, usually at reduced dosage. Criteria for blood transfusion might be a hematocrit below 10%, cardiac failure or failure to improve; however, we think that blood transfusion is very seldom indicated in falciparum malaria.

Table 1. Patients with Falciparum Malaria and Hematocrits below 15%. Blood Transfusions not Given.

Case No.	Initial Parasite Count*	Lowest Hematocrit	Final Result
1	577,850	14%	Radical Cure
2	22,194	13%	Radical Cure
3	24,240	13%	No Follow-up
4	138,200	12%	Radical Cure
5	4,212	11%	No Follow-up
Average	153,339	13%	-

* Per cmm

Intravenous Overload, Pulmonary Edema and Coma in Falciparum Malaria

It has been reported that pulmonary edema is a specific complication of falciparum malaria; however, most cases of pulmonary edema

reported in recent years and those seen by us at Trad have followed the administration of large volumes of intravenous fluids. We have studied fluid balance in seriously ill malaria patients.

During the last 15 months at Trad we have seen 5 malaria patients who developed pulmonary edema and coma. We considered that these patients may have been given excessive amounts of intravenous fluid in the first 24 hours (Table 1); on average they received 2490 ml (range 2000-3000 ml). In most instances each 500 ml contained 10 gr quinine. Intravenous fluid overload may have contributed to the pulmonary edema and coma, and quinine overdosage probably also occurred in some instances.

Table 1. Falciparum Malaria with Coma. Pulmonary Edema Attributed to Intravenous Overhydration in First 24 Hours.

Case No.	Initial Parasite Count**	Age (Years)	IV Fluids (ml) Infused in Time Interval (Hours) 0-8	8-15	16-24	Total (ml) 24 Hours	Comment
1	317,844	20	1450	0	1000	2450	
2	94,640	30	2000	0	1000	3000	death
3	601,640	30	1650	350	1000	3000	
4	318,000	16	1500	0	500	2000	death
5	777,600	22	1000	500	500	2000	
Average	421,600	24	1520	170	800	2490	

** per cmm

Subsequently, we decided to limit intravenous fluid therapy to not more than 500 ml in any 8 hour interval. Five patients were treated with this regimen (Table 2), and pulmonary edema did not occur. One death occurred, but this can be attributed to the poor condition of the patient upon hospital admission. The average intake of fluid in the first 24 hours was 1500 ml (range 1000-2000 ml).

Nevertheless, with this limited intravenous fluid regimen, 30 gr of quinine are still being infused in 24 hours. We think this may be too much in patients with very high parasite counts or who are in deep coma. 20 gr may be a more suitable daily dose in these patients.

We conclude that coma and pulmonary edema in falciparum malaria are often caused by or exacerbated by excessive intravenous infusion of fluids.

Table 2. Falciparum Malaria with Coma. Absence of Pulmonary Edema Attributed to Optimal Intravenous Hydration in First 24 Hours.

Case No.	Initial Parasite Count**	Age (Years)	IV Fluids (ml) Infused in Time Interval (Hours) 0-8	8-16	16-24	Total (ml) 24 Hours	Comment
6	109,382	35	1000	0	1000	2000	
7	2,997	16	900	100	0	1000	
8	307,638	30	630	370	500	1500	
9	26,568	45	500	500	500	1500	death*
10	22,295	24	500	500	500	1500	
Average	93,776	30	706	294	500	1500	

* Patient moribund on admission.

** Parasites per cmm.

B. LABORATORY STUDIES

1. Specific Antibody Levels in Individuals from a Malarious Region as Estimated by Radioimmunoassay

BACKGROUND: A solid-phase radioimmunoassay has been developed for estimation of immunoglobulin class specific antimalarial antibody (11). The assay was refined by replacing the sheep erythrocyte as an immunoadsorbent to remove the antibody from the test serum. The assay proved to be sensitive and the reproducibility was high; therefore, it became necessary to evaluate it using individual sera collected from persons in a malarious region. This study describes the results of such an evaluation.

DESCRIPTION: The radioimmunoassay (RIA) was performed as previously described (12) with the following modifications. The third phase of the assay, the competitive binding portion, was replaced with a direct binding system as illustrated in Figure 1. Briefly, the tube containing the bound antigen-antibody complex was washed 5

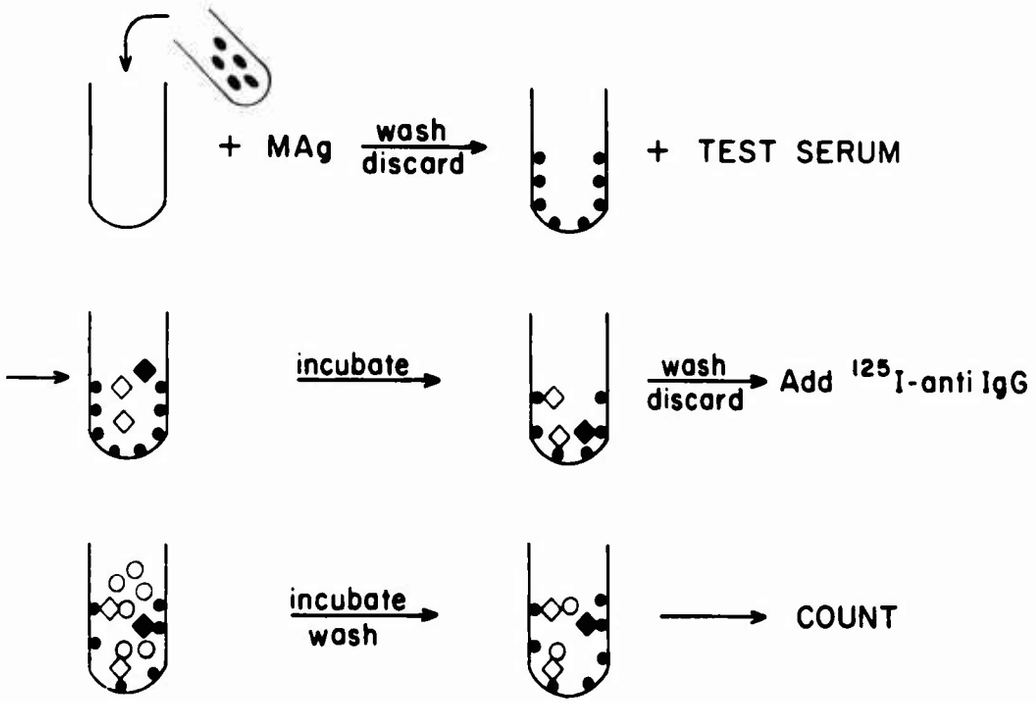
times with PBS, pH 7.2; and ^{125}I-labelled, purified rabbit anti-human IgG antibody was added (32 µg/ml)and allowed to incubate for 3 hours. After incubation, the antibody was aspirated and the tube washed 5 times, or until the wash no longer contained radioactivity. The tube was then counted in a gamma spectrometer. The quantity of specific anti-IgG antibody present in the test sera was estimated by comparing results from the test sera with those obtained from a standard curve developed by substituting purified human IgG for the antigen-antibody complex.

Purified anti-human IgG antibody was produced in the following manner. Human IgG, purified by batch DEAE procedures, was injected into a goat and the serum retrieved was shown to contain precipitins by immunoelectrophoresis and gel-diffusion. The antibody was purified by ammonium sulphate precipitation and by passing the dissolved precipitate across an immunoadsorbent column of human IgG linked to sepharose with cyanogen bromide. The antibody was eluted with glycine-HCl,pH 2.3 with 10% dioxane and dialyzed overnight in TRIS buffer, pH 8.0. The antibody was rendered mono-specific by passing the dialyzed material through another immuno-adsorbent column of myeloma IgM linked to sepharose with cyanogen bromide. Monospecificity was demonstrated with immunoelectropho-resis and radioimmunoelectrophoresis.

All sera were assayed a minimum of 2 times with duplicate prepara-tions in each assay. Control positive serum and normal serum were included in each assay and if deviations from the mean for these control sera were too great, as determined by a quality control system(13), that particular assay was considered invalid and the data were not included.

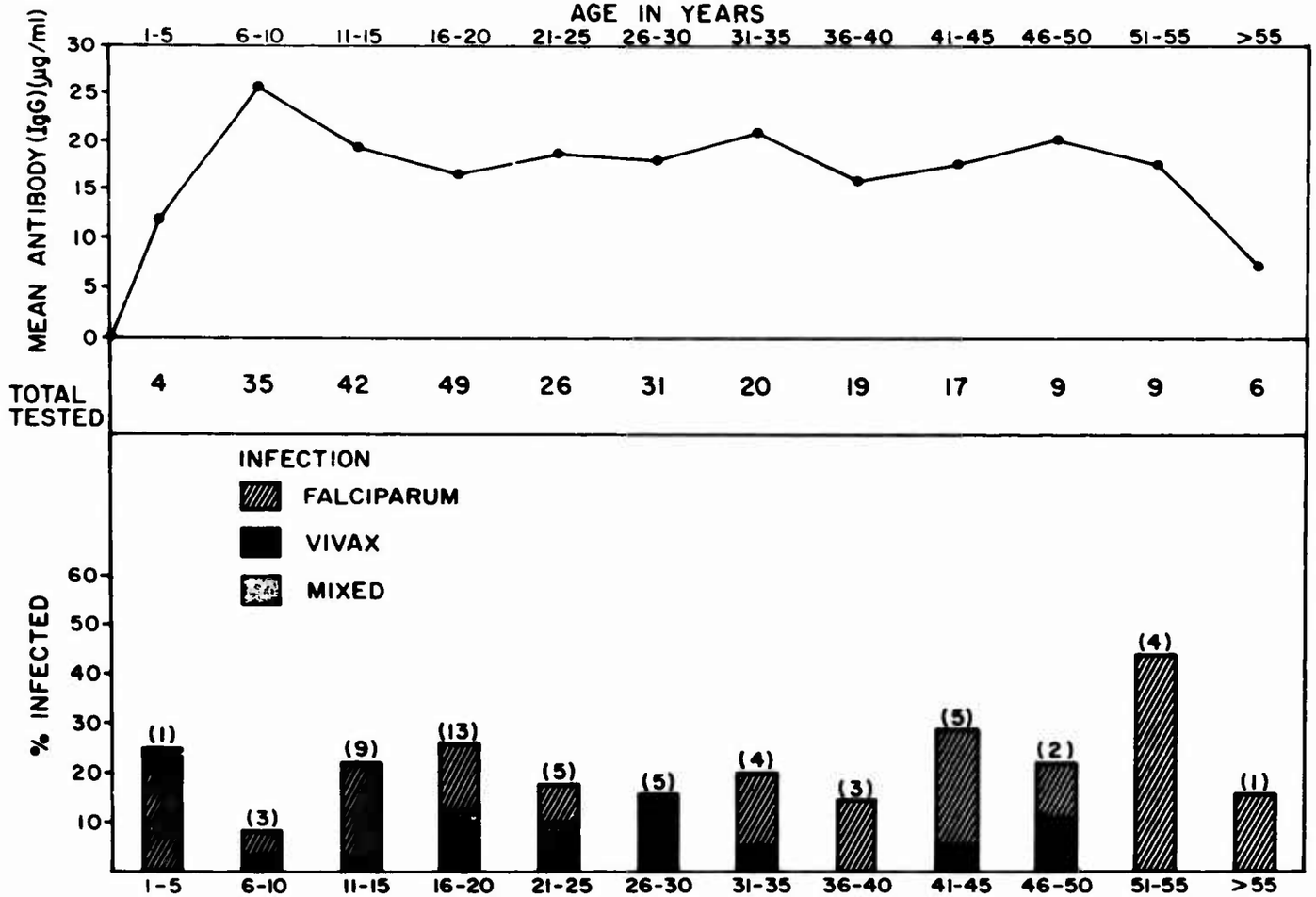
RESULTS: Repeated experiments testing the amount of ^{125}I-antigen and purified ^{125}I-IgG bound to the polypropylene tube indicated that the amount of malarial antigen or human IgG bound to the tube was consistent in each assay with less than 1% error between assays. This provided the basis for developing the ^{125}I-labelled purified antibody system described above. Retesting of the same sera after the initial adsorption demonstrated that no detectable quantities of IgG antibody could be found. 262 serum samples were assayed with 179 demonstrating detectable antibody (Table 1). The mean antibody level of the 179 individuals was 27.07 µg/ml. Individuals who had malaria, as demonstrated by examination of stained blood smears, had a mean of 9.36 µg/ml with 27 of 59 having detectable antibody

FIGURE I : SCHEMATIC REPRESENTATION OF RADIOIMMUNOASSAY



- • Malaria antigen
- ◇ IgG antibody
- ◆ IgM antibody
- ○ ^{125}I-labelled anti-human IgG antibody

FIGURE 2. ANTIBODY (IgG) LEVELS AND PER CENT INFECTED INDIVIDUALS



(Table 2). A total of 17 had palpable spleens, but no correlation was observed between detectable antibody and spleen rate. Antibody levels and the infection rate by age group are presented in Figure 2.

DISCUSSION: The modified RIA system has proven to be as sensitive and as accurate as the previously used inhibition system and the time required to perform the assay has been reduced by approximately 50%. Serum antibody levels to this antigen were detectable in 179 of 262 samples assayed (68%). Antibody was detected in all age groups assayed with a maximum peak in the 6-10 age group, which also had the lowest rate of infection for P. falciparum (Fig. 2). Of the infected group, 22 of 59 (37%) did not have detectable antibody to this antigen. Data are insufficient to make any correlations between the presence of the antibody to the antigen used and immunity to malaria; however, there does seem to be a trend indicating a positive correlation. This study is continuing and additional data may provide an answer to this question. The RIA has been shown to be an effective and accurate method of determining IgG antibody to specific malarial antigen in serum drawn under field conditions.

Table 2. Mean Antibody (IgG) Levels in Infected Individuals

Species	Number	With Detectable Antibody	Mean Antibody Levels µg/ml	No. with Palpable Spleens
P. falciparum	34	18	10.29	14
P. vivax	13	8	15.25	3
Mixed	2	1	2.1	0
TOTAL	59	27	9.36	17

2. Evaluation of Experimental Antimalarial Drugs in Rhesus Monkeys Infected with Plasmodium cynomolgi

OBJECTIVE: To evaluate the effectiveness of selected experimental antimalarial drugs in suppressing Plasmodium cynomolgi parasitemias in rhesus monkeys. Experimental drugs were selected and furnished by the Division of Medicinal Chemistry, Walter Reed Army Institute of Research.

TABLE 1: Specific Antibody (IgG) Levels in Individuals Living in a Malarious Region of Thailand

Age	Sex	Number of Samples	Number with Antibody	Mean Antibody Level (µg/ml)	Range (µg/ml)
1-15	M	24	20	28.4	6.2-79.4
	F	16	14	25.0	0.8-52.0
11-15	M	22	17	25.4	2.4-56.0
	F	20	12	30.8	2.0-75.0
16-25	M	35	23	27.0	4.0-54.8
	F	30	18	29.3	0.4-69.6
25-40	M	42	28	30.2	0.8-81.4
	F	33	23	21.7	0.6-72.4
40+	M	27	13	20.7	8.2-54.2
	F	13	11	24.9	8.6-91.4
Sub-Total		150	101	27.07	0.8-81.4
Total		262	179	27.07	0.6-91.4

DESCRIPTION: Experimental antimalarial drugs being developed by the US Army Antimalarial Drug Program were evaluated in rhesus monkeys for suppressive activity against Plasmodium cynomolgi var. bastianelli. After a 6 week conditioning period in the laboratory, healthy, malaria-free Indian rhesus monkeys weighing 1.5 - 3.5 kg were infected by intravenous injection of 5×10^8 parasitized erythrocytes from a splenectomized donor monkey. On day 4, when parasitemia was at the peak (200-900,000/cmm), a 7-day course of daily drug administration was initiated. Blood parasites were counted daily for the first 15 days and every two days thereafter. At the end of thirty days, monkeys with negative blood smears were splenectomized. Only those monkeys which showed no recrudescence of parasitemia for 30 days after splenectomy were classified as cured. Clinical signs of drug toxicity or intercurrent disease which appeared during the course of study were recorded. Necropsy examinations were performed terminally.

Drugs were dissolved or suspended in a vehicle containing 0.3% methylcellulose in distilled water within one hour before administration. In most cases, drugs were administered by nasogastric incubation after an 18 hour fast, but parenteral routes have been elected in special situations. Each drug was tested over a range of doses to determine a minimum curative dose, a minimum effective dose and, sometimes, a maximum tolerated dose. Normally two monkeys were treated at each dose level, with doses spaced 0.5 log 10 apart (ie. 316, 100, 31.6, 10.0, 3.16, 1.0 mg/kg, etc). Usually 6 to 10 monkeys were sufficient to permit evaluation of a single drug. Two vehicle-treated controls were included with each group of 10 drug-treated monkeys.

PROGRESS: Eighteen (18) experimental antimalarial drugs were evaluated during the year. These compounds and their minimum curative doses are listed in Table 1. Seven of these compounds are "standard" antimalarials. These baseline data will be used for comparative purposes when structurally related drugs are evaluated.

Two antimalarial drugs were tested in combination with sulfadiazine. WR 122455 (a phenanthrenemethanol) and sulfadiazine did not exhibit additive antimalarial activity when administered in combination. WR 93133 and sulfadiazine administered in combination had additive or synergistic antimalarial activity.

Table 1. Minimum Curative Doses of Antimalarial Drugs in Plasmodium cynomolgi Infected Rhesus Monkeys

Type of Drug	WRAIR Drug Number	Minimum Curative Dose (mg/kg/day)
2, 4-Diaminoquinazolines	WR 135403	0.1
	WR 151341	0.1
	WR 154907	0.1
	WR 162877	0.1
	WR 179214	0.316
Quinolines	WR 2977 (Amodiaquine)	10.0
	WR 4234 (Plasmochin)	31.6
	WR 7295 (Endochin)	NC [1]
	WR 187005	100.0
	WR 198557	10.0
Sulfones/sulfonamides	WR 448 (Dapsone)	10.0
	WR 7557 (Sulfadiazine)	100.0
Pyridinemethanols	WR 151312	10.0
Miscellaneous	WR 1543 (Atabrine)	31.6
	WR 2978 (Pyrimethamine)	1.0
	WR 3090	31.6
	WR 93133	31.6
	WR 194916	NC [1]

[1] Not curative. Compound ineffective at the highest dose tested (100 mg/kg).

C. VECTOR STUDIES

1. Studies on Malaria Vectors

OBJECTIVE: To investigate the bionomics and population dynamics of known and potential vectors of human malaria in Southeast Asia, their relationship to the dissemination of chloroquine-resistant strains of P. faciparum, and to apply the information acquired to the development of effective methods of control for these vectors.

DESCRIPTION: Specific factors being studied in the process of defining actual and potential vector species in Thailand include the following: incidence of malarial parasites in wild anopheline populations, susceptibility of colonized strains of Anopheles to infection with P. falciparum, patterns of biting activity of suspect anopheline species, and ovipositional habits of proven vector species.

2. Malaria field studies in Prachinburi Province.

Entomological field studies were continued in the Bu Phram valley of Kabinburi District. Major emphasis was on a continuation of the survey for dry season breeding sites of A. balabacensis. As reported in previous years, ovipositional sites of this malaria vector have been located during the dry season along stream beds on the forested slopes of the hills surrounding Bu Phram valley. Water trapped in rock holes from the previous rainy season or fed by persistent springs on the slopes provide sites for low density breeding of A. balabacensis during the dry season. Because of the time and effort required to locate these sites in the difficult terrain of this area, application of larval control measures based upon ground surveys alone would be impractical. The possibility of applying infrared aerial photography as a survey technique for locating dry-season breeding sites was explored during this reporting period. Aerial photographs of jungle areas taken with infrared film can delineate small areas of water beneath the forest canopy because of differences in the infrared reflectivity of the adjacent vegetation. Through the cooperation of the 432 Technical Reconnaisance Wing, 7/13 Air Force, USAF, aerial photographic surveys of the entire Bu Phram valley and adjacent hills were flown during January and March 1974, using Kodak 2424 infrared aerographic film. A third survey of this area is planned for after the onset of the monsoon rains. Processing and interpretation of these aerial surveys is being carried out by the School of Public Health, University of Texas, through contract with the National Aviation and Space Agency, Houston.

3. Susceptibility of A. balabacensis and A. minimus to infection with P. falciparum prior to and after standard chloroquine therapy.

Results of studies given in the last Progress Report (14) indicated that when A. balabacensis and A. minimus were fed on patients with chloroquine-resistant falciparum malaria prior to and 2 and 7 days after chloroquine therapy there was no increase in the proportion of mosquitoes of either species infected after the initiation of therapy;

however, the oocyst indices (mean number of oocysts per infected mosquito) for A. balabacensis were 1.7 and 1.8 times greater, respectively, on days 2 and 7, than on day 0 (Table 1). A similar increase in oocyst indices was not observed for A. minimus. Since gametocyte densities in the donor patients on days 0, 2 and 7 were not significantly different, it is difficult to explain the observed increases in oocyst indices on that basis. These observations do, however, agree with observations on the enhancement of infectivity of P. berghei for A. stephensi fed on mice infected with chloroquine-resistant strains of the parasite after the mice had been treated with 1 or 10 mg/kg body weight of chloroquine (15). The apparent enhancement of the infectivity of chloroquine-resistant strains of P. falciparum for A. balabacensis (but not A. minimus) lends support to the hypothesis that this mosquito species has been a contributing factor to the rapid increase of chloroquine-resistant strains of P. falciparum in Southeast Asia.

4. Determination of volume of blood meals ingested by A. balabacensis and A. minimus.

Comparison of the infection rates occurring in colonized strains of A. balabacensis and A. minimus fed simultaneously on falciparum malaria patients indicated that a significantly higher proportion of A. balabacensis became infected and also that the mean number of oocysts developing in the gut of infected A. balabacensis was higher than in A. minimus. These differences could be related to the volume of blood ingested by each species or to differences in the ability of each to concentrate the cellular components of their blood meals.

The volumes of blood meals taken by laboratory-reared A. balabacensis and A. minimus were compared by feeding them simultaneously on rabbits whose RBC and serum had been labelled with Cr^{51} and I^{125}, respectively. In order to collect excreta discharged by the mosquitoes during engorgement, they were individually housed in filter-paper lined vials whose nylon screened mouths were placed against the shaved sides and back of the donor rabbit. Blood meal volumes and the composition of mosquito excreta were determined by comparing the radioactivity of the engorged mosquitoes and the filter papers containing their excreta with that of a sample of blood from the donor rabbit taken at the time of feeding.

Four separate feeding experiments were completed in which a total of 72 A. balabacensis were fed on rabbits with radioisotope-labelled

blood. The mean volume of blood ingested by this species was 2.69µl (range 0.14-4.95µl). The excreted portion of the blood meal was measured for 8 A. balabacensis and averaged 0.12 µl per individual; this excreted portion consisted almost entirely of serum globulins. The mean volume of blood ingested by 17 A. minimus, on the other hand, was 1.38 µl. Excreta discharged during feeding, for 8 of these mosquitoes, was approximately the same as for A. balabacensis--0.11 µl per mosquito. These initial experiments suggest that differences in rate of infection observed between the two anopheline species can at least partially be explained by these differences in blood-meal volumes.

5. Bionomics of laboratory-reared Anopheles minimus.

A colony of A. minimus has been successfully maintained at SMRL since 1970. Observations on this colony made during the present reporting period are given below. The SMRL strain of A. minimus was established from the progeny of 32 engorged females collected feeding on buffaloes in Saraburi province and returned to the field insectary at Phra Phutthabat. The insectary was maintained at 24-28°C and 55-90% relative humidity. In our insectary, these mosquitoes laid a total of 1572 eggs (mean: 49/female), ninety per cent of which were fertile. Adults reared from the offspring of the wild-caught females were forced-mated and their progeny formed the basis of the present colony, which has been maintained by the same forced mating technique used with the A. balabacensis colony (16). After several generations were fed on human blood, adaptation to feeding on hamsters was accomplished. As with the colonized strain of A. balabacensis, female A. minimus are mated after they have taken a blood meal. Oviposition is on the surface of water in paper cups, in preference over moist filter paper or sand. An average of 90 eggs per female are deposited between 3 and 15 days following forced-mating. Eggs hatch two to three days after oviposition. The larvae were fed a mixture of two parts Bacto Liver Powder and one part each of ground laboratory guinea pig and rat-mouse food. First and second instar larvae were fed twice and third and fourth instars three to four times daily. The duration of the larval stages ranges from 8 to 26 days, with a mean of 3-4 days for each instar (Table 2). Fifty per cent of A minimus larvae in this laboratory strain pupate by the 13th day, and 96 per cent of larvae reaching the pupal stage complete development to adults. Average larval mortality for the past year has been approximately 3 per cent. The sex ratio of emerging adults during

Table 1. Median and Ranges of Oocyst Indices for Mosquitoes which Fed on Infective Patients on Days 0, 2 & 7

Day	Number Inf. Pts.	Median (Range) Oocyst Indices	
		A. balabacensis	A. minimus
0	13	3.3 (0-120.2)	2.8 (0-24.9)
2	11	5.7 (0-29.2)	2.4 (1.0-9.5)
7	6	6.1 (2.1-46.7)	3.1 (1.0-32.9)

Table 2. Summary of the Duration of the Immature Stages of Colonized Anopheles minimus

Stage	Day Present	Day 50 Percent Attain a Given Stage	Percent Mortality
1st instar	0-7	-	1.9
2nd "	2-11	3	0.7
3rd "	5-18	7	0.1
4th "	6-25	10	0.7
Pupae "	8-26	13	5.8

this period has been 44 per cent male and 56 per cent female. The average production from the colony was 3045 males and 3501 females per month. Mortality of emerging adults has been approximately 6 per cent (Table 2). Sixty-four per cent of pupation occurs between 1800 and 0600 hours, while the majority of adults (59%) emerge during the same period.

Project 3A762759A829 MALARIA 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	74 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 / A. WORK UNIT
73 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A829	00	337
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			CH FR 134

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)
PRECEDING FISCAL YEAR 74	9	352
CURRENT FISCAL YEAR 75		352

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER [redacted]

ASSOCIATE INVESTIGATORS

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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) 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 systheses.

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) 73 07 - 74 06 The main thrust of the synthesis program for this year has been in the area of prophylactic and curative agents and has centered on the 8-aminoquinolines, lepidines and quinadines. The effort has been very successful. Work on the few remaining classes in the aminoalcohol area, viz, the anthracenes, naphthalenes and naphthothiophenes has been phased out because objectives have been reached. The remaining synthesis areas are probing actions designed to develop weaker leads. A broad spectrum of structure activity information in support of the rationale systhesis continues to be obtained from the no-dollar agreement. During FY 74 875 compounds were obtained from the rational synthesis program, of which 477 were targets. An additional 5558 compounds were forthcoming from the no-dollar agreement, 607 as gifts and 81 purchased. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* 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

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The Contract Chemical Synthesis Program

At the end of FY-74 there were 24 active synthesis contracts to be carried into FY-75. During the year 5 new contracts were let and 14 were terminated. 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 and will be carried over into FY-75. A fourth preparations laboratory was terminated during the year. There has also been one contract to synthesize radioactively tagged compounds and one to analyze and confirm the 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 compounds. Six new agreements were signed and two terminated. Thirty-four papers in the chemical literature were published during the year as the result of work done in the synthesis program.

With the advancement of selected quinoline-, pyridine-, and phenanthrene methanols to the stage of clinical testing, a selection of a naphthalene- and anthracene methanol for advanced screening, work on the amino alcohol type antimalarials was in the phasing out process during the year; there is no work planned in this area for FY-75.

The emphasis on prophylactic and curative agents has been maintained throughout the year. This work has centered on the synthesis of 8-aminoquinolines, lepidines and quinaldines. Some other types of compounds in which prophylactic action was indicated, e.g., the 2-phenyl-2-(4-piperdyl)tetrahydrofuranes, the 2-hydroxy-3-alkyl-1,4-naphthoquinones and the thioalkyl quinolinequinones, were shown to have suppressive activity only and were dropped. Some 6-aminoquinolines have shown prophylactic activity but, in general, are more toxic than the 8-amino analogs. The best compounds in the latter class have been shown to have a therapeutic index four times that of primaquin in the monkey. Because of demonstrated activity, work in the related 1,5-naphthyridine series has also been carried on. The 1,2,3,4-tetrahydro-8-aminoquinolines were disappointing and have been phased out.

Work in the antimetabolite area has also been vigorously pursued during the year, although the emphasis has shifted from the synthesis of compounds designed to inhibit p-aminobenzoic acid incorporation and dihydrofolic acid reductase to compounds designed to interfer with the interconversions of coenzymes beyond the reductase stage, interfer with end product utilization, and inhibit thymidylate synthetase. Toward these ends, efforts were directed, for example, to the synthesis of isomeric pyridopyrimidines and pyrimidopyrimidines, as well as pteridines, all substituted in the 6 position with arylthio, benzylamino or anilinomethyl moieties. An appreciable number of aminopurine nucleosides were synthesized as potential purine and pyrimidine end product inhibitors as well as several related miscellaneous types which are to be looked at but not pursued in depth unless they proved to be of biological interest. Work on the related 7-substituted-1,2,4-benzotriazine-1-oxides has been continued. Because of the inability to demonstrate sufficient antimalarial activity, or improve on existing activity, several synthetic programs in the antimetabolite area have been dropped. These include the 2,4-diaminopyrrolo[3,4-d]pyrimidines, the 2,6-diamino-8-substituted purines, the pyrimido[5,6-c]pyridazines, the 5H-pyrrolo[3,2-d]pyrimidines, the 6-substituted-2,4-diaminoquinazolines, and the 6-aza analogs of the 2,4-diaminopyrimidines, the diamino-1,2,4-triazines.

With the selection of a Mannich base for advanced studies, work in this synthesis area has been phased out.

Work on the synthesis of Clindamycin analogs has continued during the year.

Several miscellaneous synthesis programs have been or are being phased out because the compounds do not appear to have sufficient promise. These include the amino acid antagonists, the orotic acid analogs, the bis amidines, the substituted piperazines, and the thionicotinamide analogs.

Acquisition of Compounds

A total of 875 new compounds were submitted by the rational synthesis contracts during FY-74, 477 of which were target compounds. An additional 607 new compounds were received as gifts, 5558 were received under the cooperative industry-government agreement and 7086 were purchased for a total of 7086. The WRAIR bottling team made 17 trips during the year and collected 4485 compounds. A total of 49 compounds were obtained from the preparations laboratories, including 34 small quantities, 11 in medium quantities and 4 in large quantities.

The Organic Laboratory Synthesis Program

During the fiscal year, the Organic Synthesis Section of the Department of Organic Chemistry has prepared compounds of several

classes as candidates for antimalarial screening. A total of 11 thiosemicarbazones containing adamantyl, pyridyl, and aryl groups were prepared and submitted. Of the two compounds for which data are available, one is active. Compounds similar to these are the pseudodithiobiurets, of which two examples were synthesized. The preparation of novel antimalarial compounds containing the 1-azaadamantane nucleus is underway, and to date, seven intermediates of these materials have been submitted for testing. Interest in acridine analogs of the 8-aminoquinoline antimalarial class has prompted a synthesis program for these materials; a total of nine intermediates have been prepared to date. As a follow-up to some work funded under the antimalarial program (R.A. Coburn and B. Bhoosnan, J. Org. Chem., 38, 3868 (1973), contribution No. 1179 in the U.S. Army series of publications on malaria research), a series of six thiazolotriazinonethiones and their analogs were synthesized and submitted for screening. Finally to investigate further the effect on antimalarial activity of replacing the hydroxyl group of an active compound with a thiosulfuric acid group, the Bunte salt analog of the IND compound, WR-122,455, was prepared and submitted for testing.

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 OE 6441	74 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	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 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	62117A	3A162110A830	00	055
b. CONTRIBUTING				
c. 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
67 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 74	3.5	289
FISCAL YEAR CURRENT 75	3	308

19. RESPONSIBLE DOD ORGANIZATION

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ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Bio Sensor Research
ADDRESS: Aberdeen Proving Ground, MD 21010

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Cantelberry, COL M. W.
TELEPHONE: 301-671-3312
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Hamlin, CPT, M.H., II
NAME: Leighton, 1LT, F. A. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence not considered

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) 73 07 - 74 06. Forty-five litters produced 255 weaned puppies. Present kennel population is 221. During the year 114 dogs were transferred to other government agencies including DOD Dog Training Center, Lackland AFB, US Army Scout Dog Platoon, Ft. Benning, GA, and USAMEDDAC, Ft. Belvoir, VA. An additional 30 young dogs were retained as breeders. The number of willing, aggressively self-confident puppies born during the past year has continued to increase markedly. Eighteen of the 28 litters which have completed puppy evaluation are considered superior in this respect. The comparable figure last year was 14 of 35 litters. Progress continued in reducing the rate of experienced hip dysplasia. During the period of this report 337 different dogs 5, 8 or 11 months of age were radiographed; of these, 43 were dysplastic. By comparison, of the 298 dogs radiographed last year, 62 were dysplastic. The improvement in temperament and dysplasia is primarily attributable to the selective breeding program. Consultant visits were made by nationally recognized authorities in the fields of genetics, canine behavior, and hip dysplasia.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*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.

1116

Project 3A162110A830 MILITARY DOG IMPROVEMENT

Task 00 Military Dog Improvement

Work Unit 055 Development and evaluation of improved biological sensor systems

INVESTIGATORS.

Principal - COL M. W. Castleberry, VC
Associates - CPT Melvin H. Hamlin, II, VC
1LT Edlin A. Leighton, MSC

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 enemy than is now generally available.

BACKGROUND. This study is being made in response to the approved US Army QMDO, "Detector System, Military Dog," (USACDC Action Co Number 12527). Seven breeds of dogs, including crosses, were st by the University of Maryland for behavioral evaluation and selec for army breeding and training (Army Contract No. DADA 17-68-C-8015). As recommended in the final report of that study, and because of the years of military experience gained with the German Shepherd Dog, this breed was selected for primary breeding emphasis.

APPROACH. 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.

PROGRESS.

A. Breeding Program

1. Forty-five litters produced 303 weaned pups.

2. Present kennel population is 221 dogs.

3. Disposition of 244 dogs was made as follows:

Walter Reed Army Institute of Research	132
Retained for breeding	30
DOD Dog Training Center, Lackland AFB, TX	28
USA Mobility Equipment Rsch & Dev Center	22
Scout Dog Detachment, Ft. Benning, GA	15
Letterman Army Institute of Research	7
Civilian Police Departments	4
National Park Service	2
The Seeing Eye, Inc.	2
Security Police Squadron, Andrews AFB, MD	2

B. Special Projects

1. Heart rates (telemetered) and plasma cortisol levels of 93 puppies representing 19 different litters were measured. The possibility of correlating these physiological values with the aggressiveness and confidence subsequently exhibited by each maturing test puppy was studied statistically. It was concluded that neither heart rate nor plasma cortisol levels are reliable indicators of the future temperament of the individual puppy. The work was accomplished by CPT J. M. Linn, VC, who will shortly rejoin this Division. The data were used for partial fulfillment of the requirements of the MS degree in Animal Behavior, the Washington University, St. Louis, MO.

2. A study concerning the problem of the under-aggressive dog was completed in collaboration with the USA Land Warfare Laboratory. Under-aggressiveness is one of the primary rejection factors in the procurement and training of dogs for military purposes. The efficacy of food reinforcement in correction of this trait was tested. This was basically accomplished by stealing the hungry dog's food by a human "aggressor." The least sign of resentment shown by the dog was abetted and praised by his handler. All eight of the dogs so handled eventually responded in varying degrees by barking or even biting the protected arm of the "aggressor." Only one dog responded with sufficient elan to be subsequently trained as a patrol dog. The remaining seven lacked the basic dependable courage required of a military working dog. The procedure has been adopted as a remedial measure by this Division.

3. Panosteitis - This Division is continuing to cooperate with the Veterinary Division, Letterman Army Institute of Research (LAIR) in their study to define the biological parameters, radiographic findings, and etiology of eosinophilic panosteitis in dogs. During the past fiscal year this organization shipped seven study dogs to LAIR for use in this program. In addition, serum samples from dogs which could not be released were collected and sent to LAIR for evaluation. Additional study dogs are

obtained by LAIR by breeding the dogs previously provided by this Division. While Panosteitis is not a major concern, its sporadic occurrence within our colony stirs continued interest and support of this study.

4. A program was initiated with the Veterinary Division of Walter Reed Army Institute of Research (WRAIR) to evaluate reoccurring episodes of puppy diarrheas. Various parameters were selected for evaluation including fecal bacteria and viruses. Wet smears and McMaster's determinations were employed to identify and estimate the numbers of ascarids, coccidia and giardia present in stool specimens. In addition, absorption tests and intestinal biopsies are under evaluation.

5. A statistical reevaluation of our current puppy procedures has been initiated to determine the value of each procedure as an indicator of the puppy's potential as a future military working dog. Several tests, such as response to "come," "sit," and "stay," are better accomplished by the meeker puppies while "rag play" and "fetch" appeal to the more dominant type puppy. The new experience test and the maze test are also undergoing critical review.

C. Veterinary Medicine

Provision of an effective preventive medicine program is a basic requirement. The canine vaccination regimen is an important facet of the overall effort. Starting with the puppy's fourteenth day of life, measles vaccine is administered to provide an early cross-immunity to canine distemper. Distemper and hepatitis vaccine is administered at eight weeks of age. A combination distemper, hepatitis and leptospirosis vaccine is administered at eleven and fourteen weeks of age, with boosters annually thereafter. The rabies vaccination program has remained essentially the same with one exception. As phenolized rabies bacterin has become more difficult to obtain, and in view of recent recommendations, the dogs are immunized at thirteen weeks of age, six months of age, one year of age and annually thereafter with a modified live virus rabies vaccine. Attesting to the efficacy of this immunization program is the fact that this kennel has experienced none of these diseases during the past five years.

The reduction of the incidence of hip dysplasia continues to be an integral part of the objectives of this Division. Dogs are routinely radiographed at five, eight and eleven months of age to detect this condition. Indicative of progress, but not of rate, is the fact that during the past fiscal year 337 dogs were so radiographed; of these, 43 were dysplastic. By comparison, in FY73, 288 were radiographed and 63 were dysplastic.

The Orthopedic Foundation of America (OFA) has recently changed the age at which a dog is certifiable from one year to two years. Because of this, a growing number of our dogs designated as breeders will not be OFA certified until they reach this two-year age requirement. They will, however, continue to meet the original standard.

Surgical procedures during the past fiscal year included twenty ovariohysterectomies, seven cosmetic surgeries, one cesarean section, one correction of a persistent right aortic arch and a number of routine minor procedures.

The ability of the Division to care for the neonatal puppies was enhanced recently by the acquisition through WRAIR surplus property of an Isolette. This unit is capable of more precise control of the temperature, humidity and, if necessary, oxygen content of the new-born or sick puppy's incubator environment.

A rigorous program of detection, treatment and elimination of parasites continued to be maintained. Only two external parasites were of any consequence. One of these, demodectic mange, is occasionally encountered and responds well to treatment. The other is the American Dog Tick which is controlled through spraying of wooded exercise areas and by periodically dipping the dogs in a 0.025% Lindane solution. Intestinal parasites are detected by programed fecal examinations. The primary parasites are ascarides, coccidia and giardia. Occasionally the presence of strongyloids is noted. The presence of canine heartworm, Dirofilaria immitus, in the breeding stock is routinely checked. Periodic titer determinations for the presence of Brucella canis in the breeding stock are also made.

D. Visits

1. One hundred and fifty-five visitors toured this installation during FY74. These included representatives of the Ministry of Defense, United Kingdom; the German Army; the Philippine Constabulary; various city canine corps police officials; The Seeing Eye, Inc.; the German Shepherd Dog Club of America; and others.

2. Consultant visits were made to this Division by Dr. W.H. Riser (hip dysplasia); Dr. D. F. Patterson and Dr. R.L. Willham (genetics); and Mr. E.H. Hart (breeding and blood lines).

E. Equipment

An infant incubator was received.

DISCUSSION.

The number of willing, aggressively self-confident puppies born during the past year has continued to increase markedly. Eighteen of the 28 litters on which puppy evaluation was completed were considered superior in this respect. The comparable figure last year was 14 of 35 litters.

The improvement in temperament, trainability and absence of dysplasia is primarily attributable to the selective breeding program. In obtaining these improvements the co-efficient of inbreeding has increased to 4.68%. As might be expected, a small increase in the number of genetic anomalies was experienced. Of primary importance were 21 cases of inguinal hernia (14 by same sire), 10 cases of embryonal cardiovascular persistences, and 4 cases of monorchidism. None of these puppies, including their littermates, are used as breeders. Depending upon the results of the pedigree review, one parent may be continued as a breeder. Outbreeding is not a consideration.

Implimentation of the canine remount service is expected to begin this coming spring with the placement of a few of our older breeding dogs with reputable civilian breeders of the German Shepherd dog.

PRESENTATIONS.

Presentations concerning this Division's activities were made by the principal investigator to the organizations listed below:

1. Weimaraner Club of Greater Washington, Washington, DC
   8 November 1973.

2. Fifth Annual Council of Army Veterinarians, Alexandria, VA
   21 May 1974.

3. Army Reserve Mobilization Detachment 1615, APG-EA, MD
   10 June 1974.

CONCLUSION.

Man's ability to successfully breed for a desired result is well known. The development of a truly superior military working dog is completely feasible and is progressing well.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6444	74 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
73 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	3A162110A830	00	056
b. CONTRIBUTING				
c. CONTRIBUTING	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 74	7	61
FISCAL YEAR CURRENT 75	6	180

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

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

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Huxsoll, D. L. LTC
TELEPHONE: 202-576-5194
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: T. J. Keefe, LTC
NAME: L. N. Binn, Ph.D.
NAME: E. H. Stephenson, MAJ DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security classification code) (U) Military Dogs; (U) Tropical Canine Pancytopenia; (U)Ehrlichia canis; (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 tropical canine pancytopenia (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) 73 07 - 74 06 The peripheral hematologic response of German Shepherds to concurrent infection with E. canis and B. canis showed that dogs infected with B. canis alone experience an Ehrlichia-like pancytopenia of short duration. Dogs chronically infected with Ehrlichia and superinfected with Babesia experience only a Babesia-like hematologic response. E. canis will propagate to higher titer in bovine fetal spleen cells and canine spleen cells than in previously utilized macrophage culture, thus enabling comparative study of the Chlamydiacea and Rickettsiaceae. Parainfluenze SV-5 was recovered from and serum antibody rises noted during an outbreak of respiratory disease among military dogs at Lackland and Kadena AFB. A new canine virus associated with serum antibody conversions was isolated during the Lackland epizootic. Initial studies on a diarrhea syndrome affecting military dogs have disclosed sprue-like lesions in the small intestine, apparently unassociated with viral, bacterial, chlamydial, or coccidial agents. Dogs experimentally infected with R. rickettsii were rickettsemic for 14 days, developed disease similar to that described in man, and may provide a useful disease model. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 73 - 30 June 74.

* 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 3A162110A830 MILITARY DOG IMPROVEMENT

Task 00 Military Dog Improvement

Work Unit 056 Diseases of military animals

Investigators:
Principal: LTC David L. Huxsoll, VC
Associate: Leonard N. Binn, Ph.D.; COL Earl W. Grogan, VC; LTC Thomas J. Keefe, VC; LTC Paul K. Hildebrandt, VC; MAJ Edward H. Stephenson, VC; MAJ Michael G. Groves, VC; MAJ John L. Brown, VC; MAJ Charles Montgomery, VC; CPT Kevin P. Keenan, VC; CPT George E. Lewis, Jr., VC; CPT Ralph C. Giles, VC; Helen J. Jervis, Dr. Nat. Sc.; Irene E. Hemelt, AB; Ruth L. Marchwicki, BS; Robert E. Sims, BS; Ernest S. Windham, MS; Albert R. Warner, Jr.; Raymond E. Hayhoe; CPT Melvin Hamlin, VC; COL Merida W. Castleberry, VC; SFC Lynn E. Youmans; SP5 Alan Kleiman; SP5 Arshad Husain; SP5 Delores White; SP4 Denise Peak; SP4 Bruce Dunn; SP4 Merle Peterson; SP4 Dennis Bornstetter; PFC Leonard Beller; PFC Roy Barrier; PFC James McGough

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 tropical canine pancytopenia (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.

During the reporting period, research activities have included: (1) comparative studies between Ehrlichia, Rickettsiae, and Chlamydiae; (2) the peripheral hematologic response of German Shepherd dogs concurrently infected with Ehrlichia canis and Babesia canis; (3) studies on the pathogenesis of Rickettsia rickettsii infection in the dog; (4) respiratory disease studies in military dogs; (5) studies on the pathogenesis of a coronavirus gastroenteritis in neonatal dogs; (6) studies on a diarrhea syndrome in military dogs; and (7) the comparison of MHG virus with mouse encephalomyelitis viruses. Some of the investigations reported here have been done jointly with Dr. Miodrag Ristic and other investigators at the University of Illinois.

1. Comparative studies between Ehrlichiae, Rickettsiae, and Chlamydiae.

Ehrlichiae have been tentatively placed in the family Rickettsiaceae. The primary bases for this classification are the arthropod transmission and the intracytoplasmic replication of the agents. Ultrastructural morphology of the organisms suggested a taxonomic position intermediate between the families Rickettsiaceae and Chlamydiaceae (1,5,8). Additional characteristics required to elucidate the relationship of Ehrlichiae with other microorganisms and to clarify the taxonomic location of the organisms.

Until the development of the monocyte cell culture system (2), Ehrlichia studies were limited to in vivo studies. Use of the monocyte culture technique afforded a means for development of the indirect immunofluorescence test, to study the ultrastructural morphology of Ehrlichiae and to compare the organisms in lung tissue of the dog with organisms propagated in vitro, and to provide inocula of increased infectivity for pathogenetic studies. Further elucidation of the replication cycle, ultrastructure, taxonomy, and composition of Ehrlichiae, however, requires larger quantities of the agents than can be obtained by propagation in monocyte cultures. Studies have been initiated to determine a cell line or cell type, other than the monocyte, in which Ehrlichiae will replicate readily and retain all properties of the agents propagated in vivo. Several cell lines and cell types have been employed. E. canis did not replicate in LLCMK2 cells, L929 mouse fibroblasts, A72 cells, or canine kidney cells. It currently appears that bovine fetal spleen cells and canine spleen cells are susceptible to infection with Ehrlichiae and will provide the desired propagation system. Studies on adapting and establishing E. canis in these cell types are continuing.

After a means of propagation of E. canis in large quantities has been obtained, Ehrlichiae will be compared with Rickettsiae and Chlamydiae. Agents to be used include known rickettsial strains, such as Rickettsia rickettsii, Neorickettsia helminthoeca and the Elokomin fluke fever agent, and Chlamydia psittaci strains originally isolated from diverse disease syndromes. Several techniques are to be used to compare the three groups of agents, including protein profile determination by polyacrylamide gel electrophoresis, DNA homology, and antigenic reactivity and relatedness.

Protein profiles of Chlamydiae have been used to group the agents isolated from different disease syndromes. Chlamydia psittaci strains isolated from cases of polyarthritis or conjunctivitis are closely related, while those strains isolated from cases of

enteritis and abortions form a second group (7). These findings suggested that composition may be a determinant of the disease expression induced by a given agent. A specific polypeptide profile may designate the primary cell receptor sites upon which an agent may initially be adsorbed, thereby resulting in a specific disease syndrome. Secondary adsorption to other cell types would give rise to the systemic chlamydial infections encountered on occasion.

The protein profile of an organism is an indirect depiction of the genome of the agent; therefore the protein profiles of different organisms provide an excellent means of comparing between closely related agents.

Antigenic reactivity provides another means of demonstrating relatedness between microorganisms. Based on the results of immunodiffusion tests, chlamydial strains have been separated into groups, which are identical to those determined by protein profile determinations (6). Comparison of the antigenic relatedness between Ehrlichiae and Chlamydiae is of interest due to a recent observation. Two horses had been inoculated with Chlamydiae; one received Chlamydia isolated from a case of bovine pneumonia and the other was inoculated with Chlamydia isolated from a turkey. Preinoculation and postinoculation serum samples were tested for Ehrlichia antibodies using the indirect fluorescent antibody test. Preinoculation sera was negative, but postinoculation sera were positive for E. canis antibodies. The cross reaction between Ehrlichia and Chlamydia was not expected, and resolution must await additional studies.

It is anticipated that the results of the above studies will provide the information necessary to place Ehrlichiae in the appropriate taxonomic group. Also, it will be possible to compare the equine and canine ehrlichial isolates with each other and with known members of Rickettsiae and Chlamydiae. Rickettsia, such as R. rickettsii, and Chlamydia (3,4) are capable of inducing disease in both man and animals; therefore, indications of the zoonotic potential of Ehrlichiae will be derived from these investigations.

2. Peripheral hematologic response of German Shepherd dogs concurrently infected with Ehrlichia canis and Babesia canis.

Natural infections of Ehrlichia canis, the etiologic agent of Tropical Canine Pancytopenia (TCP) are often complicated by concurrent infections with other hematropic agents. Concurrent infections of Ehrlichia canis and Babesia canis have been most often reported. Babesia infections have marked chronic as well as acute

stages of TCP in the dog, and the reverse situation has also been observed. During early studies of TCP in this laboratory, four of the first 11 isolants of E. canis, recovered from German Shepherd dogs infected in Southeast Asia, were contaminated with B. canis.

The acute stage of B. canis infection generally lasts for only a few days and is characterized by anemia, hematuria, high fever, ataxia,and detec' ble parasitemia. Dogs infected with B. canis usually recover from the acute disease but remain carriers which are clinically indistinguishable from Babesia-free dogs.

Concurrent infections of E. canis and B. canis are not unexpected. Both agents are distributed worldwide. Also distributed throughout the world is a vector for both agents, the brown dog tick, Rhipicephalus sanguineus. Experimental infection with E. canis have been studied extensively, but little attention has been given to concurrent infections with E. canis and B. canis. Dogs surviving infection with either agent usually become carriers, thus providing ample opportunity for superimposed infection with either agent.

These studies were designed to examine selected physical, hematologic (including bone marrow aspirates), serum enzymes, and immunologic (humoral and cellular) responses of German Shepherds infected with both E. canis and B. canis, and to compare these responses to those elicited by each agent singly. Current progress reported herein includes physical and peripheral hematologic changes observed during the first 45 days of infection.

The isolant of E. canis used was recovered from a German Shepherd which was naturally infected in Southeast Asia, and was maintained by passage in laboratory beagles. Passage in splenectomized dogs showed this agent to be free of contaminating Babesia spp. and Hemobartonella spp. The isolant of B. canis used had a similar history, and was also free of contaminating E. canis or Hemobartonella spp.

Included in the study were 13 adult German Shepherds, separated into groups I, II, and III. The five dogs in group I had been infected with E. canis one to three years previously. Four of these dogs had high serum antibody titers to E. canis, and their blood was capable of infecting normal dogs with E. canis by inoculation. Blood from the fifth dog in group I was non-infectious, and its serum showed only a low antibody titer to E. canis. The five dogs in group II, and the three dogs in group III had never been infected with E. canis or B. canis.

All dogs on this study were observed daily. Rectal temperatures, buffy coat smears, erythrocyte sedimentation rates (ESR), packed cell volume (PCV), red blood cell (RBC), while blood cell (WBC), and platelet counts were conducted every other day starting the week before and continuing for 4 weeks after experimental infection. Thereafter, these examinations were conducted thrice weekly on all dogs. All laboratory tests were conducted within 2 hours after specimens were collected. RBC and WBC counts were obtained with an electronic cell counter, and platelet counts were obtained with an electronic platelet counter. A microhematocrit centrifuge and Wintrobe tube were used for all PCV and ESR values, respectively. Hemoglobin (Hb) values were obtained with the cyanomethemoglobin method. With buffy coat smears, a minimum of 2,000 monocytes were examined to detect morulae of E. canis.

Each of the 14 study dogs were inoculated with 5 ml of fresh whole blood collected from a dog acutely infected with B. canis. In addition, the dogs in group III received 5 ml of fresh whole blood collected from a dog acutely infected with E. canis.

Erythrocytes containing B. canis were observed in all dogs on the second day post-inoculation (2 PI). By 7 PI, the mean PCV for dogs in groups I, II, and III (Table 1) had declined from 46% to 28%, 46% to 33%, and 47% to 31%, respectively. By 45 PI, the mean PCV for dogs in groups I and II approximately normalcy (43%). However, the mean PCV for dogs in group III continued to drop until 30 PI (26%), and by 45 PI had risen only slightly to 31% (Table 1).

By 13 PI, the mean RBC (millions/mm^3) for dogs in groups I, II, and III had declined from 6.5 to 4.5, 6.6 to 4.6, and 6.2 to 2.4, respectively. By 45 PI, the mean RBC for dogs in groups I and II approximated normalcy (6.4), but remained low (4.7) for dogs in group III (Table 1).

By 16 PI, the mean WBC in thousands/mm^3 for dogs in groups I, II, and III had declined from 10.0 to 6.6, 10.9 to 9.7, and 12.4 to 5.6, respectively. By 45 PI, the mean WBC for dogs in all groups approximated normalcy (Table 1).

By 18 PI, the mean platelet count in thousands/mm^3 for dogs in groups I, II, and III declined from 118 to 22, 275 to 33, and 237 to 18, respectively. The initial low platelet count for dogs in group I (118) reflects the chronic carrier state for E. canis. By 45 PI, the mean platelet count for dogs in groups I and II had risen appreciably (121 and 127, respectively), but remained low (45) for dogs in group III (Table 1).

The mean ESR (mm/hr) for all dogs was normal and uniformly low (1 to 2) before inoculation with B. canis. Between 6 and 12 PI, the mean ESR for dogs in groups I, II, and III had risen to 57, 18, and 22, respectively. From 12 to 45 PI, mean ESR value for dogs in groups I and III fluctuated weekly from lows of 7 to 12 to highs of 36 to 44 (Table 1).

Morulae of E. canis, exhibited by buffy coat examinations, were never observed from dogs of group II (never infected with E. canis) or dogs from group I (chronically infected with E. canis).

Morulae of E. canis were first observed within monocytes of dogs from group III (concurrently infected with E. canis and B. canis) between 12 and 16 PI, and were observed in all of these dogs through 30 PI. By 3 PI, and continuing through 30 PI, RBC parasitized with B. canis were observed from all dogs. Following inoculation with B. canis, the only death (2 PI) occurred in the only dog whose parasitemia (8%) considerably exceeded that of the other dogs (2%).

Rectal temperatures (^{o}F) for all dogs prior to experimental inoculation averaged 102.5. Between 3 and 6 PI, temperature elevations to 105 were first noticed. Temperatures then gradually declined. However, dogs spiked fevers above 104 on 12, 22, and 24 PI.

The peripheral hematologic responses observed in these three groups of dogs infected with E. canis and/or B. canis permit the following conclusions: (1) Dogs can be simultaneously infected with E. canis and B. canis and show a hematologic response equal to that evoked by infection with E. canis alone, and more severe than that evoked in dogs infected with B. canis alone. (2) The thrombocytopenia, leukopenia, and anemia initially evoked by B. canis infection is qualitatively similar to that produced by E. canis infection, but return to normalcy occurs more rapidly. (3) Dogs chronically infected with E. canis and then infected with B. canis manifest a hematologic response similar to that evoked by B. canis alone. In addition, superimposed infection with B. canis failed to stimulate the appearance of morulae to E. canis. On the basis of peripheral hematologic response, chronic Ehrlichiosis is not exacerbated by superimposed Babesia infection.

Studies are continuing on the humoral and cellular immunologic responses as well as bone marrow and serum enzyme changes provoked in these three groups of dogs.

3. Studies on the pathogenesis of Rickettsia rickettsii infection in the dog. A model for Rocky Mountain Spotted Fever.

Rocky Mountain Spotted Fever (RMSF) accounts for more than 90% of the reported cases of rickettsial disease in the United States and is the only rickettsial disease with a serious mortality rate. Case histories of human infections that indicated a tick bite following exposure to tick-infested dogs suggested that the dog may be involved as a carrier of the disease. This study was undertaken to determine: (1) the infectivity of Rickettsia rickettsii for dogs; (2) the levels and duration of rickettsemia; (3) the immunogenicity of R. rickettsii in dogs; (4) the severity and character of the disease by clinical, clinical pathological, and pathological studies; and to evaluate the primary monocyte culture technique as a means of isolation of R. rickettsii.

Thirty-four adult Beagle dogs were used. The dogs were of both sexes and 10 to 24 months of age. All dogs were vaccinated for canine distemper, infectious canine hepatitis, leptospirosis, and rabies. At the time of experimental inoculation the dogs were serologically negative for R. rickettsii antibodies, as determined by the indirect fluorescent antibody test (9). Adult male Hartley guinea pigs were used for isolation procedures. Yolk sac suspensions of the Sheila Smith strain of R. rickettsii were utilized for inoculum.

The dogs were inoculated intravenously (I.V.) with yolk sac suspensions of R. rickettsii containing the following concentrations of organisms: one dog was given 1×10^2 $GPID_{50}$; one dog was given 1×10^3 $GPID_{50}$; four dogs were given 1×10^4 $GPID_{50}$; eight dogs were given 1×10^5 $GPID_{50}$; four dogs were given 1×10^6 $GPID_{50}$; and eight dogs were given $1 \times 10^{7.25}$ GPID. Two dogs were inoculated with doses of 1×10^4 $GPID_{50}$ and $1 \times 10^{7.25}$ $GPID_{50}$ of R. rickettsii yolk sac suspension that had been formalin inactivated. Six dogs were used as controls and were inoculated I.V. with 1 ml of sterile, brain-heart infusion (BHI) broth, the media used for dilution of rickettsial preparations.

All animals were examined daily for two weeks. Daily or every other day, venous blood was collected in EDTA vacutainers (Vacutainer, Becton-Dickinson, Rutherford, NJ) for hematological and biochemical evaluation. While blood cell (WBC) and red blood cell (RBC) counts were performed on an electronic cell counter (Model B, Coulter Electronics, Inc., Hialeah, Florida). Platelet counts were also performed electronically (MK-4 Platelet Counter, General Sciences Corp., Bridgeport, CT). Packed cell volumes were determined by standard microhematocrit methods. One hour erythrocyte sedimentation rates (ESR) were determined in Wintrobe tubes. Hemoglobin was determined by the cyanmethemoglobin method. Routine

differential counts were done on blood smears stained by a modified Wright's technique. On selected groups of dogs, serum and citrated plasma was collected and the following procedures were run using standard methods: sodium, potassium, chlorides, carbon dioxide combining power, inorganic phosphorous, calcium, magnesium, alkaline phosphatase, serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total cholesterol, glucose, BUN, creatinine, prothrombin time, partial thromboplastin time, and fibrinogen.

Sera were collected at weekly and monthly intervals from dogs surviving the acute disease. The sera were frozen at -70°C until tested for the presence of antibodies to R. rickettsii using the indirect fluorescent antibody test (9).

Isolation of R. rickettsii from inoculated dogs was done by inoculating guinea pigs. Whole blood was drawn from the dogs every second or third day and heparinized (20 units/ml). Serial ten-fold dilutions were made in cold BHI broth. Three guinea pigs each were inoculated with whole blood and the next two serial ten-fold dilutions. Each guinea pig was inoculated intraperitoneally with 1.0 ml of the appropriate blood dilution. Rectal temperatures were recorded for 10 days and the guinea pigs were observed for typical signs of RMSF (10). At 28 days post inoculation (PI) the guinea pigs were bled and sera were tested for antibodies by the indirect fluorescent antibody test.

Monocyte cultures were prepared from 10 ml of heparinized (20 units/ml) blood drawn every third day from the dogs. The cultures were prepared and examined according to methods previously described (11-13).

Complete necropsies were done on all dogs that died or were killed at various stages of disease. Tissue samples were taken and preserved for histopathological examination with the light and electron microscopes.

Dogs inoculated with viable rickettsiae developed a clinical syndrome that varied in severity with the dose of rickettsia given. Control dogs and dogs inoculated with formalin-inactivated rickettsiae demonstrated none of these signs.

All dogs were febrile, anorectic, and lethargic two to three days PI. Fevers continued for 12 to 14 days PI, and rose as high as 105°. Hypothermia occurred in acutely fatal cases. Other general signs were lymph node enlargement, weight loss, and dehydration.

By the third to fourth day of fever, cutaneous signs were evident. These included generalized hyperemia of oral, ocular, and genital mucus membranes and non-pigmented skin, especially on the abdomen and ears. Petechiae developing into ecchymoses became evident shortly afterward, and were particularly pronounced in the ocular, oral, and genital mucus membranes. Edema of the lips was common at this time, and in males edema was seen in the sheath and scrotum.

As the fevers peaked the pulse was accelerated, but full and regular. Several acute deaths occurred at the stage when hemorrhages were most pronounced. Presumably the deaths were due to circulatory failure, resulting in hypoxemia, shock, cyanosis, delirium, and death. Associated with these acute deaths, epistaxis and a sudden development of ecchymoses, especially in the oral mucus membranes, were observed.

Most of the dogs developed rhinitis and a dry non-productive cough. Several animals developed epistaxis as part of the generalized signs of bleeding. All had increased respirations. Several developed a productive cough, with purulent nasal exudate. Dry rales were commonly detected. Moist airway sounds were less common, although several dogs did develop acute bronchopneumonias.

All animals became anorexic and later constipated. Melena or rectal bleeding was not a frequent sign but was observed.

A scrotal reaction, not unlike that seen in male guinea pigs, was observed in several male dogs with more severe signs. Edema and hyperemia of the scrotum was first seen, followed by a macular rash which became purpuric with necrosis and ulcers on the scrotal skin. Palpation demonstrated painful, swollen epididyides, although the testicular tissue seemed normal. Also in these males, edema of the prepuce was seen associated with petechiae and ecchymoses of the glands, penis, and other genital mucus membranes. Oliguria was common in the more severely affected dogs, with azotemia and anuria in a few acutely fatal cases.

At the onset of fever, all the dogs developed scleral infection and conjunctivitis. Chemosis and a more severe conjunctivitis with mucopurulent to purulent discharge followed. Petechial hemorrhages were then commonly noted in the conjunctivae and retinae. Several dogs developed acute anterior uveitis with deep circumcorneal and cilliary congestion, contracted unresponsive pupils, and cloudy anterior chambers and corneal opacity.

Nervous disfunction was usually generalized in its manifestation, with coma and convulsive seizures being very grave signs and always associated with cyanosis and death. One dog developed a head tilt and nystagmus to the right (slow component toward head tilt). A limited number of cerebrospinal taps were clear with cell counts in the normal range.

Anemia, as manifested by decreases in RBC counts, hematocrit, and hemoglobin concentrations, was evident in the infected dogs as compared to controls.

At the onset of fever, some animals developed a mild leukopenia, but this was quickly followed by a leukocytosis which increased in proportion to the severity of the individual case. Neutrophilia, eosinopenia, lymphopenia, and monocytosis was the most common pattern seen in the differential count. By the third or fourth day PI a left shift and toxic granulation of the neutrophiles, which is a rare finding in the dog, was evident in many cases. Toxic granulation was seen as vacuoles along the periphery of the cells, toxic granules, basophilia of the cytoplasm and condensation of nuclear chromatin. This finding correlated with rickettsemia in the respective cases.

A marked elevation of the erythrocyte sedimentation rate (ESR) was seen during the febrile period and was associated with an increase in plasma fibrinogen levels.

Thrombocytopenia was a consistent finding and this data correlated with evidence of hemorrhage. Prothrombin times and partial thromboplastin times were usually not prolonged as compared to controls.

A group of 10 animals were studied via the above mentioned biochemistry examinations. After inoculation with 1×10^5 $GPID_{50}$ organisms, the dogs developed a spectrum of clinical disease that ranged from acute death in 6 to 8 days PI to a moderate response with subsequent recovery. The magnitude of the chemical changes depended on the severity of the disease. Taken as a group, the significant changes were elevations of serum alkaline phosphatase and cholesterol values. They also developed decreases in sodium, chloride, and carbon dioxide levels.

Rickettsemias were determined by inoculation of adult male guinea pigs with heparinized (20 unit/ml) whole blood and 10^{-1} dilutions and 10^{-2} dilutions made in brain heart infusion broth. Controls were likewise inoculated with whole blood from uninfected dogs. The inoculated guinea pigs were observed for typical signs of RMSF and rectal temperatures were taken for 10 days. They were bled at 28 days PI and tested by the indirect fluorescent antibody test. Results indicated that the dogs in this series were rickettsemic for a period of 10 to 14 days PI.

Primary monocyte cultures were prepared from the same dogs to evaluate the cell culture method as a means of detecting rickettsemia. Cultures were examined on days 3, 5, 7 and 10 of culture. Initial studies indicated a good correlation between guinea pig positives and monocyte culture positives; however, subsequent studies have been less encouraging.

Serial serum samples obtained from 12 animals were tested for antibodies to R. rickettsii by the indirect fluorescent antibody test. Titers of 1:40 to 1:320 were first detected 14 to 30 days PI. These titers reached peak values of 1:160 to 1:2560 at 23 to 46 days PI. By 160 days PI no detectable antibody could be found. An interesting exception to these data was one dog that developed a titer of 1:40 at 268 days PI, and 1:160 at 300 days PI. Dogs that had been inoculated with formalin inactivated R. rickettsia failed to develop detectable antibody titers at any time PI.

Eight of the above dogs were rechallenged with 1×10^7 $GPID_{50}$ of R. rickettsii. Although serological studies have not been completed, the dogs that had recovered from prior infection with R. rickettsii have shown little or no clinical or hematological evidence of disease. Several of these dogs were 360 days PI when rechallenged. This is in contrast to an acutely fatal disease seen in the four susceptible dogs similarly inoculated. It appears that dogs that survive RMSF infection are solidly immune for at least a year.

Results of necropsy and histopathologic examinations of all dogs dying or killed in extremis are summarized below. Electron microscopic studies are currently being done.

Necropsy lesions consisted of edema of the ears and muzzle, ulcerative glossitis and dermatitis of the scrotum. Petechiation was seen on the conjunctiva, oral mucus membranes, genital mucus membranes, skin of the flanks, and posterior abdominal wall. There was petechiation of the stomach wall, a hemorrhagic colitis, and accentuation of Peyer's patches. There was a generalized hemorrhagic lymphadenopathy and the spleen was enlarged and dark in color. Microscopically, the prominent lesion was a necrotizing vasculitis of the small veins and arterioles with perivascular accumulations of polymorphonuclear and lymphoreticular cells. This vasculitis was seen in vessels of the skin, epididymus, testicle, gastrointestinal tract, lung, liver, and was particularly extensive in the meninges, retina, and skeletal muscle. Small focal glial nodules were present in the cerebral hemispheres. Focal disseminated necrosis was also present in the liver and, to a lesser extent, in the myocardium. Moderate glomerulonephritis was present in the kidneys.

The observations and data obtained in this study indicate that the dog may be of more than epidemiological interest in the study of Rocky Mountain Spotted Fever. The canine system may represent a model for the study of human disease.

Dogs inoculated with 1×10^2 to 1×10^7 $GPID_{50}$ of the Sheila Smith strain of R. rickettsii developed rickettsemias of 10 to 14 days duration. These animals have a clinical syndrome which appeared to be dose related, and is compatible with the human disease on a clinical, hematological, biochemical and pathological basis (10, 15-19). Serologically, the dogs responded typically to this infection, and seemed to acquire an immunity to reinfection.

The monocyte culture technique has been successfully used in the guinea pig and monkey system (14) and should be explored further in the canine system. The obvious advantage to the monocyte culture system versus guinea pig inoculation is that a more rapid (as early as 3 days of culture) assessment of rickettsemia can be made.

In reference to the serological findings, one interesting observation was the reoccurrence of a detectable titer in one dog that was followed for a year. While this may simply represent a generalized immune stimulation from other sources, the suggestion that this dog may have been chronically infected as in other Rickettsial diseases (20) is interesting. It has been suggested that the dog is a reservoir for Sao Paulo typhus (21) and Boutonneuse Fever (21), both rickettsia of the spotted fever group.

The clinical pathology findings were comparable with the human disease. Anemia, leukocytosis, toxic left shift, elevated ESR, hyperfibrinogenemia, and thrombocytopenia are all findings in the human clinical literature (22, 23). The findings of elevations of serum alkaline phosphatase and cholesterol can be explained by the generalized damage to endothelial cells. Hyperlipemia, with associated hypercholesterolemia, has been experimentally produced in a number of generalized vascular phenomena (24).

The fundamental lesion seen in these dogs was a wide-spread vasculitis involving capillaries and small arteries, and is compatible with the lesions seen in man. Two forms of the vascular lesions were observed, depending on the acuteness of the disease. The lesion seen in dogs dying acutely represented primarily a non-specific endothelial damage, which was probably due to a toxic activity of the rickettsiae. The later stage lesion was more of a necrotizing vasculitis that resembled the arteritis seen in hypersensitivity processes. This concept has been suggested by others (25), and experimental evidence for an Arthus phenomenon-immediate

type of hypersensitivity reaction has been demonstrated in experimental RMSF in the guinea pig (26). Thus, the dog may provide an interesting model for basic and applied studies of RMSF and other rickettsial diseases.

4. Respiratory disease in military dogs.

From 1966 to 1968 extensive epizootics of respiratory disease occurred at military dog procurement and training centers. These epizootics seriously disrupted the training and deployment of dogs. Parainfluenza SV5 virus was recovered from affected dogs and the virus was found to be highly communicable (27-29). In 1971 and 1972 the incidence of respiratory disease and SV5 infections declined to a very low rate. However, on the basis of serological test findings 88% of newly recruited dogs were susceptible to SV5 infection.

In mid-June 1973 an extensive epizootic of respiratory disease began at the Lackland Air Force Base (LAFB) military dog procurement and training centers. The present report summarizes laboratory studies on this epizootic and subsequent cases of disease at LAFB. In addition, studies were conducted on an epizootic of respiratory disease in military dogs at Kadena Air Force Base, Okinawa.

At LAFB the first case of respiratory disease was recorded on 14 June 1973. The next day four additional dogs were affected and a sixth dog on the following day. On the fifth to ninth days 6 to 10 new cases were seen each day. Additional cases of disease occurred continuously through 6 August when the 103rd dog developed signs of disease. The most frequent signs of disease were cough, nasal discharge, loose stools and elevated rectal temperatures. The duration of illness was approximately two weeks. Affected dogs were given nitrofurantcin alone or in combination with neomycin or chloromycin. A second series of cases involving 15 dogs occurred between 25 September and 6 November. The latter outbreak may have originated from a newly procured dog which arrived with signs of disease.

An epizootic of respiratory disease in military dogs also occurred at Kadena Air Force Base, Okinawa. Twenty-two cases of respiratory disease occurred in a population of 51 dogs between the 13th and 22nd of June. On the second of June, 25 new dogs had arrived from LAFB. Two new and two resident dogs were affected on the first day signs were observed.

Bacterial cultures of nose, throat and rectal specimens did not reveal any potential pathogenic organisms. These studies were conducted at WRAIR and Brooks Air Force Base, Texas.

The virus studies were facilitated by a prospective serum collection program in which newly procured dogs were bled on arrival and again before assignment. The latter specimens were collected 1 to 6 months later. These specimens were examined for SV5 antibody. After onset of the epizootic at LAFB, virus isolation specimens were collected from the nose, throat and rectum of selected affected and control dogs. The specimens from the sick dogs were usually collected on the first day of disease. Acute and convalescent blood specimens were collected from each dog at LAFB. However, only convalescent serum specimens were available from Kadena Air Force Base and the serum specimens obtained in the prospective study described above were used in lieu of the acute serum specimens. The procedures used for virus isolation and serological tests are fully described in previous reports (27,30,31).

Three viruses were recovered from 29 affected dogs (Table 2). Two parainfluenza SV5 viruses were isolated from nasal and throat specimens in both primary dog kidney (PDK) and Walter Reed Canine Cell (WRCC) line. Each isolate was neutralized by specific, rabbit anti-SV5 serum and both dogs developed high antibody titers for the reference canine SV5 and to the Tramp LAFB 1973 SV5 isolate. The remaining virus, W191R, was recovered from fecal specimens of a third dog in the WRCC line. On passage, cytopathic effects were evident within 24 hours and often rapidly spread to involve the entire cell monolayer. A similar cytopathic agent was isolated from a fecal specimen obtained the next day. The dog developed significant neutralizing antibody to this isolate.

The properties of the Tramp SV5 isolate were similar to other strains of this virus. Chloroform treatment completely inactivated the virus and the virus was filtrable through 220 nm but not through 100 nm membrane filters. Guinea pig red blood cells were hemagglutinated by virus preparations and infected cell cultures hemadsorbed guinea pig red blood cells. Infected cell cultures contained multinucleated cells with cytoplasmic eosinophilic inclusions.

Following recovery of the W191R, the agent was purified by three successive terminal dilutions and virus pools prepared. Except for the WRCC line, attempts to produce transmissible cytopathic effects in primary and continuous cells were unsuccessful (Table 3). The W191R virus was not lethal for suckling or weanling mice. However, neutralizing antibody was present in 3 week post inoculation serum of inoculated mice. The level of antibody was related to the dose of virus inoculated.

Studies on the chemical and physical properties of W191R virus are summarized in Table 4. The W191R virus was resistant to chloroform treatment but was significantly inactivated at pH 3.0. The virus passed through membrane filters of 220, 100, and 50 nm. The virus was not stabilized at 50°C by molar magnesium. Treatment of infected WRCC with 5-iodo-2-deoxyuridine (IUDR) did not inhibit the growth of the virus. These findings indicated that the W191R virus contained ribonucleic acid, was naked, and less than 50 nm in diameter.

Partially purified virus was prepared by differential centrifugation for electron microscopic examination. Mr. Walter Engler of the Armed Forces Institute of Pathology collaborated in these studies. The virus preparations were negatively stained with phosphotungstic acid (PTA) and observed in the Hitachi HU 12 electron microscope at 100 KV. The virus particles were usually round to oval with projecting capsomeres. The virions were naked and averaged 30 nm in diameter with a range of 25-35 nm. The PTA stain penetrated the virions resulting in a variable number of dark areas within the virions. The presence of dark areas are somewhat similar to the vesicular exanthema virus of swine and feline rhinoviruses. The latter viruses have been reclassified from the rhinoviruses to a new group designated caliciviruses. Further studies are in progress to further define the structure of the W191R.

In previous annual reports four calici-like canine viruses similar to W191R have been described (1969-70, 1971-72). Each of these isolates is antigenically distinct in neutralization tests. Antiserum to purified W191R virus was prepared in rabbits and guinea pigs and cross neutralization tests carried out (Table 5). Both the rabbit and guinea pig antiserum to the W191R virus did not neutralize the other four agents. Similarly, antiserums to 3 of the 4 viruses did not neutralize the W191R isolate. The cross reactions obtained with the fourth virus, A358, varied from animal to animal and did not exceed 1:64 of the homologous titer (Table 5). The findings clearly indicate that W191R is antigenically distinct from the other isolates.

Serological studies were carried out to assess the importance of parainfluenza SV5 and the W191R isolate in the disease episodes at LAFB and Kadena AFB (Table 6). SV5 was highly communicable in each of the outbreaks of disease, as 75-100% of the dogs had a rise in titer. With one exception, all sick dogs had SV5 antibody in the convalescent serum specimens. Infections with W191R occurred only at LAFB in the initial epizootic where 31% of the dogs had a rise in titer. It is of interest to note that no serological evidence of SV5 or W191R infection was obtained in all nine dog handlers or veterinary personnel examined.

To detect infections with other canine viruses paired serum specimens from 37 dogs were selected for study. Approximately one-half the study group had a rise in titer to the reference canine parainfluenza virus (Table 7). It is of interest to note that these same dogs also had a rise to the LAFB 73 Tramp SV5 isolate. No serological evidence of infection with infectious canine hepatitis, Toronto A26/61 canine adenovirus, canine rhinovirus A128Thr, canine calici-like A128T and A358 virus, reovirus types I, II, and III was detected. Canine herpesvirus, the minute virus of canine and canine coronovirus 1-71 infections were comparatively infrequent as 1 or 2 dogs had increased antibody titer. Three dogs (11%) had increased antibody titers to transmissible gastroenteritis virus, one of which had a rise in titer to the 1-71 related coronavirus. Infections with the calici-like virus L198T was observed in 4 dogs (15%), one of which also had a rise to the LAFB-W191R isolate. These results indicate that other viruses were infecting dogs at LAFB but at a comparatively low rate. The role, if any, of these viruses in the disease signs observed is unknown.

Parainfluenza SV5 antibody studies were also conducted on paired serums of apparently healthy newly recruited dogs obtained in the routine bleeding prospective study. Prior to May 1973, conversions to positive SV5 antibody status were not detected. Two dogs converted during May, the first had been at LAFB for 90 days, prior to the May 15 second bleeding. The second dog, converted between 22 and 28 May. This dog was purchased during a California buying trip from which 25 dogs were sent to Okinawa. A second dog from this group also converted to positive antibody status between 21 May and 15 June 73. These findings indicate that SV5 was circulating in dogs purchased in California and could explain how SV5 infections were introduced into the Kadena AFB colony. During the first 10 days of the June-July epizootic only 3 of 11 (27%) dogs developed SV5 antibody, however 18 of 20 (90%) present more than 15 days converted. After the first epizootic SV5 infections were not detected in unexposed dogs as none of 16 dogs converted. However, once respiratory disease began again in September inapparent infections occurred in 10 of 10 (100%) dogs present more than 10 days.

The findings in this study offers further evidence for the role of SV5 in respiratory disease of the dog. The introduction of this virus into a susceptible dog population led to cases of respiratory disease and a high degree of virus communicability was observed. The role of the new virus (W191R) in the epizootic is unknown, however the serological tests indicated that this agent was not as communicable as SV5. Studies on the pathogenesis of W191R will be required to ascertain its pathogenicity for dogs. Several parainfluenza SV5 commercial vaccines are being developed and consideration should be given to the use of a safe and potent product. The

large number of susceptible dogs arriving at the reception center warrants its use of a vaccine to prevent the disruption of training and deployment of dogs. Furthermore, there is always the problem of shipping dogs which are inapparently infected and subsequent introduction of the virus to a new susceptible population.

Studies were conducted on the etiology and epizoology of respiratory disease in military dogs at Lackland Air Force Base, Texas, and Kadena Air Force Base, Okinawa in 1973. From 14 June to 6 August 1973, 103 cases of respiratory disease occurred in the reception and training centers at Lackland AFB. Fifteen additional cases occurred between 15 September and 6 November at Lackland. At Kadena AFB, 22 cases of respiratory disease occurred between 13 and 22 June 1973. Parainfluenza SV5 and an apparently new canine virus (W191R) were recovered from 2 stick dogs at Lackland. W191R virus had similar properties to the calicivirus group. Serological test findings indicated that SV5 infected 75-100% of affected dogs at Lackland AFB and at Kadena AFB. The W191R virus infected 31% of the dogs at Lackland during the first epizootic and infections were not detected at Kadena AFB or in the subsequent cases at Lackland. In addition, a small number of infections were detected with other canine viruses. Consideration of use of a parainfluenza SV5 vaccine in military dogs was discussed.

5. Studies on the pathogenesis of 1-71 Coronavirus gastroenteritis in neonatal dogs.

Introduction: Members of the coronavirus group have caused respiratory, hepatic and enteric disease in poultry, mice, rats, swine, cattle and man. This laboratory has isolated two coronaviruses, L198R and 1-71 from diseased dogs. (Annual Reports 1969-70 and 1970-71). The 1-71 virus was isolated from fecal specimens of military dogs in Germany during an outbreak of severe gastroenteritis. A study of this isolate in dogs and swine established that the 1-71 coronavirus is a distinct infectious agent that can cause gastroenteritis in neonatal dogs (Annual Report 1972-73). This report summarizes further studies on the pathogenesis of this disease.

Experimental design: The litters of four Beagle dogs, seronegative for L198R, 1-71 and TGE coronaviruses, and one control Beagle's litter comprised the study group. The bitches whelped naturally and nursed their litters. The puppies of the four litters were inoculated with 3×10^4 to 2×10^6 $TCID_{50}$ of the 1-71 isolate per os at either the fourth or twelfth day of life. Virus isolation tests were made by standard methods on daily fecal swab specimens. At selected times puppies were euthanized and necropsy examinations

were done during the course of disease and samples were obtained for virological and pathological examinations. Viral titers of selected tissues were obtained. Histopathology and histochemical studies were completed. Immunofluorescence and electron microscopic studies are presently underway.

Results: This report summarizes clinical observations and virus isolations from fecal swabs and tissues, and the morphological and histochemical findings of control and infected puppies. For illustration the results of two litters (1700 and 2SP5) are used; however, the same general pattern has been seen in other litters.

The clinical manifestations in these animals are similar to those reported previously (Annual Report 1 July 1972-73). Typical signs of gastroenteritis were observed. The neonatal pups became weak, and crying was more frequent. A moderate to severe diarrhea was observed, yellow to green in color and of such a volume that the bitch was unable to keep the pups clean. Dehydration followed, and infected litters failed to gain weight as compared to controls. No mortality occurred, and all pups that were allowed to survive made a complete recovery.

Rectal swabs were taken daily on all animals, prepared for culture by standard methods, inoculated on primary dog kidney cell cultures (PDK), and observed for typical coronavirus cytopathic effect (CPE), and the formation of giant cells. All primary cultures were passed twice.

Virus isolations from two litters are summarized in Table 8. Most of the positive samples were obtained at the onset of clinical signs. In the 1700 litter the incubation period was two days, with signs lasting for six days. In the 2SP5 litter signs occurred as early as day one, but fecal samples were not positive until day three.

Samples of duodenum, jejunum, ileum and colon were processed and titrated on PDK to determine the approximate titer (Log 10) per 0.1 gram of tissue. Stomach, liver, spleen, pancreas, kidney, bladder, lung, and heart tissues and mesenteric lymph nodes were also processed from some litters and titers obtained. The results obtained on the 1700 and 2SP5 litters are summarized in Table 9. Titers of 0 or < 1.0 were considered negative or non-specific. Due to the need for speed in obtaining histochemistry and electron microscopic samples, pancreas, kidney and bladder were contaminated with enteric bacteria; therefore viral titers in these tissues constitute a "no test" finding. These same organs from other litters not presented in Table 9, without evidence of enteric

bacteria, have shown no virus. To date the duodenum, jejunum, ileum, colon and mesenteric lymph nodes have consistently contained virus from two to nine days post infection (PI).

Bacterial cultures and identification were done on all samples prior to processing for virus isolation. E. coli, fecal Streptococcus spp. and Micrococcus spp. were the principal isolates from gut samples. No Salmonella or other possible aerobic enteric pathogens were isolated.

To establish temporal baselines on morphology and histochemistry of the neonatal dog gut, uninoculated pups were sacrificed at the same ages as the infected pups. Three levels of small intestine (duodenum, jejunum, and ileum) and colon were studied. Morphology, mucosal thickness and mucin histochemistry examinations were done on paraffin sections of tissues fixed in cold neutral formalin. Enzyme histochemistry and the distribution of fat were studied on fresh frozen sections.

In the small intestine the villi in the control neonatal pups are long and thin, three to five times as long as the crypts. There are individudal variations in the length of the villi as well as differences between the different portions of the gut. Villi tend to be longer in the jejunum than in the ileum or the duodenum. The lamina propria contains very few cellular elements on day two of life, resembling the gut of a germfree animal, but with time it becomes increasingly cellular.

The epithelium in the duodenum is normally tall and columnar with no or minimal vacuolization. Lower in the gut, especially in the jejunum, the columnar cells in the villi contain very large vacuoles and inclusions are usually supranuclear, but may also be basal, displacing the nuclei toward the surface. They are largest and most numerous on day two of life, decreasing in number and size till day six. The inclusions are PAS positive. As they disappear, they are replaced by a finely granular PAS positive cytoplasm.

The Peyer's p tches, which are greatly developed in the adult dog are represented in the young puppy by poorly defined loose masses of lymphoid tissue occupying an area below the crypts. By the sixth day of life, however, the adult pattern of patch organization is recognizable.

The techniques used for mucin histochemistry allowed discrimination between neutral and acid mucins and also between nonsulfated (sialomucins) and sulfated (sulfomucins). In the crypts, the neutral mucins were more abundant than the acid mucins, while in the

villi the reverse was the rule. There were, however, great individual differences in the relative amounts of sulfomucins and sialomucins present in the small gut goblet cells. Half of the control pups showed only traces of sulfomucins, while the others contained large amounts of sulfomucin. As the same variability has been found in the goblet cells of adult dogs of various breeds, changes in the composition of the mucin in the small intestine goblet cells may not be a reliable indication of possible gut abnormalities.

Fat was abundant in the duodenal and jejunal mucosa of all control pups examined. The stomachs of these pups were full of milk curd. In the younger puppies, the epithelial fat, especially toward the tips of the villi, was present in large globules probably corresponding to the large vacuoles seen in paraffin sections. The size of the fat inclusions decreased toward the base of the epithelial cells. Fat was also abundant in the duodenal and jejunal lamina propria and in the lymphatics. In the ileal mucosa fat was present in small amounts and was limited to the tips of a few villi.

Enzyme histochemsitry demonstrated that the brush border alkaline phosphatase (ALPase) and cytoplasmic DPNH diaphorase showed a marked drop in activity from the duodenum to the ileum. Glucose-6-phosphatase (G-6-Pase), which is associated with the endoplasmic reticulum, appeared more uniformly distributed in the epithelial cells of the villi, but even G-6-P activity decreased in the ileum as compared to more proximal locations. Acid phosphatases (ACPase), activity in the supranuclear region of the epithelial cells, increased sharply in the ileum. Luminal macrophages were larger and show a stronger ACPase activity in the jejunum than in the duodenum and ileum.

The colon of the control neonatal pup was morphologically similar to the adult colon. The lamina propria, however, appear less cellular in the neonate. The goblet cells of the colonic mucosa contained mostly sulfomucins mixed with lesser amounts of neutral mucins and sialomucins. Fat inclusions, at times quite large, were present in the luminal epithelial cells. In the crypt cells, the fat distribution resembled that observed in the small intestine. Of the enzymes studied in the colon ALPase shows activity in the brush border of the luminal cells and those in the upper part of the crypts, while ACPase and DPHN Diaphorase show little activity, and G-6-Pase shows practically no activity in this area.

The most obvious morphological changes seen in the small gut of the pups infected with I-71 coronavirus was a shortening of the villi and lengthening of the crypts, as seen in Figure I. These changes

occurred earlier (day 2 PI) in the younger pups than the older pups (day 4 PI). Maximum lesions were seen between day 2 and 4 in the younger animals, and on day 4 and 7 in the older ones. Shortening of the villi appeared at the same time in the duodenum and jejunum and later in the ileum. Recovery was delayed in the ileum, especially in the older pups.

Histologically the first lesions were seen two days PI. The mucosa was edematous; the cellularity of the lamina propria had increased as compared to controls; and there was some transmigration of leukocytes. The epithelial cells had lost many of the large inclusions and contained more finely vacuolated cytoplasm. In the crypts there were occasional large epithelial cells with large nuclei and prominent nucleoli.

Four days PI histologic changes were more conspicuous. The cellularity of the lamina was greatly increased. The villi were not only shortened but often fused at various points. The epithelial covering of the villi was irregularly flattened and heavily vacuolated focally. The elongated crypts were occasionally cystic and were associated with an abnormally large number of mitoses. Again there were cells with abnormally large nuclei, multiple nucleoli and polynuclei.

Seven days post infection there were signs of recovery. The villi were finger-like, without fusion, and were covered by normal columnar epithelium. However, the crypts were still abnormally deep, with many mitoses. The cellularity of the lumina was still increased and some edema was present The cellular architecture was further improved 10 days after infection.

In the older pups, infected at 12 days of age, the control gut was like that of an adult except for scant cellularity of the lamina. The inclusions and vacuoles present in the younger puppies had disappeared during the maturation processes, except at the tips of the villi. Not until four days post infection were the most noticeable pathological changes seen, such as fusion of the villi, flattening and vacuolization of villus epithelium, accelerated epithelial extrusion and edema, and increased cellularity of the lamina. Villous fusion, increased mitotic activity in elongated crypts, epithelial cell vacuolization and isolated crypt abcesses were less pronounced than in the younger litters.

Changes due to coronavirus infection in the colon were slight. In the younger puppies, there was some dilation of the crypts on day 2 and vacuolization of the surface mucosal epithelial cells on day 4 with accelerated epithelial shedding and goblet cell discharge. The epithelium returned to normal on day 7 and the crypts were normal by day 11. The colons of the older pups demonstrated the same changes but to a lesser degree.

Studies of mucin histochemistry were completed on sections of small intestine and colon. Considering the variety of acid mucins distributed in the control pups, it is not possible to attribute any significance to the different pattern of mucin distribution in the small bowel goblet cells observed during these experiments. On the other hand, the colon, which showed only slight morphologic changes, demonstrated a noticeable loss of sulfomucins in the goblet cells. This was quite consistent in the infected pups, starting on day 4 in the younger, and on day 7 in the older pups. On the 11th day post-infection, normal colonic sulfation was returning in the younger but not yet in the older pups.

Enzyme histochemistry demonstrated that brush border ALPase had a marked drop in activity in sections of duodenum, jejunum and ileum that was progressive from day 2 PI through day 4 PI. ALPase activity returned to normal by day 7 PI. G-6-Pase activity, associated with endoplasmic reticulum, was diminished as early as day 3 PI, and markedly depressed by day 4 PI. Like ALPase, G-6-Pase activity returned to normal by day 7 PI. The lysozomal enzymes of ACPase were very depressed in activity in the epithelial cells by day 4 PI, but gradually returned to normal with recovery by day 7 PI. The luminal macrophages increased in size and number early as day 2 PI. They persisted and showed a marked increase in ACPase activity that was still very evident by day 7 PI. The mitochrondrial enzyme DPNH diaphorase was very decreased in activity by day 3 or 4 PI but increased in activity with recovery by day 7 PI.

These studies show definite but reversible histologic and biochemical alterations in the small intestine and colon during acute coronavirus 1-71 gastroenteritis of neonatal pups. These pathophysiologic lesions are consistent with the clinical signs and the duration of illness. Further studies of the canine coronavirus 1-71, with both in vivo and in vitro experimental systems, should provide additional information about the pathogenesis of acute infectious non-bacterial gastroenteritis.

6. Diarrhea syndrome in military dogs.

A "diarrhea" syndrome has been present for approximately five years in the closed colony of 225 German Shepherd dogs at the Division of Biosensor Research at Aberdeen Proving Ground, Maryland. At any one time there are approximately 26 brood bitches, 4 studs, 145 young adults in evaluation and training, and 50 dogs less than 2 months of age. All dogs are housed on concrete and, with exception of the unweaned dogs, are exercised daily in wooded areas. Pens are washed daily, with whelping area pens being additionally blow torched biweekly.

At the start of the third week of life, mother's milk is supplemented by a mixture of 2/3 milk supplement and 1/3 high protein cereal. During the fourth week the cereal is replaced by water softened commercial dry puppy food. Homogenized whole meat is also gradually added until by the fifth week the supplement consists of one-half water softened dry puppy food and one-half homogenized meat. Free choice dry puppy chow is made available beginning the fifth week. Weaning is completed during the seventh week.

All dogs are protected by vaccination against preventable canine diseases, which are no current problem. Examinations for parasites are conducted thrice per week on puppies, weekly on young dogs, and monthly on adult dogs. Ascarid ova are constantly observed, as are coccidial oocysts and giardial trophozoites. Strongyloides larvae are rarely observed. Medication for ascarids starts at two weeks of age with piperazine, then Vermiplex, and Dichlorvos, if necessary. Quinicrine HCl and sulfadimethoxine are used to control giardia and coccidia, respectively. Dizan is used for control of strongyloides. Appropriate medication starts at the first signs of an abnormal stool, such as a positive fecal examination, abnormal looseness, blood, and/or mucus. As necessary, these medications are usually employed when the pup is between 2 to 8 weeks of age. In addition, brood bitches are vaccinated with an Escherichia coli bacterin before breeding, and orally medicated with Furacin for the first two weeks after whelping.

Diarrhea is more frequently observed during the two whelping periods (December to January, July to August) when the population of neonatal puppies is highest. The severity and frequency of diarrhea decreases with age, but particularly affects neonatal puppies (3 to 8 weeks old), regardless of season. Almost all of the puppies are affected at some time during this age range. Adult dogs are occasionally affected with overt diarrhea and the overall impression is that "normal" stools in adult dogs are more loose than desired. The nature of the diarrhea ranges from runny, normal colored stools to mucoid and very bloody stools, with all variations in between. Although associated mortality is rare, the syndrome results in an unacceptable number of small and unthrifty puppies. The provisional diagnosis was coccidiosis.

On 1 February 1974, a prospective microbiological study was started on three (3), one week old puppies and their mothers in each of five (5) litters - 20 dogs. Weekly rectal swabs were collected in appropriate holding medium for aerobic bacteriology, virus isolation (primary dog kidney and WR-3873D cell line) and chlamydia isolation (chicken yolk sac inoculation). Periodic blood samples were collected from each dog for hemograms and sera for subsequent assay.

Fresh fecal smears were examined for giardial trophozoites and undigested fat (Oil Red O) and starch (Lugols). Composite stool samples were examined weekly from each litter.

Over a 10 week period, the "diarrhea" syndrome appeared different from that originally described. Tables 10 through 14 list the appearance of the stool during the third through tenth week of age for each of the five study litters (bitch plus three puppies), respectively, in association with the presence of coccodial oocysts and giardial trophozoites. Infection with Isospora rivolta (unsporulated oocyst measuring 15 to 20 μ wide by 20 to 25 μ long) regularly occurs at about 3 weeks of age, peaks between 5 and 8 weeks of age, and declines rapidly thereafter. Frequently this infection is immediately followed by Isospora canis (unsporulated oocyst measuring 30 to 35 μ wide by 35 to 40 μ long) at 8 to 9 weeks of age, which rarely achieve the degree of infection (maximum infection observed: 2900 oocysts/gm) observed for I. rivolta. Both species of coccidia have been observed in the same dog. Finite episodes of diarrhea or dysentery were difficult to define since they occurred sporadically in individual dogs and lasted from 1 to 7 days. The diarrhea experienced by Xenette (Table 15) illustrates these episodes. Typically however, after approximately five weeks of age, the puppies stool would frequently alternate between semi-formed and mushy. These mushy, abnormal stools coincided in time of appearance with the augmentation of coccidial infection at five to nine weeks of age. However, there was no direct correlation between the degree of stool abnormality and numbers of detectable coccidial oocysts. In addition, 12 week old dogs showed the same degree of stool abnormalities over a 1 to 2 month period during which time coccidial oocysts were either not detected or too few to count in concentrated fecal specimens (Tables 15 and 16). Finally, during two days in March, single fecal specimens were collected from 82 older dogs (3 to 12 months old) housed outdoors on a concrete pad. Soft, mushy stools were collected from 48 (58%) dogs, nine (18%) of which contained giardial trophozoites, whereas only three (6%) contained coccidial oocysts. These observations indicate that coccidia, although probably contributing to intestinal upset at an early age, are not the principal reason for the abnormal stools. The consistent association of giardial trophozoites with abnormal stools, particularly in dogs with episodes of diarrhea or dysentery without evidence of heavy coccidial infection, suggests but does not substantiate a causative role for giardia. An important corollary to the abnormal stools was the observation that a high proportion (6/31 - 19%) of puppies in the study litters were small and unthrifty.

The following bacteria were aerobically cultured from 153 rectal swabs or stools collected during the first ten weeks of age from the 20 study puppies or other puppies with frank diarrhea or dysentery: hemolytic E. coli (25%), Proteus sp. (22%), Providence

sp. (10%), atypical E. coli (7%), Pseudomonas sp. (6%), and Citrobacter sp. (2%). Salmonella spp. were never isolated, and anaerobic bacteriology was not performed. Additionally, enterococci were isolated from all specimens. Episodes of diarrhea or dysentery, or the appearance of particularly mushy stools were not associated with particular bacterial species or numbers. Three selected strains of hemolytic E. coli lacked tissue invasiveness as determined by their failure to produce conjunctivitis in guinea pigs when the latter were exposed to broth cultures containing at least 10^6 organisms/ml (Sereny test). Consequently, the occurrence of these bacteria presumably reflect "normal" bacterial flora which are not causally related to the diarrhea syndrome.

Rectal swabs for isolation of chlamydia were held in 2.0 ml of cold (4^0C) sucrose phosphate buffer, which was clarified by centrifugation and filtered (0.45μ)to remove bacteria. The filtrate and next two decimal dilutions were inoculated in 0.5 ml amounts into the yolk sacs of 70 day old chicken embryos. No Chlamydia were isolated from 25 specimen, eleven (11) of which were from dogs with mushy or watery stools.

Ninety (90) rectal swab specimens from the five (5) study bitches and seventeen (17) puppies were inoculated into primary dog kidney (PDK) cells and the Walter Reed Canine Cell (WRCC) line for virus isolation. Twenty-six (26) of these specimens were obtained from dogs having abnormal (semi-formed, mushy, or watery) stools. Additionally, tissues obtained at necropsy from two study puppies (Table 8, 1497 and 1550) yielded no viruses. Two, reo-like viruses were isolated in PDK cells. The first isolate was recovered from a fecal specimen of an apparently "normal" puppy (1490); the second was recovered two weeks later from a palatine ulcer in the puppies' mother (1102). Both viruses produced similar cytopathic effects in PDK cells, including cytoplasmic inclusions, and agglutinated human "O" red blood cells. A third, dissimilar virus was recovered from a fecal specimen of puppy 1501 (Table 8). This virus produced cytopathic effects in the WRCC line, and intranuclear changes resembling inclusions were observed in H&E stained cultures. Studies are continuing to identify these viruses, and to clarify their epizootiology by serology.

Examinations for intestinal pathology were performed on four study puppies by Dr. Helen Jervis of the Department of Experimental Pathology. Relevant features of each of the four dogs at the time of necropsy are shown in Table 17. Grossly, the mucosa of the entire small intestine of the unthrifty dogs showed short, finger-like villi. The jejunum contained tongue and leaf-shaped villi, whereas those in the ileum were relatively flat and aligned in ridges. Microscopically, there was an enteritis progressing from moderate

to severe from the duodenum to the ileum, the mucosal epithelium being increasingly flattened. The villi were often clubbed and fused, and the lamina propria showed increased cellularity progressing from the duodenum to the ileum. The villus/crypt ratio was 1:1 or less (Figure 2), whereas the expected normal ratio would approximate 3:1. These changes were less striking in dog 1497. Giardia were present in decreasing numbers from the duodenum to the jejunem, and were not observed in the ileum. Relatively few giardia were observed, since most would have been lost during fixation of the tissue. The most severe lesions were observed in dogs 1550 and 1552, and coccidia were only rarely observed in the jejunum and ileum of these dogs. Dog 1501 showed less severe mucosal changes, but more readily observed coccidia in the epithelial cells of the villi. Importantly, oocyst counts from 1501, 1550, and 1552 at the time of necropsy reflect peak levels of coccidial infection. Bacteria colonized the ileum and often the jejunum of all dogs, and in 1550 appeared to penetrate the epithelial cells. The cause(s) of these sprue-like lesions is not apparent from the histopathology. The minimal evidence of coccidial invasion is not consistent with the degree of pathology noted, and indicates that the diarrhea syndrome is not due to uncomplicated coccidiosis.

These studies have shown that the diarrhea syndrome is associated with persistently abnormal stools in most dogs under one year of age. Episodes of diarrhea have no clear cut onset or termination, and no bacteria, viruses, or chlamydia have been shown to be causally related to the diarrhea. The persistent occurrence of abnormal stools in dogs up to one year of age, and the sporadic episodes of diarrhea or dysentery do not correlate with the known cycling of coccidial infections nor with the observed paucity of histopathology attributable to coccidia. The role of giardia in the syndrome is not clear. The number of small and unthrifty puppies and the sprue-like lesions suggest that intestinal malabsorption and/or host abnormalities may be more important than microbial or parasitic agents. Studies are continuing in this direction.

7. Comparison of MHG virus with mouse encephalomyelitis viruses.

In 1964 (33) the isolation and characterization of MHG virus was described. The virus was isolated from tissues of laboratory rats with signs of encephalitis. Physical properties of MHG virus were similar to those of the mouse encephalomyelitis viruses, and suckling mice inoculated intracranially with MHG virus developed lesions characteristic of mouse encephalomyelitis. On the basis of complement fixation tests, MHG virus shared some antigens with members of the mouse encephalomyelitis group. However it was concluded that MHG virus differed from mouse encephalomyelitis viruses by cross neutralization test, lack of infectivity for weanling mice and its

inability to hemagglutinate human O cells. The investigators concluded that although MHG virus possessed some antigens in common with mouse encephalomyelitis viruses, it did not meet all requirements for inclusion in this group. Thus a definitive classification of MHG virus was not made.

The development of new techniques has permitted a more definitive comparison of MHG virus with the mouse encephalomyelitis viruses.

Germfree suckling and weanling mice from Charles River Breeding Laboratories were used for preparation of seed viruses and for preparation of immune antisera. These animals were maintained in filter cages with chlorinated water and steam sterilized feed. Specific pathogen free mice from the Walter Reed Colony, which have been shown to be serologically free from mouse encephalomyelitis, were used to prepare complement-fixing (CF) and hemagglutinating (HA) antigens and to determine animal infectivity. Suckling and weanling rats from the Walter Reed Colony, which are serologically negative for mouse encephalomyelitis, were used to prepare antisera and to determine animal infectivity.

For animal infectivity studies a 10% suckling mouse brain suspension of each agent was diluted tenfold in Hanks' balanced salt solution (BSS). Suckling mice and rats were inoculated intracranially with 0.02 ml of virus and intraperitoneally with 0.05 ml. Weanling mice and rats were inoculated intracranially with 0.02 ml of virus and intraperitoneally with 0.1 ml. The LD_{50} titers were calculated by the method of Reed and Muench (34).

MHG virus, isolated by McConnell, et al (33) and passed 5 times in suckling rat brain was used to prepare the seed virus in suckling mice. TO virus (Yale Strain) and FA virus were obtained from the American Type Culture Collection. A tissue culture adapted strain of GDVII virus was obtained from Dr. John Parker, Microbiological Associates. Seed and working virus pools, as well as HA and CF antigens for all 4 agents, were prepared by inoculating 1 day old suckling mice with 0.02 ml of virus. Brain material was harvested when the mice showed signs of encephalomyelitis. Twenty percent tissue suspensions were prepared in Hanks' BSS with 100 units of penicillin and 100 ug of streptomycin per ml and centrifuged at 2,000 rpm for 20 minutes. The supernatant fluid was removed and stored frozen at -80°C. Complement fixing antigen was prepared as above but sterile phosphate buffered saline, pH 7.2 was used as the diluent. Normal antigen was prepared by collecting brain material from uninoculated germfree mice.

Weanling, germfree mice were used to prepare immune sera. Antisera to GDVII and FA viruses were prepared by injecting intraperitoneally 0.5 ml of a brain suspension diluted 10^{-5} followed 1 week later by 0.5 ml of the brain suspension diluted 10^{-2}. This was followed by 2 injections, one week apart, of 0.5 ml of the 10% mouse brain suspension. Antisera to TO and MHG viruses were prepared in germfree mice by 4 weekly intraperitoneal injections of 10 brain suspensions. Antisera to GDVII, TO, MHG and FA viruses were prepared in weanling rats by 4 weekly intraperitoneal injections of 0.5 ml of 10% brain suspension. Sera were collected 7 to 10 days after the last injection. Normal mouse serum was obtained from uninoculated germfree mice and normal rat serum was obtained from uninoculated rats.

Two cell lines, BHK-21 and L929, were used for growth and plaque assay of GDVII, TO, and MHG viruses. The L929 cells were obtained from Microbiological Associates. Monolayer cultures of these cells were prepared with the use of Earle's BME supplemented with 10% heat inactivated fetal bovine serum and 100 units of penicillin and 100 ug of streptomycin per ml. Earle's BME containing 2% fetal bovine serum was used as maintenance medium. The BHK-21 cells were originally obtained from the Department of Virus Diseases, Walter Reed Army Institute of Research. These cells were grown in monolayer cultures in Medium 199 with 10% heat inactivated fetal bovine serum and 100 units of penicillin and 100 ug of streptomycin per ml. Medium 199 containing 2% fetal bovine serum was used as maintenance medium. All cultures were grown in 32 oz prescription bottles at 35°C. When monolayers formed, the cells were trypsinized, centrifuged and suspended in the appropriate growth media. This cell suspension was used to seed 32 oz prescription bottles, Leighton tubes and round tubes.

For plaque assay L929 cells were grown in plastic 25 cm^2 tissue culture flasks. Cultures were inoculated when a complete monolayer formed. The inoculated cultures were incubated 1 hour at 35°C. Flasks were rotated gently every 15 minutes during this adsorption period. The cell monolayers were then overlaid with medium consisting of 1% Difco purified agar in Earle's BME with 2% heat inactivated fetal bovine serum and 100 units of penicillin and 100 ug of streptomycin per ml. The infected cell cultures were inverted and incubated at 35°C. Plaques were evident 3 days post inoculation. To facilitate counting the plaques, 0.5 ml of neutral red diluted 1:1000 was added to each flask, and the flasks were then incubated for 1 hour at 35°C.

The complement fixation test was performed according to the methods of Kent and Fife (35) with modifications for the microtiter system. The sera were heat inactivated and diluted twofold. The optimum antigen dilutions for the tests were predetermined with homologous serum. Final incubation of the microtiter plates was for 1 hour at 37°C with shaking every 15 minutes.

The hemagglutination-inhibition test was set up in the microtiter system. All reagents were kept at 4°C and the test was set up and incubated at 4°C. The sera were heat inactivated and diluted in phosphate buffered saline, pH 7.2. Mouse brain suspensions were trypsin treated (36). Eight units of antigen and a 0.5% suspension of human O erythrocytes were used.

For the plaque reduction test sera were heat inactivated, diluted fourfold, and each dilution was mixed with an equal amount of virus diluted to contain approximately 100 pfu per 0.1 ml. This serum-virus mixture was incubated at room temperature and shaken every 15 minutes. After a 1 hour incubation period 0.2 ml of the serum-virus mixture was added to each of 3, 25 cm^2 plastic flasks containing monolayers of L929 cells. Flasks were incubated for 1 hour at 35°C. During this time the flasks were rotated gently. The monolayers were overlaid with the above plaque media. After the agar solidified the flasks were inverted and incubated at 35°C. Plaques were counted 3 days post inoculation for GDVII virus and 4 days post inoculation for TO and MHG viruses.

The strain of GDVII virus used in this study produced CPE on the first passage in BHK-21 cells and L929 cells and could be passed readily in each of these cell lines. However, the titer of GDVII virus in L929 cells was 10 times higher than in BHK-21 cells (Table 18). In both cell lines CPE consisted initially of focal areas of rounded cells. Eventually the CPE spread throughout the cell monolayer and the cells became detached from the glass. When inoculated into suckling mice this tissue culture adapted strain of GDVII virus produced typical signs of disease, including encephalitis, paralysis and death.

TO and MHG viruses grown in suckling mouse brains did not produce CPE on primary inoculation of BHK-21 and L929 cells; however, after 1 blind passage in both cell lines CPE appeared and was similar to that produced by GDVII. As in the case of GDVII, both TO and MHG produced higher titers in L929 cells (Table 18). TO and MHG viruses grown in tissue culture produced typical disease when inoculated into suckling mice.

FA virus did not produce CPE after 3 blind passages in both BHK-21 cells and L929 cells. Attempts to grow FA virus in tissue culture, including a series of blind passages in both BHK-21 and L929 cell lines, were unsuccessful.

GDVII, TO and MHG viruses produced plaques in monolayers of L929 cells overlaid with agar (Table 18). The plaques were easily recognized 3 days post inoculation; however plaque size varied with the respective viruses. Plaques produced by GDVII ranged in size from

2-4 mm, whereas the plaques produced by TO virus were smaller and ranged in size from 1-2 mm. MHG virus was characterized by a plaque size distinctly smaller than those of either GDVII or TO. Plaques of MHG virus measured 0.5 to 1 mm in diameter.

The hyperimmune mouse and rat sera to GDVII, TO, MHG, and FA viruses suppressed plaque formation by 100 pfu of GDVII, TO, and MHG viruses. The 80% plaque reduction titers of both rat and mouse sera are given in Table 18. The data presented in Table 19 shows a complete cross neutralization of GDVII, TO, and MHG viruses by immune sera of GDVII, TO, MHG, and FA.

Results of serological comparison of GDVII, TO, MHG, and FA viruses by the complement fixation test are given in Table 20. Although cross reactions occurred between all 4 agents, differences in homologous and heterologous titers were evident.

GDVII and TO viruses propagated in the brains of 1 day old suckling mice agglutinated human O erythrocytes. FA and MHG viruses prepared in the same way as GDVII and TO viruses failed to hemagglutinate human O erythrocytes. Table 21 summarizes the results of the hemagglutination-inhibition test with TO and GDVII antigens. Anti MHG mouse sera inhibited the hemagglutination reaction with TO virus at a relatively low titer of 1:40 but did not inhibit hemagglutination with GDVII virus. Anti MHG rat sera had no inhibitory effect.

In animal infectivity studies suckling and weanling mice and rats were inoculated with tenfold dilutions of GDVII, TO, MHG, and FA viruses. The results are summarized in Table 22. All 4 agents produced signs of disease and death in 1 day old suckling mice inoculated intracranially and intraperitoneally. When inoculated intracranially, GDVII, TO, and FA viruses produced disease and death in 3 week old weanling mice but MHG virus provoked no disease in weanling mice. None of the agents caused death in weanling mice when inoculated by the intraperitoneal route. In suckling rats only TO virus did not produce death when inoculated intracranially or intraperitoneally. It is also significant that the titer of FA virus in suckling rats inoculated intracranially was significantly lower than that of either GDVII or MHG viruses. None of the 4 agents produced disease in 3 week old weanling rats inoculated by both routes.

The development of the plaque assay technique for GDVII, TO, and MHG viruses and the subsequent development of a plaque reduction test for these same viruses permitted additional evaluation of the serologic relationship of MHG virus to viruses of the mouse encephalomyelitis group (37). In the plaque reduction test complete cross neutralization of GDVII, TO, and MHG viruses by immune sera of

GDVII, TO, MHG and FA was demonstrated. This finding supports the close antigenic relationship of MHG virus to GDVII, TO and FA viruses. It further shows that the neutralization test cannot be used to separate members of the mouse encephalomyelitis group.

Results of complement fixation tests support the findings of the plaque reduction tests in that cross reactivity between all 4 agents was noted. Although differences were observed in homologous and heterologous titers, the complement fixation test does not provide a means of separating the 4 viruses.

Although other investigators (38) have reported that all members of the mouse encephalomyelitis group of viruses agglutinate human O erythrocytes, a hemagglutinin could not be demonstrated in brain suspensions of suckling mice infected with either FA or MHG viruses. A hemagglutinin could readily be demonstrated in suckling mouse brains infected with GDVII and TO viruses. These findings support the observations of Morris (36), who reported that FA virus, although treated with trypsin, does not hemagglutinate human O erythrocytes. The findings also confirm the previous report (33) that MHG, unlike GDVII, does not hemagglutinate human O erythrocytes. It is apparent from findings in this current study that the lack of a demonstrable hemagglutinin does not exclude MHG from the mouse encephalomyelitis group.

Anti MHG mouse sera inhibited hemagglutination by TO virus but did not inhibit hemagglutination with GDVII virus. This observation may be significant in the routine serological screening of rodent colonies for evidence of infection with the mouse encephalomyelitis viruses, particularly MHG. If only GDVII is used as the antigen in hemagglutination-inhibition tests, antibodies to MHG may not be detected.

MHG virus could readily be separated from GDVII and TO viruses on the basis of plaque size in L929 cells. Since plaque size is frequently used to differentiate virus strains (39-41), the variation in plaque size observed between GDVII, TO and MHG provides evidence that plaque size may also serve as a means of separating the mouse encephalomyelitis viruses.

The titration of the viruses in suckling and weanling mice and rats revealed differences between the 4 viruses studied. MHG did not produce disease in 3 week old weanling mice when inoculated intracranially. On the other hand, GDVII, TO and FA viruses all produced disease and death in weanling mice inoculated intracranially. TO differed from the other viruses in its inability to produce disease and death in suckling rats. One might speculate from the high susceptibility of suckling rats to MHG virus inoculated by both

the intracranial and intraperitoneal routes that MHG virus is a strain of the mouse encephalomyelitis group which has been adapted to rats. The strain was initially recovered from adult rats with signs of encephalitis (33).

The current studies support the inclusion of MHG virus in the mouse encephalomyelitis group of viruses. However on the basis of animal infectivity and serological studies it appears to be distinct from GDVII, TO and FA viruses.

Table 1. Mean peripheral hematologic values on three groups of German Shepherds experimentally infected with Babesia canis.

	Group I[1]	Group II	Group III[2]
PCV[3]			
Day 0	46	46	47
Low (day)	28 (7)	33 (7)	26 (30)
Day 45	43	43	31
RBC[3]			
Day 0	6.5	6.6	6.2
Low (day)	4.5 (13	4.6 (13)	2.4 (13)
Day 45	6.5	6.4	4.7
WBC[3]			
Day 0	10.0	10.9	12.4
Low (day)	6.6 (5)	9.7 (10)	5.6 (16)
Day 45	8.8	12.0	14.5
Platelets[3]			
Day 0	118	275	237
Low (day)	22 (6)	33 (12)	18 (12)
Day 45	121	127	45
ESR[3]			
Day 0	1	2	1
Low (day)	57 (7)	18 (10)	22 (12)
Day 45	3	2	18

1. Dogs had been infected with Ehrlichia canis 1 to 3 years before day 0.

2. Dogs were infected with Ehrlichia canis at the time of infection with Babesia canis.

3. Packed Cell Volume (PCV) percent; Red Blood Cells (RBC), millions/mm^3; White Blood Cells (WBC), thousands/mm^3; Platelets, thousands/mm^3; Erythrocyte Sedimentation Rate (ESR), mm/hr.

Table 2. Viruses recovered from military dogs with respiratory disease at Lackland AFB, Texas Summer 1973.

Dog	Specimen	Cell culture* Used (Passage)	Neutralization titer of isolate against reference canine SV-5 Serum**	Serum neutralization test against		
				WRAIR canine SV-5 viruses***	Lackland 1973 SV-5 reference isolate****	Lackland W191R isolate
Tramp***	Nasal	PDK(2), WRCC(2)	64, 16	0/ $\bar{>}$ 256 ≠	0/ $\bar{>}$ 256	0/0
73-1548	Nasal	PDK(2), WRCC(2)	ND 16	0/ $\bar{>}$ 256	0/ $\bar{>}$ 256	0/4
	Throat	PDK(2), WRCC(2)	64, 16			
W191	Fecal	WRCC(1)+	0	64/16	ND	0/64

* PDK = primary dog kidney, WRCC = Walter Reed Canine Cell line, ND = not down
** Homologous titer ranged from 16-64
*** Ft. Benning 1966 3 x 84 reference virus
**** Lackland 1973 Tramp isolate.
\+ A similar cytopathic agent recovered from throat and rectal swab specimens obtained next day.
≠ Titer in acute serum specimen/Titer in convalescent serum species.

Table 3. Primary and continuous cell cultures resistant to cytopathic effects of W191R Calici-like isolate.

Primary cell cultures	Serial cell cultures
Primary Dog Kidney	LLCMK2
Porcine Embryonic Kidney	PK15
Human Embryonic Kidney	Vero
African Green Monkey Kidney	
Rhesus Monkey Kidney	
Feline Kidney	
Swine Kidney	

Table 4. Chemical and physical properties of W191R isolate from Lackland Air Force Base - 1973.

Treatment	Virus	1/TCD50 (log) Not treated (A)	Treated (B)	Change (A-B)
Chloroform	W191R	4.5	4.5	0
	ICH	6.3	6.5	+ 0.2
	C. herpes	5.0	< 1.0	> 4.0
pH 3.0 for 3 hrs - at 25°C	W191R	5.8	2.3	2.5
	ICH	5.3	5.8	+ 0.5
	C. herpes	4.8	< 2.0	$\geq$ 2.8
Filtration - 0.45 nm	W191R	5.8	5.3	0.5
0.22			4.8	1.0
0.10			4.3	1.5
0.05			$\leq$ 4.8	$\geq$ 1.0
Magnesium Stability at 50° for Hr IM Mg	W191R	6.0	< 2.0	> 4.0
Saline		5.8	2.3	3.5
IM Mg	Polio	6.0	6.0	0
Saline		6.0	< 2.0	> 4.0
IUDR ($10^{-3.5}$M)	W191R	6.2	5.8	0.4
	ICH	4.5	2.3	2.2
	SV-5	6.0	5.7	0.3

*Membrane filter (Millipore Corp.)

Table 5. Antigenic relationship of W191R and Canine-Calici-like isolates.

Antiserum (Animal #)	1/ neutralizing antibody titer to	
	W191R	Homologous virus
W191R (Guinea pig #6)	16,384*	-
(Rabbit #379)	4,096*	-
3-68 (Guinea pig #425)	16	65,536
(Rabbit #967)	16	256
L198T (Guinea pig #424)	< 16	1,024
(Rabbit #13)	< 16	256
A128T (Guinea pig 2362)	< 16	4,096
(Rabbit #207)	< 16	≥ 1,024
A358 (Guinea pig # 6)	1,024	≥ 256,144
(Guinea pig #10)	< 16	≥ 65,536
(Rabbit #847)	< 16	16,384
(Rabbit #848)	1,024	≥ 65,536

* W191R antiserums did not neutralize 3-68, L198T, A128T and A358 at a dilution of 1:16.

Table 6. Parainfluenza SV5 and W191R antibody studies of military dogs with respiratory disease in 1973.

Location	Dates	No. with increased neutralizing antibody titer/total tested (%)	
		SV5	W191R
Lackland AFB	14 Jun - 6 Aug	76/102 (75)*	31/101 (31)
	25 Sep - 6 Nov	15/15 (100)	0/15 (0)
Kadena AFB, Okinawa	13 Jun -22 Jun	13/15 (87)**	0/15 (0)

* 1 sick dog did not have SV5 antibody in convalescent serum.
** All dogs had SV5 antibody at end of epizootic

Table 7. Neutralization (N) and hemagglutination-inhibition tests for Virus Infections In military dogs at Lackland AFB, Texas

Virus	Test	No. with increased titer total tested	(%)
Parainfluenza SV5 (3x84)*	N	20/37	(54)
Tramp**	N	16/31	(51)
Infections Canine Hepatitis	N	0/30	(0)
Toronto A26/61 Adenovirus	N	0/30	(0)
Canine Herpesvirus	N	1/30	(3)
Transmissible Gastroenteritis of Swine	N	3/27	(11)
Canine Coronavirus 1-71	N	2/31	(6)
Canine Rhinovirus A128THR	N	0/26	(6)
Canine Calici-like Isolates			
W191R***	N	8/33	(24)
3-68	N	2/26	(8)
L198T	N	4/26	(15)
A128T	N	0/26	(0)
A358	N	0/26	(0)
Minute Virus of Canines	HI	1/31	(3)
Reovirus Type I	HI	0/31	(0)
II	HI	0/31	(0)
III	HI	0/31	(0)

* Canine reference parainfluenza SV5 (3x84 Ft. Binn 66 isolate)
** LAFB 1973 SV-5 (Tramp isclate)
*** LAFB 1973 Calici-like isolate

Table 8. Virus isolations and clinical signs of puppies insulated with Coronavirus 1-71.

Dog No.	Day post-infection			
	Virus recovered	Diarrhea	Dehydration	Dog sacrificed
1700,P7[1]	0	0	0	0
P6	0	0	0	2
P1	3	3	0	3
P4	3	2,3	0	3
P2	3,5	3-5	4-5	5
P5	3,6	3-7	5-7	7
P3	6	2-8	6,8	9
2SP5,P52	0	0	0	0
P3	0	0	0	2
P4	0	1	0	3
P1	5	0	0	5
P2	6,7	3-8	4,7,8	8
P6	3,5,7	1-3, 6-8	6,7	9

1. Dogs infected with 1-71 coronavirus at 4 days of age.

2. Dogs infected with 1-71 coronavirus at 12 days of age.

Table 9. Virus Titers (Log 10) in Tissues of Pups inoculated with 1-71 Coronavirus.

Dog No.	duod.	jeju.	ileum	colon	mesent. L. node
1700P7 (c)	0	0	0	ND	ND
1700P6 (2)	3.5	0	0	0	ND
1700P1 (3)	3.5	> 4.5	> 4.5	2.5	ND
1700P4 (3)	3.0	3.0	> 4.5	> 4.5	ND
1700P2 (5)	> 4.5	3.5	2.5	ND	ND
1700P5 (7)	3.5	1.5	3.5	0	ND
1700P3 (9)	3.5	0	1.0	0	ND
2SP5P5 (c)	0	0	0	ND	ND
2SP5P3 (2)	2.5	2.0	2.0	2.0	ND
2SP5P4 (3)*	4.0	> 4.5	> 4.5	2.5	> 3.5
2SP5P1 (5)*	2.0	2.5	2.0	2.0	> 3.5
2SP5P2 (8)	0	0	2.0	0	ND
2SP5P6 (9)*	2.0	2.0	2.0	2.0	> 3.5

(c) = control
(n) = day post inoculation sacrificed
* = lung, heart, liver, spleen, stomach - negative for virus

Table 10. Selected stool characteristics by weeks for 3 to 10 week old puppies of "B" litter.

Age in weeks	Coccidia	Giardial trophozoites	Undigested Fat	Undigested Starch	Stool characteristics
3	none	none detected	none	none	yellow-green
4	none	"	none	slight	brown-green; mushy
5	*Isospora rivolta*[1]	"	none	slight	brown, formed
6	*I. rivolta*[1]	"	slight	moderate	brown, mushy or formed
7	*I. rivolta*[1] 7,680/gm	"			brown, mushy or formed often mucus
8	Insufficient sample				
9	*I. rivolta*[1]	none	not done	not done	brown, mushy or formed
10	*I. canis* 6,800/gm	2 to 8/HPF	none	moderate to heavy	brown, mushy or formed often mucus

[1]Unsporulated oocysts by concentration/flotation were too few to count.

Table 11. Selected stool characteristics by weeks for 3 to 10 week old puppies of "C" litter.

Age in weeks	Coccidia	Giardial trophozoites	Undigested fat	Undigested starch	Stool characteristics
3	*Isospora rivolta*[1]	none detected	none	slight to moderate	brown-green semi-formed
4	*I. rivolta*[1]	none detected	none	slight to moderate	yellow-green or brown semi-formed or formed
5	*I. rivolta*[1]	none detected	none	slight to moderate	brown, semi-formed or formed
6	*I. rivolta*[1]	occasional	none	slight to moderate	brown, semi-formed fromed, or mushy
7	*I. rivolta* 2,800/gm	1 to 2/HPF	none	moderate to heavy	brown, formed or mushy, some mucus
8	none	2 to 4/HPF	none	moderate	brown, formed or mushy
9	none	1 to 2/HPF	none	heavy	not recorded
10	*I. canis*	2 to 10/HPF	none	heavy	brown, mushy or watery

[1]Unsporulated oocysts by concentration/flotation were too few to count.

Table 12. Selected stool characteristics by weeks for 3 to 10 week old puppies of "D" litter.

Age in weeks	Coccidia	Giardial trophozoites	Undigested fat	Undigested starch	Stool characteristics
3	*Isospora rivolta*[1]	none detected	none to slight	slight to moderate	yellow-brown formed to semi-formed
4	none	none detected	none to moderate	slight to moderate	brown, formed to semi-formed
5	none	none detected	none to moderate	slight to moderate	brown, formed to semi-formed
6	*I. rivolta* 7,600/gm	none detected	not done	not done	Insufficient sample
7	*I. rivolta* 7,100/gm	none detected	not done	not done	Insufficient sample
8	*I. rivolta* 13,800/gm	none detected	none to slight	heavy	brown, formed or mushy
9	*I. rivolta* 2,900/gm *I. canis* 2,900/gm	2 to 10/HPF	none to slight	heavy	brown, mushy
10	*I. rivolta* 600/gm *I. canis*[1]	2 to 10/HPF	none	moderate	brown, mushy to watery

[1] Unsporulated oocysts by concentration/flotation were too few to count.

Table 13. Selected stool characteristics by weeks for 3 to 10 week old puppies of "E" litter.

Age in weeks	Coccidia	Giardial trophozoites	Undigested fat	Undigested starch	Stool characteristics
3	*Isospora rivolta*[1]		none	none to slight	brown-green, yellow semi-formed
4	*I. rivolta*	1 to 2/HPF	none	slight to heavy	reddish-brown, formed, semi-formed
5	*I. rivolta*	2/HPF	none	not done	brown, semi-formed or mushy
6	*I. rivolta* 46,600/gm	none detected	none to moderate	moderate to heavy	brown, formed, or mushy with mucus
7	none	2 to 10/HPF	none	moderate	brown, semi-formed or mushy
8	*I. rivolta*[1] *I. canis*[1]	1 to 2/HPF	none	moderate	brown, semi-formed or mushy
9	*I. rivolta*[1] *I. canis*[1]	1 to 2/HPF	none	moderate	brown, watery mush with mucus
10	none	1 to 2/HPF	none	moderate	brown, mushy or watery with mucus, some blood

[1]Unsporulated oocysts by concentration/flotation were too few to count.

Table 14. Selected stool characteristics by weeks for 3 to 10 week old puppies of "F" litter.

Age in weeks	Coccidia	Giardiol trophozoites	Undigested fat	Undigested starch	Stool characteristics
3	none	none detected	none	none	yellow-brown semi-formed
4	*Isospora rivolta* 13,300/gm	none detected	none to moderate	none to slight	brown, formed to semi-formed
5	*I. rivolta* 112,600/gm	1/HPF	none to moderate	moderate to heavy	brown, formed and mushy, some mucus
6	*I. rivolta* 1600/gm	1/HPF	slight	heavy	brown, mushy
7	*I. rivolta* 7,600/gm	1 to 2 HPF	slight	heavy	brown, mushy, sometimes watery
8	*I. rivolta* 2,600/gm	1/HPF	none	heavy	brown, mushy to watery
9	Not sampled				
10	Not sampled				

Table 15. Selected stool characteristics for a twelve week old puppy (Xenette) by dates of collection.

Collection date	Coccidia	Giardiol trophozoites	Stool characteristics
18 March 74	none	8 to 10/HPF	light brown, fetid watery to mushy with some blood
19 March	none	8 to 10/HPF	light brown, fetid watery to mushy with some blood
20 March	none	none	light brown, fetid watery to mushy with some blood
21 March	*I. rivolta*[1]	occasional	light brown, fetid mushy with some blood
8 May[2]	none	1 to 2/HPF	brown, mushy to watery with some blood and mucus
15 May[2]	none	occasional	brown, watery to mushy

[1]Unsporulated oocysts by concentration/flotation were too few to count

[2]Intervals between specimen collections indicate periods when stools were not watery or bloody; they were formed, semi-formed, or mushy

Table 16. Selected stool characteristics for twelve week on "Z" puppies by date of collection.

Collection date	Coccidia	Giardial trophozoites	Stool characteristics
3 April 74	Isospora canis[1]	1 to 2/HPF	brown, mushy to watery
4 April	I. canis[1]	1 to 2/HPF	brown, watery to mushy with some mucus
5 April	I. canis[1]	1 to 2/HPF	brown, mushy to watery with some blood and mucus
15 April	I. rivolta[1]	1 to 2/HPF	brown, watery to mushy
30 April	none dected	1 to 2/HPF	brown, watery with spots of blood
8 May	I. canis[1]	1 to 2/HPF	brown, watery to mushy
13 May	none detected	1 to 2/HPF	brown, watery to mushy
15 May	none detected	1 to 2/HPF	brown, watery to mushy

[1]Unsporulated oocysts by concentration/flotation were to few to count.

Table 17. Selected characteristics of four study puppies at time of necropsy.

Dog	Age	Weight	Anatomical anomoly	I. rivolta	Giardial trohpozoites	Stool
1497 "normal" size	6	1.5	none	100/gm	occasional	mushy
1501 stunted	9	1.6	achalasia	7,600/gm	8/HPF	semi-formed
1550 stunted	5	0.8	PRAA[1]	none[2] detected	2/HPF	watery
1552 stunted	5	0.7	PRAA[1]	none[2] detected	2/HPF	watery

[1]Persistent right aortic arch

[2]Two days before necropsy, a stool sample from these puppies pen contained 46,600 I. rivolta/gm.

Table 18. Titration of GDVII, TO and MHG viruses in BHK and L929 cell cultures.

Virus	TCID*$_{50}$ log 10/0.1 ml		Plaque Assay PFU** log 10/0.1 ml
	BHK-21	L929	L929
GDVII	5.7	6.7	7.0
TO	4.5	5.5	6.5
MHG	4.0	4.7	5.9

*Tissue culture ID_{50}

**Plaque forming units

Table 19. Serological comparison of GDVII, TO, MHG and FA viruses by the cross neutralization test.

Virus	Reciprocal of antibody titer with indicated serum*				Normal
	GDVII	TO	MHG	FA	
GDVII	1024 (1024)	256 (1025)	64 (16)	256 (1024)	0 (0)
TO	256 (1024)	1024 (1024)	64 (16)	256 (1024)	0 (0)
MHG	256 (1024)	1024 (1024)	64 (16)	256 (256)	0 (0)

*= Highest dilution at which 80% plaque reduction occurred; non bracketed values = mouse sera; bracketed values = rat sera; 0 = < 1:10.

Table 20. Serological comparison of GDVII, TO, MHG and FA viruses by the complement-fixation test.

Virus	Reciprocal of antibody titer with indicated serum				Normal
	GDVII	TO	MHG		
GDVII	160 (160)	80 (160)	40 (160)	80 (160)	0 (0)
TO	640 (160)	320 (160)	160 (80)	320 (160)	0 (0)
MHG	80 (80)	80 (80)	640 (640)	40 (80)	0 (0)
FA	160 (160	80 (160)	40 (160)	160 (320)	0 (0)

Non bracketed values = mouse sera; bracketed values = rat sera; = < 1:10.

Table 21. Serolocial comparison of GDVII, TO, MHG and FA viruses by the hemagglutination-inhibition test.

	Reciprocal of antibody titer with indicated serum				
Virus	GDVII	TO	MHG	FA	Normal
GDVII	320 (320)	80 (80)	0 (0)	80 (40)	0 (0)
TO	80 (16)	640 (320)	40 (0)	20 (40)	0 (0)

Non bracketed values = mouse sera; bracketed values = rat sera; 0 = < 1:20.

Table 22. Titration of GDVII, TO, MHG and FA viruses in mice and rats by the intracranial (IC) and intraperitoneal (IP) routes of inoculation.

	Mice				Rats			
	Suckling		Weanling		Suckling		Weanling	
Virus	IC	IP	IC	IP	IC	IP	IC	IP
GDVII	8.1*	2.2	6.6	0	7.1	2.4	0	0
TO	8.1	3.5	4.7	0	0	0	0	0
MHG	6.4	2.3	0	0	6.8	4.5	0	0
FA	6.4	2.6	6.7	0	2.0	1.7	0	0

*= $1/LD_{50}$ (log 10)

0 = No deaths

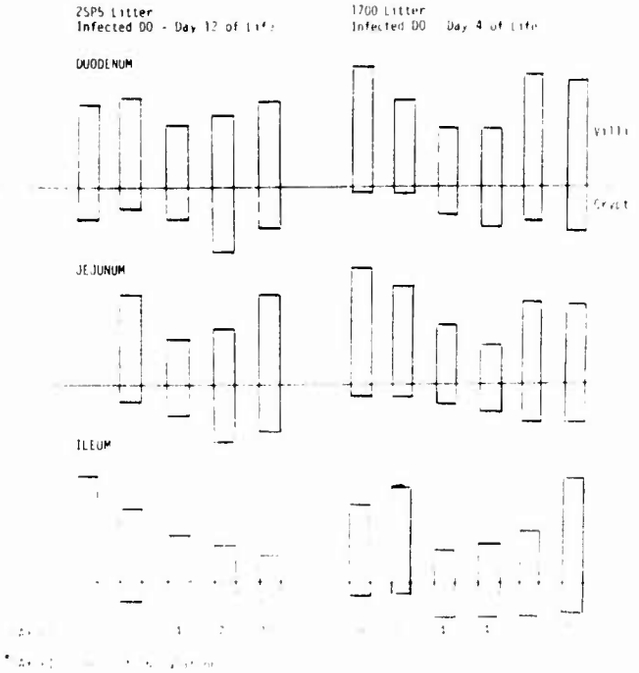

Figure 1. Relative heights of villi and crypts in small intestines of dogs infected with coronavirus 1-71.

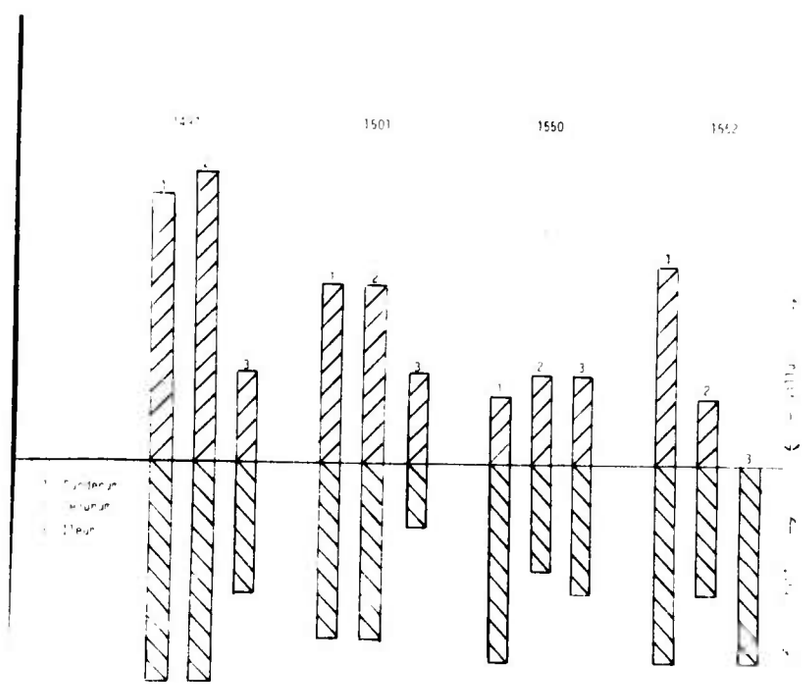

Figure 2. Height of villi and crypts of small intestine from four study puppies.

Project 3A162110A830 MILITARY DOG IMPROVEMENT

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 0B 6525	74 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
73 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	6.759A	3A762759A831	00	070
b. CONTRIBUTING				CH FR 051
c. [redacted]	CARDS 114F			

11. TITLE (Procede with Security Classification Code)

(U) Anti-Schistosomal Drug Development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010000 Pharmacology 020000 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 74	1.1	77
FISCAL YEAR — CURRENT 75	1.1	127

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC [illegible]

RESPONSIBLE INDIVIDUAL
NAME: Bueher, [illegible]
TELEPHONE: [illegible]

21. GENERAL USE

Foreign Intelligence Not Considered

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

22. KEYWORDS (Precede 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. Precede text of each with Security Classification Code.)

23. (U) Establish the Mortality Test System to screen 20 drugs weekly for prophylactic activity against S. mansoni infected albino mice. Presently no prophylactic drugs are available which prevent troops from acquiring schistosomiasis when deployed in endemic areas--South America, Africa or Asia.

24. (U) WRAIR provides drugs for prophylactic testing in mice exposed to 5000 S. mansoni cercariae. Each drug is screened for activity by the Mortality Test System using 5 mice per test dose at [illegible] mg/kg. If drug toxicity is observed, drugs are tested at [illegible] mg/kg. The 500 mg samples are adequate to cover both the routine and retest. All drugs are prepared in peanut oil and administered subcutaneously to infected mice 5 days after cercarial exposure. Drug activity is based upon an increased mouse survival. Since all infected non treated control mice are dead by the 50th day.

25. (U) 75 07 - 76 06. This research is complementary to studies being conducted under DA0B 7294, Work Unit 001, entitled Chemotherapeutic Studies on Schistosomiasis. The drug testing program is dependent upon a successful B. glabrata snail colony. A total of 500 S. mansoni cercariae shedding snails must be maintained to yield weekly 1.5 million cercariae needed for testing 20 drugs per week. When the snail colony was transferred to the Instituto de Medicina Tropical, an immediate water quality problem occurred. The water problem was identified as zinc from the galvanized water pipes. These water pipes were replaced with black iron pipes and the water problem was corrected. Snails were exposed in June to 5-10 miracidia which will provide, in July, the 500 infected snails. The Brazilian Paraiba strain of snail and S. mansoni infection will be verified to insure that the mortality response is similar to that observed with the Puerto Rico strain of snail and S. mansoni infection before prophylactic antischistosomal drug testing begins in August 1974. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*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 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 joint research program by University of Brasilia and Walter Reed Army Institute of Research was undertaken to test twenty candidate drugs weekly for prophylactic activity against schistosomiasis mansoni. Presently these are no known marketed prophylactic drugs, and there are no private, commercial and other government agencies directing resources towards finding prophylactic anti-schistosomal drugs. The Walter Reed Drug Screening Facility/Japan (1964-71) tested more than 50,000 drugs and identified four chemical classes that showed possible prophylactic activity. Subsequently, The WRAIR/Japan schistosome drug test unit was moved to Brasilia, Brazil in 1973 for the purpose of carrying out the prophylactic testing of these selected classes of compounds for anti-schistosome activity. In the event military forces are deployed in schistosome endemic areas (Caribbean, South America, Africa, and Asia) a major manpower loss will be due to schistosomiasis. This program undertakes to find an effective prophylactic drug which will conserve troop strength by preventing schistosome infections. The Mortality Test System (Radke, et al 1971) will be used to study drug activity in mice that are exposed to 3,000 S. mansoni cercariae. Drugs are administered subcutaneously and tested at 640 and 1920 mg/kg with five mice per test dosage. Drug activity is measured by increased survival time and worm burdens.

Progress.

No drugs were tested for prophylactic activity since the year was devoted to the physical move, training of personnel, and resolving the laboratory snail-water problem. Eight personnel were employed to carry-out the drug testing program. In September 1973, the 17 conex containers of drug testing equipment and supplies were moved into the newly constructed Center for Tropical Disease. The next four months were used to shakedown equipment and building defects. Sinks and special water filters were installed. However, the snail water quality problem was not resolved until March 1974 when the problem was identified as zinc which was coming from the galvanized water pipes. Since replacing the galvanized water pipe in April 1974 with black iron pipe, the laboratory snail colony has been producing large numbers of snails. Regular weekly snail exposures to miracidia started in June. These exposed snails will maintain the level of 500 cercariae shedding snails that are needed to carry out the prophylactic drug testing by the Mortality Test System. The actual drug testing will get underway in August 1974.

The University's animal facility stepped up the 18-23 gram, 38-48 day old albino mouse production to meet our weekly 400 mouse requirement.

The hot water heater was installed in June and it will be connected to the cage washer which will be delivered in July.

Literature Cited.

References:

1. Radke, M. G., Broome, P. B., and Belanger, G. S.: Schistosoma mansoni: Mouse Mortality Test System for Mass Screening for Prophylactic Drugs. Exp. Parasit. 30: 1-10, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6526	74 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
73 07 01	D. CHG	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	071
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			CH FR. 060

11. TITLE (Precede with Security Classification Code)

(U) Field Studies of Rickettsial Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 002 600 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: EXPIRATION:

b. NUMBER: NA

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	4.0	144
FISCAL YEAR CURRENT 75	4.0	250

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Inst of Research
Washington, DC 20012
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RESPONSIBLE INDIVIDUAL
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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: Singh, R. B.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Webb, C.R. COL
NAME:

21. GENERAL USE

Foreign Intelligence not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaysia; (U) Rickettsial Diseases; (U) Chiggers; (U) Mites

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 surveys in Southeast Asia in order to investigate the prevalence of scrub typhus vectors, the incidence of infection in specific tropical habitats, and to relate strain differences to degree of virulence and host response.

24. (U) A variety of modern biological techniques are employed to study the antigens present in R. tsutsugamushi and Ps. pseudomallei and to compare the host's response to these antigens. In addition, chigger vectors will be monitored on selected host species at a field study site at Bukit Lanjan, Selangor, Malaysia. Controlled chigger colonies will also be used to evaluate scrub typhus vector response to environmental factors and selected hosts.

25. (U) 73 07 - 74 06 This research is complementary to studies being conducted under DAOB 7533, Work Unit 097, entitled "Study of Tropical Diseases of Military Importance." For details see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1973 - 30 June 1974.

*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 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 071, Field Studies of Rickettsial Diseases

Investigators.
Principal: COL Charles R. Webb, Jr., MC
Associate: CPT Lyman W. Roberts, MSC, MAJ David M. Robinson, VC

1. Bionomic comparisons of infected and noninfected L. (L.) arenicola. Some bionomic data were collected on infected and noninfected L. (L.) fletcheri (USAMRU-M Annual Report 1969; Neal and Barret 1961), but no concurrently collected data were available for comparison of infected and noninfected mites. Naturally infected and noninfected L. (L.) arenicola colonies were established from collections in Mersing, Johore (USAMRU-M Annual Report CY 1973). Twenty infected and 25 noninfected females from the laboratory colonies were compared. Each female was maintained in a separate rearing container, and one noninfected male was added to each container. Data were collected on oviposition, eclosion, and sex ratios of progeny.

Infected mites produced significantly ($P < .005$) fewer eggs than noninfected mites. The infected mites averaged 55.9 eggs and noninfected ones 117.1 eggs per female. The percentage of eggs not hatching was slightly higher in the infected group (13.3 percent vs 10.9 percent for noninfected mites). This difference was not statistically significant but may be of biological significance. The sex ratio of progeny from the noninfected females was about 1.2:1 (females to male), while the infected females produced only female progeny, except for a single noninfected male. Observations are incomplete but it appears that either the rickettsia adversely affect the chiggers, or infected chiggers represent a genetic line that is lower in fecundity than the noninfected chiggers. In previous observations of infected and noninfected L. (L.) fletcheri, no such differences were apparent. If the rickettsial infection is harmful to the chigger, this would provide an explanation of the relatively low rates of infection in field collected chiggers. It would also support the contention that chiggers can become infected from vertebrate hosts, because natural selection would appear to favor noninfected chiggers. Additional bionomic data are needed from other groups of chiggers.

2. Transovarial passage and filial infection rates. Rapmund et al. (1969) found that the transovarial infection rate was 98 percent and the filial infection rate usually 100 percent over the first 5 laboratory generations of the infected L. (L.) fletcheri colony. Mouse passage and challenge results through 11 subsequent generations have shown no significant decrease in the transovarial or filial infection rates in this colony.

A previous infected colony of L. (L.) arenicola produced only 4 infected progeny over 2 laboratory generations, after which the infected line was lost (Rapmund et al. 1972). The present infected L. (L.) arenicola colony has now been monitored through 4 generations by single feedings of chiggers on mice. Results showed that 98.6 percent of 731 larvae from 43 separate females transmitted R. tsutsugamushi to mice. All of the 43 females transmitted transovarially. Thus, for the 4 generations observed, transovarial and filial infection rates in the infected L. (L.) arenicola colony were like those of the infected L. (L.) fletcheri colony. However, the rickettsial strain in the arenicola chiggers is highly lethal for laboratory mice, while the strain in the fletcheri colony is not.

3. Collection of additional vectors for colonization. Chiggers were collected from hosts and via the black plate technique from various locations in West Malaysia. Identifications were made from cast larval skins or by mounting progeny from individual females.

L. (L.) deliense was the only known vector collected from Bukit Lanjan, Selangor over a 3 year period. Scrub typhus isolation rates were relatively high in selected rodent hosts from this area (USAMRU-M Annual Report CY 1972). It seemed likely that the infection rate in L. (L.) deliense would be relatively high. However, of 1,234 larvae from Bukit Lanjan that were fed on laboratory mice, none transmitted R. tsutsugamushi. Additional mouse passage results are pending on 611 other deliense from the same area. Dissection and direct fluorescent antibody examinations of 420 L. (L.) deliense from Bukit Lanjan yielded only 8 larvae with scrub typhus rickettsia. Infections were very light in all but 2 of these 8.

L. (L.) scutellare is a proven vector of scrub typhus in Japan, but specimens collected previously in Malaysia have all been negative for R. tsutsugamushi (USAMRU-M Annual Report CY 1970). In June 1974, over 2,500 scutellare were collected on black plates from a banana grove in the Cameron Highlands, Pahang, Malaysia. All were fed in pools of 50 per mouse, and mouse passage results are pending. This species has only been reported from the Cameron Highlands area in Malaysia and may require a cooler average temperature than the other vector species. Consequently, postlarval stages are being held at an average temperature of 23°C.

Previously, L. (L.) arenicola has only been reported from sandy coastline habitats (Traub 1960; Upham et al. 1971). But recent collections from shaded, sandy soil near Kampong Sertik, Pahang, Malaysia yielded 153 specimens which conformed in appearance and range of measurements to the description of L. (L.) arenicola. The collection site is approximately 50 miles inland from the nearest coast. Ten of the specimens were mounted and the remainder were fed on laboratory mice. If adult mites can be obtained, they will be paired with L. (L.) arenicola and L. (L.) deliense from the laboratory colonies to determine if interbreeding with either species can occur.

4. Influence of temperature on R. tsutsugamushi infection in chiggers. Cavanaugh and Marshal (1972) reported that variations in climatic factors influenced the efficiency of Xenopsylla cheopis in transmitting the plague bacillus. In preliminary tests we found that vector chiggers could survive exposure to temperatures unfavorably high for growth of R. tsutsugamushi in cell culture. The possibility that infection rates in chiggers could be influenced by temperature is being investigated.

One hundred infected L. (L.) fletcheri larval from the laboratory colony were placed in a waterbath at 40°C for 72 hours. Of these, 36 survived and were fed on 3 laboratory mice. As a control, 50 other larvae from the same colony were fed on 5 additional mice. Mouse passage results showed that the heat-treated larvae failed to transmit R. tsutsugamushi to mice, while the mice fed upon by untreated larvae all became infected. Ten of the heat-treated larvae were recovered and are presently in the deutonymph stage. Progeny from these mites, if any, will be fed individually on mice to determine whether they can transmit R. tsutsugamushi.

If chiggers can be cleared of R. tsutsugamushi by temperatures unfavorably high for the rickettsiae it seems likely that temperature is a major limiting factor in the transmission of scrub typhus. Chiggers are probably exposed to host or ground temperatures of 40°C or more under natural conditions. The 72 hours exposure period used in the initial trials was chosen to simulate a typical feeding time on a host. Exposure for less time or even to slightly lower temperatures may affect transmission efficiency. These investigations will be continued to determine the temperature parameters of significance, if any.

Conclusions and Recommendations

1. Infected Leptotrombidium (L.) arenicola produced significantly ($P < .005$) fewer eggs than noninfected ones. Infected mites also had a higher percentage of eggs not hatching. Additional bionomic data are needed for comparisons of infected and noninfected mites.

2. Transovarial passage and filial infection rates in the infected L. (L.) arenicola colony are similar to those demonstrated in the infected L. (L.) fletcheri colony.

3. Infection rates in field collected L. (L.) deliense were very low in an area where high scrub typhus isolation rates in hosts was reported. L. (L.) scutellare, a vector species apparently restricted to Cameron Highlands in Malaysia, may require a cooler average temperature than other vector species. L. (L.) arenicola - like specimens were collected from an inland site.

4. Heat-treated L. (L.) fletcheri larvae from the infected colony failed to transmit R. tsutsugamushi to mice. Temperatures unfavorably high for rickettsial may be a limiting factor in scrub typhus transmission. Additional investigations are needed to determine the significant temperature parameters involved.

5. Response of Silvered Leaf-monkeys to Rickettsia tsutsugamushi. Over the past several years, we have been investigating the suitability of the Silvered Leaf-monkey (SLM) as a model for scrub typhus infections. In the last annual report, the response of a group of SLM to primary infection with the Karp, Kato, Gilliam, and TA 678 strains was described. In summary of these studies antibody responses were more promptly initiated in some strains than others, as were rickettsemias. These parameters were not related to severity of illness. All the inoculated SLM produced antibody regardless of illness, fever, or detectable rickettsemia.

During this reporting period, these SLM were challenged with homologous, closely related, and unrelated strains of Rickettsia tsutsugamushi. The responses are presented in Table 1. The TA 686 strain was not virulent for mice (not shown); and perhaps predictably, was not pathogenic in the control monkeys. None of the SLM inoculated with the TA 686 strain became febrile even though all were rickettsemic. This relationship does not apply to all the strains tested since the Kato strain which was uniformly lethal for mice produced intermittent fevers at the same dose levels in SLM, while the TA 678 strain which was avirulent for mice produced high persistent fevers at the same dose levels in SLM.

In measuring protection, the presence or absence of rickettsemia was not an acceptable criterion, since the majority of the SLM were rickettsemic for periods of time following challenge with related strains. A decrease in the febrile period to $\frac{1}{2}$ of that of control animals (Ormsbee, R.A., E.J. Bell, D.B. Lachman, and G. Tallent. 1964. The influence of phase on the protective potency of Q fever vaccine. J. Immunol. 92: 404-412) has been used as a measure of protection. When this criteria was applied, protection was afforded against challenge with most strains of any relatedness regardless of antibody status at or prior to challenge. Exceptions to this were SLM 195 which had 5 consecutive days of temperature $\geq 103^{o}$. This was identical to control animals. SLM 211 and 212 had 11 and 5 days of fever respectively while the 3 controls averaged only 2 days of fever. SLM 195 was convalescent from Kato and challenged with Kato. The other SLM were convalescent from TA 678 and were challenged with TA 586, which is a completely unrelated strain by FA techniques.

Prior to challenge, all the SLM had FA antibody titers of 10 or less to the challenge strain. The presence or absence of fluorescent antibody to the particular challenge strain was not an important factor in protection. ("Unrelated" strains were chosen because of (1) their inability to elicit antibody which cross-reacted with the original strain, as well as (2) their lack of related FA antigens on direct

Table 1. Selected responses of convalescent SLM to challenge with R. tsutsugamushi.

SLM No.	Strain Original	Strain Challenge	Febrile Response to Challenge	Rickettsemia on Indicated Day Following Inoculation 3	7	10	14	21	28
189	Karp	Karp	-[a]	0[d]	0	0	+	0	0
				0	+	+	0	0	0
190	Karp	TA 686	NA[b]	0	0	+	+	+	+
				0	+	+	+	+	+
192	Karp	TA 678	-	0	+	+	+	+	+
				0	0	0	0	+	0
193	Karp	TA 678	-	0	+	+	+	+	0
				0	0	0	+	+	0
195	Kato	Kato	+[c]	0	0	0	0	0	0
				0	+	+	+	+	0
199	Kato	TA 686	NA	0	+	+	+	+	0
				0	+	+	+	0	0
200	Kato	TA 678	-	0	+	+	+	0	0
				0	0	0	+	0	0
201	Gilliam	Gilliam	-	0	0	0	+	0	0
				+	0	0	0	0	0
202	Gilliam	TA 716	-	0	+	+	+	+	0
				+	+	+	+	+	0
204	Gilliam	TA 716	-	0	+	+	+	+	0
				+	+	+	+	0	0
205	Gilliam	TA 678	-	+	+	+	+	0	0
				0	0	+	0	0	0
206	Gilliam	TA 678	-	+	+	+	+	0	0
				+	0	+	0	0	0
207	TA 678	TA 678	-	0	0	0	0	+	+
				0	+	0	0	0	0
208	TA 678	TA 678	-	0	0	0	0	+	0
				0	0	0	0	0	0
209	TA 678	TA 716	-	0	0	+	+	0	0
				+	+	+	+	+	0
210	TA 678	TA 716	-	0	0	0	+	+	0
				0	+	+	+	+	0
211	TA 678	TC 586	+	0	0	+	+	0	0
				0	+	+	+	+	0
212	TA 678	TC 586	+	0	0	+	+	0	0
				0	+	+	+	+	0

Table 1 (Continued)

SLM No.	Strain Original	Strain Challenge	Febrile Response to Challenge	Rickettsemia on Indicated Day Following Inoculated 3	7	10	14	21	28
213,214, 215		TC 586 control		0	+	+	+	+	0
				0	+	+	+	+	0
				0	+	+	+	+	0
216,217, 218		TA 686 control		+	+	+	+	+	+
				+	+	+	+	+	+
				+	+	+	+	+	+
219,220, 221		TA 716 control		0	+	+	+	+	+
				+	+	+	+	+	0
				+	+	+	+	+	+

a - total consecutive days temperature $\geq$ 103°F less than $\frac{1}{2}$ that of control animals.

b - not applicable, no fevers in TA 686 controls.

c - total consecutive days temperature > 103°F greater than $\frac{1}{2}$ that of control animals.

d - first line - original inoculum, second line - challenge inoculum.

analysis.) SLM in which we had never detected humoral antibody to the challenge strain were protected (SLM 192, 193, 200, 205, 206, 211, and 212). SLM 195 had a peak titer of 1/80 to Kato which had decreased to < 1/10 on challenge and was not protected.

The post-challenge antibody responses of these SLM have not been completed, but results on two of the 18 challenged indicate that anamnestic responses occur to the homologous challenge antigen. The titer appears quicker and rapidly plateaus at high levels. With related challenge strains, the titer to the original strain did not appear as quickly or attain as high a level as following homologous challenge. This occurred in spite of the related strain producing titers to the original strain in control monkeys. Whether these relationships will prevail over the entire experiment is uncertain.

Summary

Silvered leaf-monkeys who had recovered from infections with selected strains of R. tsutsugamushi have been challenged with homologous, closely related, and unrelated strains. While 17 of 18 were rickettsemic, febrile responses were less than half of those of controls in 13 of 16 of the SLM. With only two of the monkey's sera antigenically typed the homologous challenge appeared to produce an amnestic response while the related challenge did not.

6. Relatedness of naturally occurring strains of R. tsutsugamushi. While antigenically identifying strains for other purposes it became apparent that those isolated from a specific species of rodent were related. The original observation was on strains isolated from Rattus argentiventer trapped in widely scattered areas of Malaysia. All of the strains isolated were identified as containing predominantly the Karp antigen with a minor component of TA 763. The present infected Leptotrombidium (L.) arenicola colony was derived from a female chigger taken from a R. argentiventer captured in Mersing, Johore; and contained a strain which was antigenically indistinguishable from the mammalian strains. The strain present in a former positive L. arenicola colony, which was lost, was retrived from storage, passed in mice, and proved to be indistinguishable from the previous strains.

With this background data, we initiated a survey to examine isolates from a broad range of mammalian and arthropod vector species. Immediately, the problem of low rickettsial titers in the mouse became apparent.

Briefly, the typing procedure involves the passage of the strain in the white mouse a variable number of times until sufficient adaption or selection occurs to allow the rickettsia to grow to a high titer. At each passage a series of antigen spots are prepared from a centrifugal pellet of the washing of the peritoneal cavity and examined by means of direct fluorescent staining with relatively

strain-specific FITC labelled antibody and unlabelled blocking antibody. If the smears contain insufficient numbers of rickettsia, the reserve mice which were inoculated at the same time the antigen spots were prepared are killed at 14 days post-inoculation and the procedure repeated. Thus, each unsuccessful procedure wastes two weeks. Some strains have been passed 3 times and titers have not yet proven satisfactory.

In virulent strains maximum titers are not attained and death does not occur prior to 8-10 days. Thus, the development of the immune response - as opposed to interferon, interference, etc. - is probably the important factor in the recovery of animals from scrub typhus infections. If this is the case we postulated that a blunting of this response by some means would allow the organisms to multiply to higher titers in the host.

The most practical method at our disposal was immunosuppressive drugs. While azothioprine and 5-fluorodeoxyuridine tended to inhibit the humoral antibody response as measured by FA, they had no effect on the virulence of the rickettsia. No increase could be seen in a subjective assessment of the number of organisms present in the peritoneal cavity following administration of the drugs. Conversely, the administration of cyclophosphamide (CYC) strongly enhanced the virulence of normally avirulent organisms and dramatically increased the number of organisms present on the prepared spots.

In order to determine the optimum time to administer the CYC the experiment depicted in Table 2 was conducted. The optimum time for administration was after 4 days, and our present standard technique calls for the administration of an 8 mg dose on day 5 post infection and a 4 mg dose on day 8. This regimen of treatment has been successful in elimination of the occasional mouse that otherwise does not die even though infected and given the single 8 mg dose.

With this background data, we resumed the identification of strains isolated from natural sources and the results were radically at variance with those observed in the work without CYC treatment. The antibody response to strains was much broader and the number of antigens detected in direct FA analysis of smears prepared from treated mice was increased. At this point, we decided to reevaluate the antigens present in each of the 9 prototype strains. This was necessary to have a basis to refer to locally isolated strains as Karp like, Kato like, etc. In Table 3 is presented a comparison of the results obtained with the TA 686 strain. The antibody titers on the cyclophosphamide treated mice were determined on 28 day bloods from survivors of a single 8 mg dose on day +4. The rickettsia were prepared from the peritoneal cavity of treated mice moribund on day 13, and the control rickettsia were harvested the same day from mice which appeared normal.

Subjective evaluation of numbers of organisms in the spots was discouraging, but significant increases in antibody titers to the TA 763

Table 2. Effect of a single dose of Cyclophosphamide on the pathogenicity on an avirulent R. tsutsugamushi strain.

Day CYC (8 mg IP) given[a]	Day Post TA 686 Inoculation												
	8	9	10	11	12	13	14	15	16	17	18	19	20
-1	100[b]	97	85	76	59	47	35	29	29	29	29	26	26
0	100	100	100	95	95	90	88	85	83	83	83	83	83
1	100	100	100	92	78	64	53	44	44	44	44	44	44
2	100	100	97	86	53	39	39	39	36	36	36	36	36
3	100	100	97	92	46	8	8	5	5	5	5	5	5
4	100	97	92	78	72	31	0						
5	100	95	95	79	59	44	8	0					
6	100	100	92	87	87	49	23	0					
7	100	100	100	83	75	68	53	8	0				
0[c]	100	100	100	100	100	97	97	97	97	97	97	97	97
None[d]	100	100	100	100	100	100	100	100	100	100	98	98	98

a - day 0 all mice were challenged with 10^4 MIPID 50 of the TA 686 strain.
b - percent survivors(survivors at 8 arbitrarily assigned 100% value)
c - CYC only on day 0, no rickettsia.
d - TA 686 only on day 0, no CYC.

Table 3. Antigenic comparison of the TA 686 strain of R. tsutsugamushi in mice administered Cyclophosphamide and controls.

		Prototype Strain Antigen[a]			
Antibody	Karp	TA 686	TA 716	TA 763	TH 1817
CYC	80[c]	80	160	40	160
No CYC	80	40	80	>10	10

		Prototype Strain Antibody[b]			
Rickettsia	Karp	TA 686	TA 716	TA 763	TH 1817
CYC	+[d]	1+	1+	2	+
No CYC	±	1	1	1+	±

a - titers were < 1/10 for the Kato, Gilliam, TC 586, and TA 678 strain antigens with or without CYC.

b - no rickettsia were detected which stained with the antibody to antigens noted in footnote a.

c - reciprocal FA titer.

d - ± a few specific staining rickettsia on the entire smear; + a few rickettsia in each 400 x field; 1 - 100 rickettsia per field; 2 - 200 rickettsia per field.

and TH 1817 strains were seen. If these antibodies had been prepared under identical circumstances, we would characterize the eliciting strains as being related but not identical. We are presently involved in repeating these observations to determine the reproducibility of the observations. At the same time we are preparing a large number of strains for typing to detect relationships among strains from the various habitats/vectors/vector hosts if they are present.

7. Assessment of the efficacy of blood spots to sample wild caught mammals for antibody to R. tsutsugamushi. In projects conducted by the Department of Medical Ecology over the past 3 years the presence or absence of antibody has been detected by the elution of material, including globulins, from blood spots dried on filter paper. In a review of this data Dr. Muul found that almost concurrent with the change from whole sera to blood spots the percentage of positives seemed to drop to 1/3 of that formerly seen. A series of experiments were designed to determine if the observed differences were real or artifactual.

Blood was drawn and paired blood spots were prepared from a series of wild trapped Rattus spp. Sera was separated and frozen. One set of spots was assayed in the normal manner after storage at room temperature. The other set of blood spots was stored in the jungle for one month prior to assay. During this time the sera were retrieved from the freezer and assayed. All samples are to be assayed at two month intervals following the initial processing

Insufficient samples have been assayed to draw conclusions over a period of time, but a total of 366 paired sera/blood spot samples have been completed using both sera and the present technique which provides for elution of the spot for $\frac{1}{2}$ hour at 37°C and all but 22 with elution overnight at 4°C. Results of this study are presented in Table 4. The group number refers to a combined pool of globulins prepared from related species of mammals and used to elicit antiglobulins in rabbits for the indirect FA.

In R. sabanus, employing the Group I conjugate, the standard filter paper spot technique detected only 76% of the animals that were positive on assay of whole sera, and 9% of the samples that were negative with sera were positive with the standard filter paper spot technique. If we combine these numbers the filter paper technique was wrong in 28 of 166 samples (17%). The overnight technique gave results that were more at variance with the sera than the standard technique. In K. muelleri using Group IV the results with either the standard or overnight techniques are not significantly different from whole sera. The Group III results are intermediate in that no false positives were detected with the filter paper spots but only 76% of the sera positives were detected by these techniques.

The only variable in the techniques was the rabbit origin anti-rattus globulins. We are comparing the antiglobulins against whole sera and eluants from blood spots by immunodiffusion and

Table 4. Comparison of whole sera and blood spots for the detection of *R. tsutsugamushi* antibody.

		SERA			
		Positive[a]		Negative	
Species	Group	Standard[b]	Overnight[c]	Standard	Overnight
Rattus sabanus	I	67/88(76)[d]	49/74(66)	7/78(9)	8/73(11)
Rattus tiomanicus jalorensis	III	28/37(76)	28/37(76)	1/107(1)	0/106(0)
Rattus muelleri	IV	42/47(89)	43/45(96)	0/9(0)	0/9(0)
Total		137/172(80)	120/156(77)	8/194(5)	8/188(4)

a - reactive at a dilution of 1/40 or greater
b - elution ½ hour at 37°C from filter paper blood spot
c - elution overnight at 4°C from filter paper blood spot
d - positive/total examined (percent positive)

immunoelectrophoresis. Preliminary results by immunodiffusion have shown an identical antigen present in both sera and eluants, and an additional antigen present in the sera which was not present in the eluants. There are also differences in the titers of the working dilutions of the antiglobulins.

It has been concluded that the filter paper technique, in addition to being a desirable technique for field work, can be an accurate reflection of the results obtained from whole sera. The key to the problem is proper production and standardization of the antiglobulin. We will continue to examine the properties of the several antiglobulins in use to detect which titration technique for standardization will yield results comparable to whole sera.

8. The incidence of scrub typhus antibodies in residents of West Malaysia (in cooperation with Dr. Donaldson, Department of Bacterial Diseases). The occurrence of scrub typhus antibody detectable by fluorescent antibody techniques in humans residing in various habitats has been investigated in earlier work by Cadigan et al. (1972) in this Institute. These studies were restricted to Orang Asli (the aboriginal inhabitants of the Malay Peninsula), and found that deep jungle dwellers had higher incidences of antibody than fringe dwellers, who in turn had higher incidencesthan village (kampong) dwellers.

Shishido (1962) in Japan reported on the incidence of complement fixing antibody in school children and adults on Hachijo Island and in soldiers at the foot of Mt. Fuji. In both areas an incidence of 5-15% unrecognized scrub typhus infections were found in adults. The prevalence in school children was much higher at approximately 50%.

Audy (1961) stated that clinical scrub typhus is an occupational disease and that differences in prevalence in age groups, between the sexes, and among occupations can all be explained by behavior or occupation. This is to say that people who develop clinical disease are those who invade infected mite islands.

We were interested in examining a number of randomly collected sera samples to ascertain three facts: (i) the incidence of scrub typhus antibody detectable by fluorescent antibody techniques in a broad spectrum of residents of Malaysia; (ii) whether the incidence is related to occupation, habitat, age, sex, etc.; and (iii) whether the incidence of antibody paralleled earlier reports on the incidence of clinical disease (Lewthwaite, 1930).

Materials and Methods

Bloods were collected by venapuncture from a wide age and occupational range of individuals. The cells were allowed to clot, and the sera was removed and stored at -20C.

Antibody assays were conducted by the indirect fluorescent antibody method of Bozeman and Elisberg (1963). The trivalent yolk

sac antigen contained a mixture of the Karp, Gilliam, and Kato strains.

Results

Sera were collected from the states of Selangor, Kedah, Perlis, and Kelantan of Peninsular Malaysia. The total number of sera collected was 1,003, and the ages of the individuals from whom samples were drawn ranged from 16 to 85. There were 586 males and 417 females in the sample (Table 5), and over $\frac{1}{2}$ of the sample was from Kelantan.

No consistent differences were detected in the percentage positive when the samples from each state were compared or when males were compared with females. The sample drawn from Perlis had a greater percentage of positives than samples from the other states, but the difference was not significant ($P > 0.05$). The male sample from Perlis did contain a significantly greater ($P < 0.025$) percentage of positives than the samples from the other states, but when the results from one kampong were deleted no significant difference ($P > 0.05$) was detected. Individual kampong differences will be discussed later.

In order to determine the influence of age on seropositivity the population was grouped in 10 year increments, and the number of positive sera was determined in each of these increments (Table 6). Although the sample which included everyone under age 25 contained a lower percent positive the difference was not significant ($P > 0.05$). The percent positive sera did not increase with age. This was a reflection of the sample distribution which did not contain any children and few young adults under 25. In comparing the geometric mean antibody titers there was a progressive increase from the youngest age group to the oldest; however,this increase was not impressive. The increase is probably a reflection of the higher titers and increased persistence of antibody following repeat infections.

Table 7 presents the results of grouping the data based on occupations. Not all of the people from which the samples were drawn could be placed in these groups e.g. military, government workers, etc. In these occupations some individuals spend a great deal of time in forest habitats while others work in offices exclusively.

As is to be expected from the literature, those people who live and work in close proximity to the habitats of the hosts for the vector species have the highest incidence of antibody (rubber tappers/ field workers) while those who work in areas where the vectors are rare have low antibody incidence (urban workers). The high incidence in a small sample of housewives was attributed to the habits of women who live in the villages who often work in padi and are responsible for the gathering of firewood if no children are at home.

Table 5. Comparison of number of individuals serologically positive for R. tsutsugamushi from each state.

State	Male	Females	Total
Selangor	23/61[a] (36)[b]	15/39 (38)	37/100 (37)
Perlis	28/59 (47)[c]	26/71 (37)	54/130 (42)
Kedah	42/149 (28)	37/95 (40)	79/244 (32)
Kelantan	101/317 (32)	80/212 (38)	181/529 (34)
Total	193/586 (33)	158/417 (38)	351/1003 (35)

a - positives/total
b - percent positive
c - $p < 0.025$

Table 6. Incidence and level of R. tsutsugamushi antibody in relation to age.

Age	Sample	Titer
< 25	14/49[a] (29)[b]	56[c]
25-34	81/213 (38)	64
35-44	112/323 (35)	72
45-54	88/255 (35)	76
55-64	41/121 (34)	112
> 65	15/42 (36)	152

a - positives/total
b - percent positive
c - reciprocal geometric mean titer

Table 7. Incidence of R. tsutsugamushi antibody in relation to occupation.

Occupation	Sample	Probability
Padi Workers	270/725 (37)[a]	> 0.05[b]
Rubber Tappers/ Field Workers	42/68 (62)	< 0.005[b]
Housewife	7/17 (41)	> 0.05[b]
Urban	29/141 (21)	< 0.005[d]
Total	348/951[e] (37)	-

a - positive/total (percentage positive)
b - no significant difference from total
c - significant increase from total
d - significant decrease from total
e - not all occupations were given or could be classified into the above groups.

Discussion

In their study of aborigines Cadigan et al. (1972) postulated that infection produces symptomatic disease in most individuals. Our results indicate that such is not the case. If we assume that antibody have been 5 to 10 years by Shishido, 1962) the annual attack rate will approximate 3% according to Muench (1959)*. The 10 year estimate yields a value of 6% for the attack rate and a 5 year persistence of antibody produces an attack rate of 11.5%. In laboratory primates the antibody elicited by a 1° infection persists for less than a year (unpublished data), but the majority of the samples from natural sources are probably 2° infections.

The variation in percent positives among the occupations parallels early reports from this Institute on the source of clinical cases of scrub. Lewthwaite (1930) emphasized the high rate of clinical illness occurring in workers on oil palm and rubber estates. In our sample the highest rate of positives were in the individuals who were employed as rubber tappers or wood cutters. Because of the nature of their occupations these people spend a great deal of time in wooded areas - plantations, scrub, and forest. The rates in padi workers were also elevated.

Traub and Wisseman (1972) have postulated that the geographical distribution of scrub typhus in the world is based on the occurrence of the Rattus rattus spp of rats. It would appear from our study that the distribution of scrub typhus within Peninsular Malaysia is coincidented with the distribution of these species which are common in forested areas, plantations, and rice padi but infrequent in urban areas. It is still unclear whether these rats serve as reservoirs for the rickettsia or only as hosts for the chiggers.

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* If y = percent positive, a = attack rate, b = reversion rate and t = time of exposure then $y = \frac{a}{a + b}(1 - e^{-(a + b)t})$

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 071 Field Studies of Rickettsial Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6527	74 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
73 07 01	D. CHG	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. CONTRIBUTING	CARDS 114F			CH FR. 061

11. TITLE (Precede with Security Classification Code)

(U) Ecological Surveys of Tropical Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 002 600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

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	108

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

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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 and North Sumatra.

24. (U) Surveys were conducted primarily on the 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. Additional surveys were conducted at selected sites in North Sumatra.

25. (U) 73 07 - 74 06 This research is complementary to studies being conducted under DAOB 7533, Work Unit 097, entitled "Study of Tropical Diseases of Military Importance." For details see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1973 - 30 June 1974.

*Available to contractors upon originator's approval

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Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 072, Ecological Surveys of Tropical Diseases

Investigators.
Principal: COL Charles R. Webb, Jr., MC
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1. Pseudomonas pseudomallei in rice growing areas of Peninsular Malaysia. This study is complete. Changes in prevalence of the organism in padi water and as expressed by human serology are described when comparison is made with studies of previous years[1-3]. A case is made for considering IHA titers of 1 in 10 and more as evidence of experience in persons from areas of endemicity. Chromobacterium violaceum is shown to be a much more common soil or padi water organism. Clinical awareness of both infections is stressed for the medical management persons from South East Asia with fever of uncertain origin.

Introduction

During the years 1964-67 several studies[1-3] were undertaken in this Unit to assess the prevalence of Ps. pseudomallei. These supported the earlier findings[4], that Ps. pseudomallei was a relatively common soil inhabitant in South East Asia.

Antibody prevalence in Thais has been studied by Nigg[5] and inapparent infection noted to be frequent.

The rice economy of Malaysia has altered radically over the last decade, the major areas of production now having been under double-cropping for 11 (Selangor) 4 (Kedah and Perlis) and 2 (Kelantan) years respectively.

Sneath et al[6] have stated that Chromobacterium violaceum is found commonly in water and soil of tropical countries and can also be isolated from temperate soils. The similarity of pathology in melioidosis and in Chromobacterium violaceum infection has been commented by Snowe[7].

Materials and Methods

Four States, representing the 3 main rice growing areas were selected for study.

Samples of padi water were taken at approximately 1 mile intervals from flooded fields in all stages of preparation up to those with well established plants. Processing of the samples was performed in

-gional hospital laboratories by USAMRU-M staff, isolation of the organisms being attempted by cultural and animal inoculation methods. Water temperature and pH were recorded. Soil cores taken at every 4th water sampling site were estimated for chloride ion. Villagers from randomly selected sites were bled of 10 ml venous blood and antibody levels measured by the IHA test of Ileri, as modified by Alexander et al[8]. Control sera were obtained from hospital personnel or blood donors.

Results

The results of water and soil examination are summarized in Table 1; those of the serologic survey, in Table 2. A comparison of the present survey and those done between 1964-67 is made in Table 3.

In Table 4 the isolation rates of Ps. pseudomallei are compared with antibody prevalence in the rice growing population of different States.

Figure 1 shows the distribution of population samples according to IHA titers of 1:10 or more.

Discussion

The prevalence of Ps. pseudomallei in surface water is reduced from former years. This reduction is greatest in areas which have been longer exposed to double cropping methods. The requirement for ample surface water for growth of the organism is well met with double cropping yet there appears to be a reduction of numbers under those conditions. If the organism is ubiquiteous it is difficult to believe in it being subject to selective wash out, especially since another soil inhabitant - Chromobacterium violaceum - does not suffer in the process. The ecologic niche of Ps. pseudomallei remains uncertain.

In an earlier serologic survey by Strauss et al[3] on small numbers of Malaysian Army recruits, the highest prevalence of antibody was found in those resident in Kedah-Perlis, followed closely by them from Kelantan. The present findings show that prevalence in Kedah-Perlis is much less then in Kelantan. Selangor, which has had the longest double cropping experience, shows the lowest prevalence. Since re-infection would serve to boost antibody titers in a population continually exposed, it can be concluded that the lower prevalence of antibody reflects well the decreased rates of isolation. At the present time this is so, as Table 4 illustrates.

Clayton et al[9] question whether an IHA titer of 1:10 or more, instead of the more usually accepted 1:40 or more, should be considered as evidence of prior exposure. If readings of <1:40 were in part due to antigens shared with other organisms, it would be expected, in any population where results are plotted as in Figure 1, that a shoulder would occur in the curve at the 1:10 and 1:20 level. That this is not the case, suggests that levels of 1:10 and more indicate prior exposure,

Table 1

Results of water examination in four Malaysian States

State	Period of sample collection	Soil Condition	Water Samples				Isolates		Soil Cl⁻ mEq/100G upper/lower	Estimate* of Hirudinaria spp prevalence
			No.	Temp° C	pH	Cl⁻mEq/100ml	Ps. pseudomallei	Chromo. violaceum		
Kelantan	May	Flooded: Late in cultivation: Much planting	180	30.9 (25.6-37.1)	5.0 (4.0-5.3)	0.09 (0.004-2.17)	30 (17%)	160 (89%)	0.013 (0.004-0.135) 0.006 (0.002-0.06)	Abundant, widespread
Kedah -Perlis	March	Flooded: Early in cultivation: Little planting	132	31.3 (26-39.5)	5.2 (4.0-6.2)	0.04 (0.004-0.52)	5* (<4%)	63 (48%)	0.115 (0.004-0.64) 0.08 (0.002-0.42)	Low, localized
	July	Flooded: Planted Good growth	61				4 (6.5%) (4.7%)	46 (75%) (57%)		
Selangor	April	Flooded: Cultivating: Some planting	62	33.5 (28.7-39.5)	5.0	0.04 (0.004-0.13)	0 (0%)	54 (87%)	0.01 (0.002-0.02) 0.012 (0.002-0.02)	None
	June	Flooded: Planted: Good growth	62				5 (8%) (4%)	58 (93%) (90%)		

Ranges shown in brackets

* One of these positive results was obtained only in culture.

* See text

Table 2. Population of both sexes sampled for antibody to Pseudomonas pseudomallei.

State	No.	No. under 30	Age Range	Avg Age	Avg Padi Years	Ps. pseudomallei IHA titer 1:40 and more	1:10 and more
Kelantan Padi	399	37	16-84	44.3	24.5	62 (15.5)	144 (35)
Kedah-Perlis Padi	292	23	20-85	43.5	24.9	18 (6.2)	52 (17.8)
Selangor Padi	100	7	21-73	41.8	22.4	2 (2)	6 (6)
Total Padi	791	67		43.7	24.4	82 (10.4)	202 (25.5)
Kelantan Control*	91	24	19-74	35.6	0	3 (3.3)	6 (6.6)
Kedah-Perlis Control	89	20	21-65	39.5	0	2 (2.2)	10 (11.2)
Total Control	180	44		37.5	0	5 (2.8)	16 (8.9)

* The Kelantan control group included 37 from a purely rubber growing village in which one of the females had a titer of over 1:40 and one each of the males and females titers of 1:20.

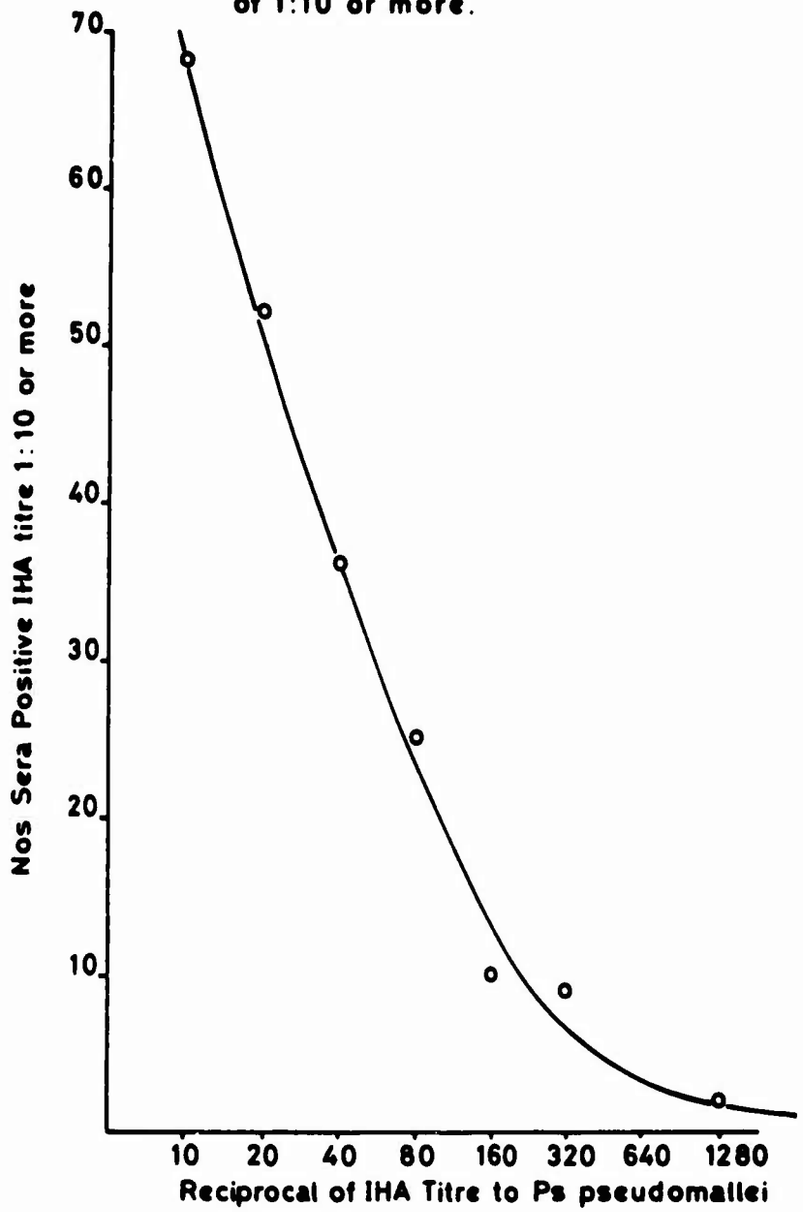
Table 3. Isolation of Ps. pseudomallei from surface water of wet rice fields in 1964-67 (1) compared to those in the present investigation.

Year	Total Samples	No. Positive
1973	455	33
1964-67 ($2\frac{1}{2}$ yr study)	753	110
1967 (short term study)	45	15

Table 4. Isolation rates of Ps. pseudomallei from water related to antibody prevalence in rice growing people.

State	% Water Samples Positive	% IHA Titers 1:40 or more
Kelantan	17	15.5
Kedah-Perlis	4.7	6.2
Selangor	4	2

Figure 1 **Distribution of Population Samples according to Ps pseudomallei titres of 1:10 or more.**



in people from an endemic area. The recrudescent nature of the disease has been commented on by Flemma et al[10] among others: persons returning from areas of endemicity should be viewed with suspicion even at IHA levels of 1:10 (especially if they are subsequently subject to severe trauma such as burning).

The recovery rates of Chromobacterium violaceum are much higher than those of Ps. pseudomallei. Despite the rarity of apparent disease caused by both of these organisms, diagnostic awareness must be preserved.

2. Ps. pseudomallei in Silvered Leaf-Monkeys. Macaques are the only primate models used thus far in experimental melioidosis[11-14]. These are frequently found on the ground whereas the Silvered Leaf-Monkey, (SLM) (Presbytis cristatus) is predominantly a tree dweller and much less often comes in contact with the habitat of Ps. pseudomallei. The numbers of macaques for research are dwindling[15] but the leaf monkeys are largely untried.

Introduction

Presbytis cristatus was used in studies on scrub typhus[16]. Recent introductions to the animal colony have had no significant IHA titer for melioidosis while positive titers have been shown in macaques.

Materials and Methods

Strains of Ps. pseudomallei which were recovered from human cases during 1964-68 and had been stored on nutrient agar were used as test organisms. Hamsters were consistently very sensitive to very small inocula; they have been used as a method of detection of Ps. pseudomallei in SLM blood, in addition to conventional cultural methods.

Antibody levels were measured by the IHA test, all monkeys having pre-injection negative readings. Suspension of Ps. pseudomallei in water were prepared and doses of from 100 to 5 x 10^3 organisms given, usually by the subcutaneous route into the test animals left thigh.

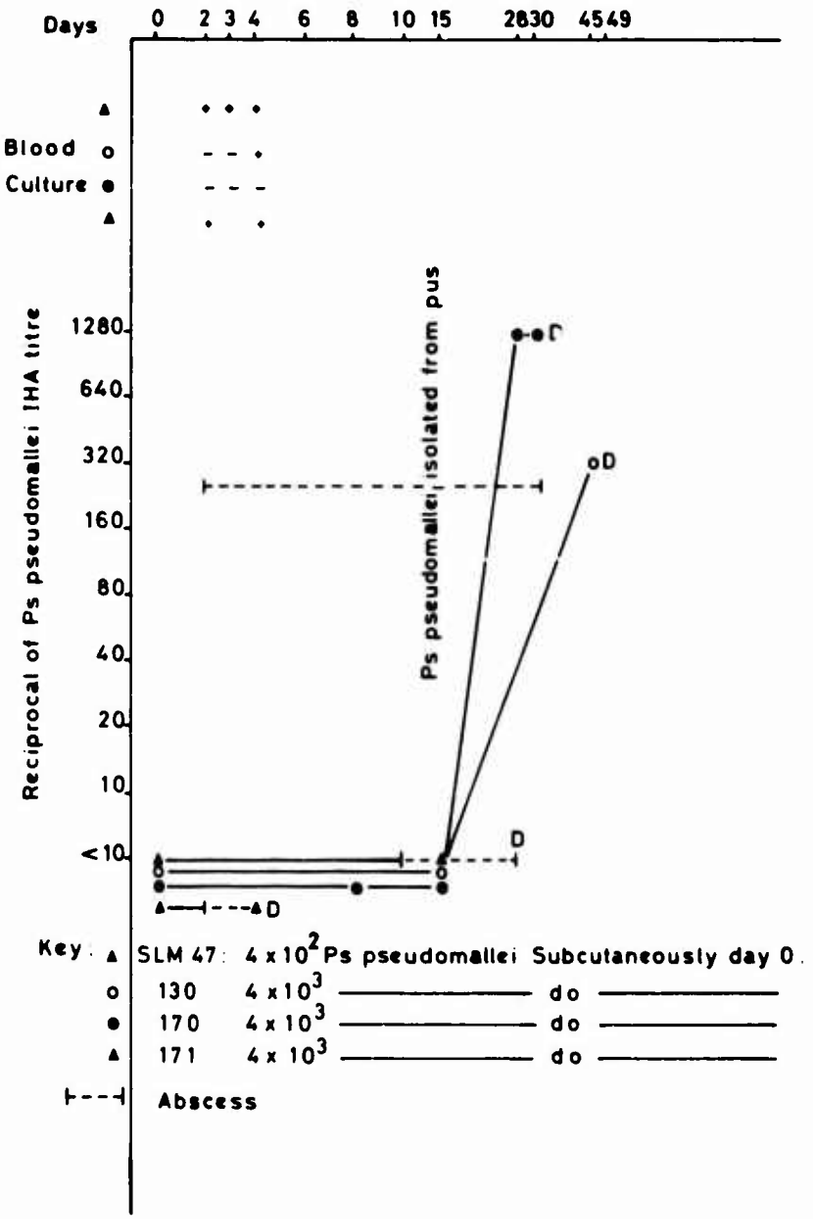
Blood cultures were performed usually on the first, second and third post exposure days and occasionally later. Abscesses which formed were cultured, directly if they ruptured or aspirated and cultured if not. Animals which died were submitted to autopsy. Histologic studies were not done.

Serologic studies were performed several times in the first two months then at approximately monthly intervals.

Results

Figures 1-3 show a selection of the results from the 22 animals used.

Figure 1 Showing the Course of infection with Ps pseudomallei in 4 SLM with death the outcome.



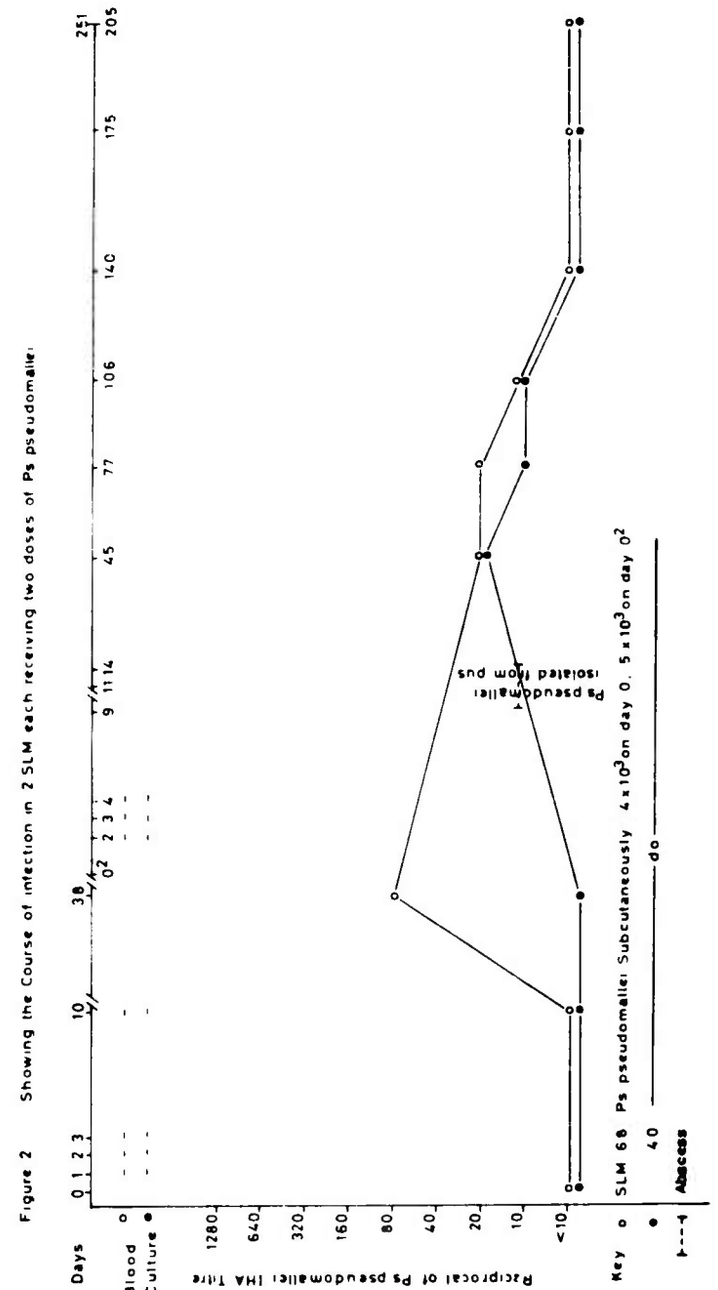
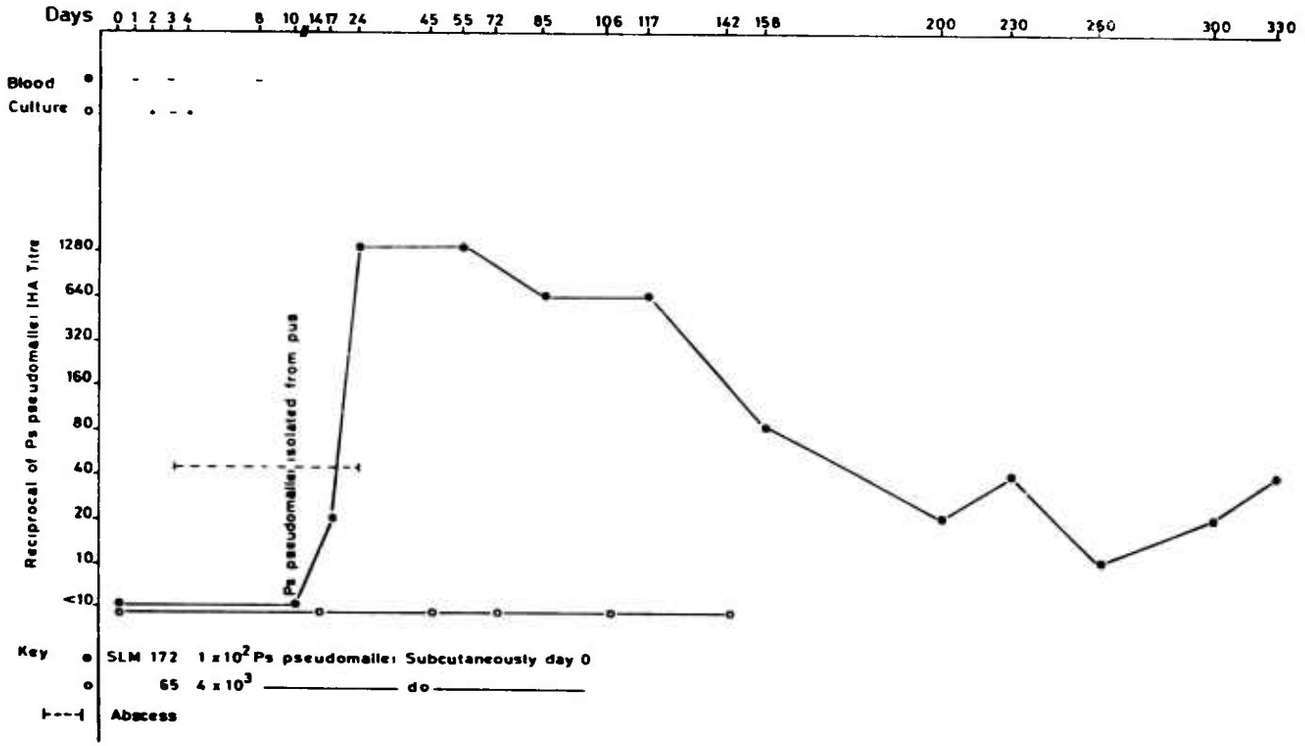


Figure 2 Showing the Course of infection in 2 SLM each receiving two doses of Ps pseudomallei

Figure 3 Showing the course of infection in 2 SLM receiving different doses of Ps pseudomallei



Discussion

There is no rigid dose-response in Silvered Leaf-Monkeys to exposure to Pseudomonas pseudomallei. Effects were seen following as few as 100 organisms given subcutaneously; none resulted from 4×10^3 given by a similar route. No pattern can be defined except the variability of response which is thought to occur in the human disease. A spectrum of effect is observed, including fulminating septicaemic (SLM 171), subacute, self limiting (SLM 40) and moderately chronic disease with a good immunologic response (SLM 172). The last may represent recrudescent disease since there is a tendency for antibody levels to show a secondary rise at about 300 days post-exposure.

Persistence of titers at varying levels up to 2-plus years in confirmed cases of human melioidosis has been shown by Alexander. The results of the present study correspond in some measure with his findings. Titers in the animals which survived have persisted over periods of from 70 to 330-plus days, the longest in persistence showing a level of 1:40.

The use of a single strain, which, by colonial morphology, gave no indication of antigenic alteration throughout the study, and which has consistently retained its capacity to cause hamster death over the same period, indicates that the variability of response shown is a characteristic of the host.

From the findings it can be concluded that the Silvered Leaf-Monkey of Malaysia (Presbytis cristatus, Raffles, 1821), as an animal model for melioidosis, reflects very well the disease processes as expressed in humans and emphasizes the great variability of this infection.

3. The leech (Hirudinaria spp.) in transmission of bacterial disease. While Ps. pseudomallei can survive in the same general environment as Hirudinaria, there is no evidence to suggest that the leech is capable of transmitting infection by this bacterium.

Inhibitory processes seem to be at work within the leech gut, which act on Ps. pseudomallei but which are not effective against the closely related leech flora. This may have implications in treatment of pseudomallei infection.

Introduction

In part of the 1965 study of leptospirosis in this Unit, land leeches (Hemadipsa spp.) were triturated and injected intraperitoneally to hamsters. One of the animals died with signs compatible with melioidosis and Ps. pseudomallei was recovered. This led, in the following year, to several projects aimed at investigating the possible role of land leeches in the transmission of melioidosis. No conclusive evidence was adduced from these studies.

The presence of Ps. pseudomallei in surface water has been established and the incidence of antibody to Ps. pseudomallei has been found to be highest in people from rice growing areas. Hirudinaria spp. is a common inhabitant of rice fields and is known to be capable of harboring rinderpest virus from infected cattle[18]. A related species transmits trypanosomes to fish[17]. To date, bacterial transmission has not been established. It appears that leeches of most genera have a specific associated bacterial flora which, in the blood sucking species, consists of a single bacterial species[20]. In Hirudo medicinalis it was suggested that the gut associated flora has the ability to destroy or inhibit other bacterial[19].

The viability of Ps. pseudomallei in Hirudinaria spp, the effect of the leech on Ps. pseudomallei with each in the same environment, and the ability of the buffalo leech to transmit Ps. pseudomallei, were investigated.

Methods

These were essentially similar to the methods of the 1966 group except that the host hamsters were not shaved before leech feeding in order to avoid skin trauma other than that inflicted by the leech. The bacteriologic media used are described elsewhere in this report. In one procedure, that of introducing known numbers into the gut of the leech, an intubation method was employed, using a Portex intravenous catheter*. In preliminary studies a weak solution of fluorescein was sodium injected transpharyngeally through the catheter. This was demonstrated, on subsequent dissection and photography under ultraviolet light, to have reached and remained in the sacculations of the gut, thus confirming that forced feeding was feasible.

For convenience, the various procedures followed will be grouped together with the results of each.

Results

Survival of Ps. pseudomallei in the same environment as Hirudinaria spp.: Cultures of a smooth strain of Ps. pseudomallei were grown in nutrient broth and concentrations varying from 1×10^2 to 1×10^5 per ml were prepared in sterile distilled water. Leeches of genus Hirudinaria which had been collected from a jungle fringe pool, were placed individually in 50 ml of sterile distilled water in a loosely capped container. Quantities of the Ps. pseudomallei suspensions were added to give final counts of 1×10^1 to 1×10^4 organisms per ml was employed. Plate counts at several dilutions were performed on NAGC agar on the day of preparation and daily thereafter, for 50 days. At approximately weekly intervals, subsamples of 1 ml were also taken for intraperitoneal injection to hamsters. On each occasion, the initial volume was made up by addition of sterile distilled water. The results are given in Figure 1.

* Portex Ltd, Hythe, England

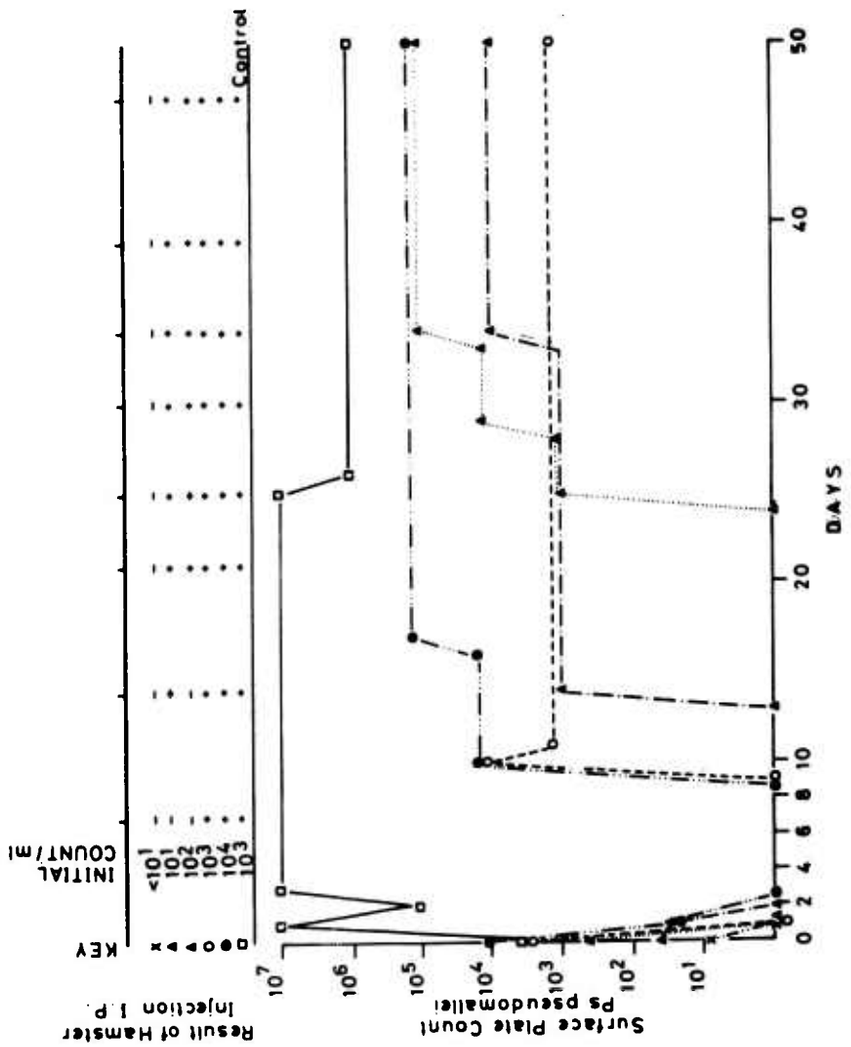


Fig.1 Surface Counts and Hamster mortality from leech containing water seeded with Ps pseudomallei

On two subsequent occasions the same procedure was followed with similar results except that the periods of occluded growth of from 10 to 25 days evident in the first experiment, did not occur.

Survival of Ps. pseudomallei within the leech, Hirudinaria spp.: Sixteen leeches of a similar size were intubated transpharyngeally and each was force-fed 0.1 ml of a suspension containing 1×10^5 Ps. pseudomallei in sterile distilled water. The leeches were distributed, in pairs, in 50 ml quantities of sterile distilled water in loosely capped jars.

Shortly following the introduction to the water, the first pair was removed and each leech of this pair sectioned at a point through the fore gut and through the hind gut. Portions of fore- and hind-gut were separately triturated in a Tenbroek tissue grinder in 1 ml sterile distilled water. 0.5 ml of the suspension was cultured on NAGC and 0.5 ml injected intraperitoneally to hamsters. The other fore- and hind- gut portions were impressed on glass slides and prepared for microscopy, using a fluorescent antibody conjugate for Ps. pseudomallei. Finally, 1 ml of the water from the jar was cultured in several tenfold dilutions on NAGC.

This process was repeated on each of the succeeding pairs on days 1, 4, 8, 12, 16, 20 and 28. All of the waters were cultured on each of these days and on days 42 and 49. Examination of the leech by fluorescent antibody technique was erroneously ommitted on day 12.

The results indicate the following:

a. Excretion of Ps. pseudomallei by the leech did not occur immediately following intubation.

b. While there was some variation in detection of Ps. pseudomallei in the leech with time, it was still present in the leeches kept for 28 days (termination of the leech phase of the experiment) and had not lost its pathogenicity for hamsters.

c. Recovery of Ps. pseudomallei from the waters tends to show a reduction following removal of the leeches from the environment.

Transmission of Ps. pseudomallei by Hirudinaria spp.: Forty leeches, grouped in 14 pairs and 4 groups of three, were fed for 10 minutes on hamsters infected intraperitoneally with Ps. pseudomallei, one group per hamster. That the hamsters were infected was confirmed by tail snip blood culture and by blood culture at necropsy following leech feeding. Counts of Ps. pseudomallei in those animals were of the order of 5×10^3 per ml blood, at necropsy.

The leeches were kept in sterile distilled water in loosely capped jars. The leeches were re-fed on normal hamsters, each batch at intervals, subsequently sacrificing the leeches and attempting recovery

of Ps. pseudomallei. The hamsters on which they had been re-fed were followed for infection by culturing those dying or at sacrifice and necropsy. Of the original 40 leeches, 18 died before the assigned day for re-feeding on normal hamsters.

The 18 dead leeches were removed from their containers on the day of death, which varied from 2 to 61 days following the infected feed. Ps. pseudomallei was not recovered from any of the waters in which they were kept.

Nine of these leeches had failed to feed as evidenced by lack of blood in their gut on dissection. From none of those which showed evidence of having fed, by virtue of an increase in girth and having free blood in their gut, was Ps. pseudomallei recovered, either by direct culture of gut contents or by intraperitoneal injection of suspension into hamsters. The blood-containing leeches had died from days 19 to 41 post infected feed.

The surviving 22 leeches, in 4 batches, were fed on normal hamsters for from 5 to 15 minutes, the intervals between feeding on infected and normal hamsters varying for each batch. Re-feeding was performed on days 0, 25, 53 and 81, the leecnes killed at termination of feeding, triturated in sterile saline, cultured for Ps. pseudomallei and, with the exception of the first batch, 1 ml of leech suspension injected intraperitoneally into hamsters.

In the first batch, fed and re-fed on the same day, all of the leech suspensions contained Ps. pseudomallei but all of the normal hamsters used in re-feeding survived until sacrifice on the 7th post feeding day and none was infected. The second batch of 6 leeches was re-fed on normal hamsters on day 28 following the infected feed. Of those, only two gave isolates of Ps. pseudomallei by intraperitoneal injection of suspension into hamsters, of which one was positive on direct culture. None of the normal hamsters on which they were fed yielded isolates.

Pseudomonas pseudomallei was not recovered by any technique from groups three and four, each of six leeches, at days 53 and 81.

None of the waters in which the leeches were kept was positive for Ps. pseudomallei.

Leech flora: This has so far been studied in only a small number of leeches. The organism most frequently isolated conforms to the definition of family Pseudomonadaceae given in Bergey's Manual, 7th Edition. However, at present, it cannot with any certainty be placed in any one genus. As Stanier et al[21] stress in their taxonomic study, there are many difficulties in specifically identifying such an organism. It would be taxonomically incorrect to name it "Pseudomonas hirudinis" as had been done for the organism isolated from Hirudo medicinalis by Büsing[19].

Discussion

The Arnulid, Hirudinaria lives closely in the same environment as several soil organisms which may give rise to human infection when introduced subcutaneously. When the combination of vector and disease agent are found and when the vector is a blood sucking animal, there are few instances where disease cannot be produced in humans. It appears, as far as bacterial infection is concerned, that the buffalo leech is incapable of harboring and transmitting the organism of melioidosis. The mechanisms of this inability are not clear but may be related to a selective inhibitory effect in the leech gut or in bacterial antagonism within its gut, due to its own resident flora. The claimed findings of earlier years are not confirmed.

4. Chromobacterium violaceum serology. Attempts to design a test for Chromobacterium antibody in human populations are in progress.

Introduction

Sneath[22] showed that the mesophilic members of the genus do not form a homogenous antigenic group when tested by bacterial agglutination methods. There is need, both in human and animal studies, for a test which would be common to all strains.

Materials and Methods

Bacterial strains used were isolated in the padi water study of Ps. pseudomallei.

Rabbits of approximately 2 kg were used to raise antibody.

Bacterial lipopolysaccharide (LPS-endotoxin) was produced by growing strains in the medium of Rice et al[23]; under the same conditions as are used for the production of pseudomallei antigen for use in the IHA test of Ileri.

Protein measurements on the product of this culture were made by Agar gel diffusion (Dr. Robinson), cellulose acetate electrophoresis and scanning spectrophotometry. Thin layer chromotography was applied in an attempt to define the LPS components. And liquid scintillation counting methods used to detect the incorporation of ^{14}C labelled glucose into the LPS molecule. The hemolytic effect of culture filtrates, described for dead cultures by Sneath[1] was assessed by use of sheep red cells.

Results

1. Lethal effect of Rice medium extract: The products of two strains, which could not otherwise be distinguished from the other 5 used, killed rabbits at significantly lower doses than similar preparations of Ps. pseudomallei. The rabbits were not protected by

large doses of Cyanocobalamin, given to ensure that the effect of CN ion, mentioned by Sneath, was not responsible. Similar doses in mice were ineffective, the lethal dose for mice being almost 5-fold that for rabbits. An endotoxin-like effect is illustrated in Figure 1.

2. Antibody production: Intravenous injection of Rice medium extract did not result in production of bacterial agglutinating antibody whereas titers of 1:250 resulted from injection of whole killed cells.

3. Application of an IHA test: When attempts were made to coat sheep's red cells with LPS antigen, as in the IHA test of Ileri, hemolysis of the cells occurred. This hemolytic activity is resistant to autoclaving, but shows an apparent reduction in activity when the extract is subjected to tryptic digestion.

4. Protein content: Polyacrylamide gel disc electrophoresis with coomassie blue staining did not reveal any protein bands whereas Ps. pseudomallei extract, similarly treated, did show bands. Cellulose acetate electrophoresis and spectrophotometry at 290 nm. confirmed the presence of protein in the Ps. pseudomallei extract and not in that of Chromobacterium.

5. LPS on thin layer chromotography: Glucose from the medium used rather vitiates the result and glucose oxidase was not available. No comment can at present be made.

6. Incorporation of ^{14}C glucose: This also is inconclusive because, by chemical methods of measurement there is enough residual glucose in the 14 day supernatant extract to make liquid scintillation readings invalid.

Discussion

Sneath[24] concludes that the hemolysis produced by mesophilic Chromobacterium is probably due to a lecithinase. The hemolysis produced in sheep cells in the present study occurs even following autoclaving of the products of growth in the medium of Rice et al. That it seems susceptible to tryptic digestion is indication of a protein structure.

If the LPS of Chromobacterium is common to all strains its use in red cell sensitization in an IHA test should enable antibody detection and remove the objections of strain specificity. This investigation will require to be continued.

Results so far obtained are inconclusive and refinements of technique in characterization of the "hemolysis" and the endotoxin and in development of an IHA test are needed.

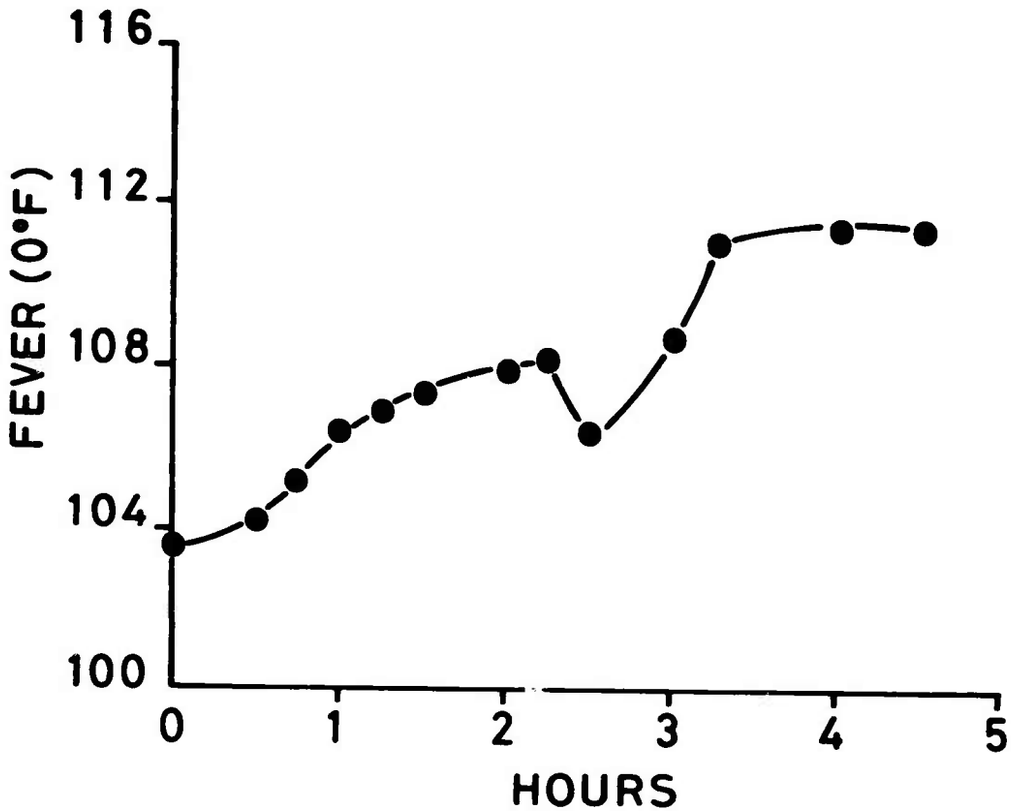


FIG. 1 FEVER CURVE FROM INTRAVENOUS INJECTION OF ENDOTOXIN IN RABBIT.

5. Streptococcal carriage rates in military personnel. Malaysian and British troops have had throat nasal and perineal skin swabs taken and some have been bled for antistrepholysis O titer. To date some of the British have had repeat examination done following a period of four to six weeks under jungle combat conditions. Data are stored on punched cards. Results at this time are preliminary, completion awaiting the second Malaysian study, definitive characterization of isolates by Dr. Maxted and full analysis of all collected results.

Introduction

Allen et al[25] in their study of pyodemia in Vietnam showed that the prevalence of this Group A Streptococcal infection was higher in American white troops than in either American negro troops or a Vietnamese, whether military or civilian. The World Health Organization in their technical series bulletin[26] indicate that little is known about streptococcal infection in developing countries and mention the implication of skin strains in production of glomerulonephritis. There is no record of the nature of skin carriage in this part of the world.

Streptococcal disease due to Str. pyogenes Group B (Str. agalactiae) has been recorded and reviewed and neonatal sepsis from the cause discussed[26]. In a personal communication, Dr. Maxted has indicated that male carriage of Str. agalactiae has as yet not been defined.

Methods and Materials

Swabbing was performed with Calcium Alginate swabs, the perineal or any skin swabs being first moistened in nutrient broth.

Plating out was immediately performed on sheep's blood agar with and without addition of crystal violet. In the earlier part of the study aerobic and anarobic inhibition methods were used but the latter was found unnecessary. Occasional swabbing was done on to Sabouraud medium.

Those troops who were likely to be available for repeat studies done had 10 ml venous blood drawn for antistreptolysis O testing.

The subjects were: (a) British troops who had been in Singapore for 2 years; (b) a group who had just arrived from U.K. and would be in this theater for 2 years; and (c) a group of Malaysians who were on active duty around the Thai border. A smaller group of British paratroops visiting the training area in Johore State were also examined.

All troops were visited and examined in their duty locations. The reading of plates and selection of colonies for study was done on return to Kuala Lumpur.

Results

These, as far as they have been tabulated, are summarized in Table 1.

The Malaysian troops were on active duty when swabbed and, apart from one small group in a duty location, were fairly free of clinical skin disease. The group specified as different were artillery and suffered from mild to moderate chronic fungal infection.

The RHF was an infantry battalion who had just come to the end of a 2 year tour in Singapore, having had their share of jungle exercise. In general attendances at sick parade, for reason of skin infection, had increased following exercise, and after one brigade exercise one third of the battalion was on sick parade for this reason, whether bacterial or fungal.

The Gordans Paratroops and the Paratroops (1) and (2) were on a short visit from Europe for jungle training and spent only 2 weeks in jungle conditions.

The Gordans (1) had just began that tour and none had been outside urban Singapore. Gordans (2) had just completed 4-6 weeks jungle training after having been about 4 months in the Far East.

Discussion

Little can be said on the basis of these crude results except that it appears that the numbers of streptococcal isolates other than Group A appear remain static. While the Group A isolates face with time in the theater. This may be the effect of striking a time of more obvious infection in the Group 1 sampled. It may be of interest to see what comparisons can be made between the untyped strains in the two groups.

Also titers in the Europeans are more prevalent than in the Malaysians. Age may be a factor as the latter group taken to be a decade older than the former. Full analysis of all the parameters chosen will be undertaken.

6. Ecology of malaria vector mosquitoes: Entomological investigations of the ecology of malaria vectors at rubber estates in Peninsular Malaysia ended in April 1974. Parasitological and human ecology studies will continue until September or October 1974.

Paroi Rubber Estate in Negri Sembilan (see USAMRU Annual Report 1973) was established as a permanent site for monitoring the human-mosquito relationships of malaria transmission.

Anopheles maculatus, considered the most important vector of malaria in the hill regions of Malaysia (Sandosham, 1959), was the

Table 1

		BRITISH					
	MALAYSIANS (1)	RHF	Gds Para	Para (1)	Para (2)	Gordans (1)	Gordans (2)
Total	210	151	65	62	62	212	314
Streptococcus A							
Throat	8	1	-	-	1	12	-
Perineum	-	-	-	-	-	2	-
Other	-	2	-	-	-	1	-
Streptococcus B							
Throat	2	-	-	-	-	6	-
Perineum	-	2	-	-	-	3	-
Other	-	-	-	-	-	1	-
Other B. hemolytic Streptococcus							
Throat	23	13	-	9	1	20	26
Perineum	7	10	1	-	-	3	6
Other	-	4	1	1	-	1	1
A.S.O. Titer							
1:250 and over	21/207	Not done	Not done	12/59	10/60	29/138	85/308

predominant anopheline in the area. This species was studied in detail during the investigation period.

Introduction

Anopheles maculatus has been documented as the most important vector of malaria in the hill regions of Peninsular Malaysia, Green 1929, Field and Reid 1956, Sandosham 1959.

Preliminary studies at Paroi Rubber Estate revealed sufficient human malaria cases and Anopheles maculatus populations to make a malaria-mosquito ecology study feasible. Entomological data on adult and larval populations, parity rates, biting patterns, and human-mosquito relationships were studied in regard to An. maculatus.

Method

The methods previously described in USAMRU Annual Report 1973 were essentially the same as this reporting period. However, the following important changes were made. Specific data was collected on Anopheles maculatus. All night biting collections were made one or two times per month. Larval collections were made on an area basis rather than by specific sites, i.e. ground pool 1, 2, etc. Number of larvae collected were compared on time units (30 minutes) per collector rather than the conventional larval per dip method.

Results and Discussion

Adult bionomics: Anopheles maculatus continued to be the predominant anopheline vector in the Paroi area. An. letifer was the only other potential vector collected and in such small numbers (3 total) it was not considered a possible malaria vector. There were 12 species of anophelines and 32 species of culicines collected at Paroi during the study period. A summary of all adult mosquito collections is given in Table 1.

Biting patterns for An. maculatus are depicted in Figure 1 and summarized by Table 2. Monthly biting totals were usually not high enough to permit a valid evaluation but overall totals do present a pattern. The peak biting times were between 2100-2400 hours with a smaller peak occurring after midnight.

Indoor and outdoor collections were made to determine biting location preferences of An. maculatus. There was slightly less than a 4:1 outdoor to indoor biting ratio for this species. A summary of these collections is given in Table 3. The study was terminated before a full year of data were available but the material in this report seems to coincide with the earlier work of Sandosham 1959, who showed that peak populations of Anopheles maculatus appeared in March and April.

Table 1. Species of mosquitoes collected at Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia from November 1973 - April 1974

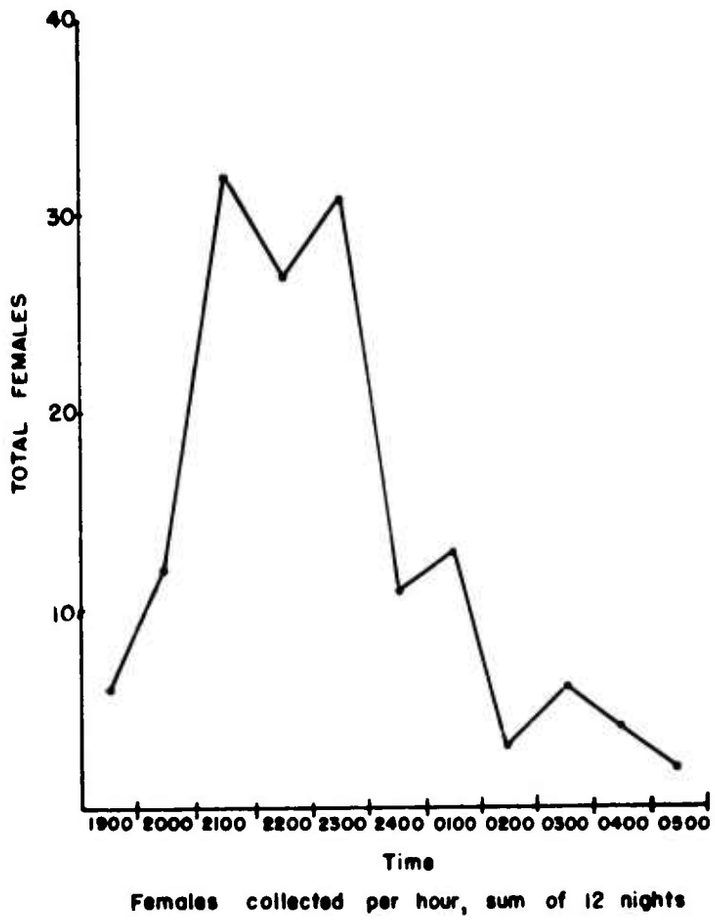
Species	Species
Aedes albopictus	Culex fuscocephalus
Aedes butleri	Culex gelidus
Aedes lineatopennis	Culex fuscanus
Aedes niveus group	Culex halifaxi
Aedes vexans	Culex (Lopho) sp.
Anopheles aconitus	Culex mimulus
Anopheles barbirostris	Culex nigropunctatus
Anopheles crawfordi	Culex sinensis
Anopheles indiensis	Culex tritaeniorhynchus
Anopheles karwari	Culex whitmorei
Anopheles kochi	Culex (Culiciomyia) sp.
Anopheles letifer	Culex pseudovishnui
Anopheles maculatus	Ficalbia sp.
Anopheles philippinensis	Mansonia annulata
Anopheles sinensis	Mansonia annulifera
Anopheles tessellatus	Mansonia crassipes
Anopheles vagus	Mansonia dives
Aedomyia catasticta	Mansonia hodgkini
Armigeres subalbatus	Mansonia nigrosignata
Armigeres pectinatus	Mansonia ochracea
Culex annulus	Mansonia uniformis
Culex bitaeniorhynchus	Uranotaenia sp.
Culex fatigans	Uranotaenia nivipleura

Table 2. Nocturnal human biting rates of Anopheles maculatus, Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia, November 1973 - April 1974. Females collected per hour, sum of 12 nights.

Month	1900	2000	2100	2200	2300	2400	0100	0200	0300	0400	0500	Total
Nov	4	1	2	8	5	5	1	1	-	1	-	28
Dec	-	-	-	-	-	2	1	1	3	-	2	9
Jan	2	1	5	5	2	2	4	-	1	1	-	23
Feb	-	1	4	-	4	-	-	1	1	-	-	11
Mar	-	8	15	6	8	2	3	-	-	-	-	42
Apr	-	1	6	8	12	-	4	-	1	2	-	34
Totals	6	12	32	27	31	11	13	3	6	4	2	147

Figure 1

Monthly average nocturnal biting rates of Anopheles maculatus, Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia November 1973- April 1974



Females collected per hour, sum of 12 nights

Table 3. Anopheles maculatus collected biting man from indoor and outdoor sites at Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia, November 1973 - April 1974.

Month	Indoor			Outdoor		
	No. Nights	No. Collected	No./man/night*	No. Nights	No. Collected	No./man/night*
Nov	8	13	0.82	8	93	5.82
Dec	6	8	0.67	6	37	3.09
Jan	3	8	1.34	3	52	8.67
Feb	3	28	4.67	3	113	18.84
Mar	4	41	5.13	4	171	21.38
Apr	2	47	11.80	2	143	35.75
Totals	26	145	24.43	26	609	93.55

* Four collectors, 2 inside and 2 outside were used each night. Collections were made from 1900-2300 hours.

Paroi Estate is unique in that the human population is probably the main host for Anopheles maculatus. At the beginning of the investigation mosquito blood studies were planned to identify the host preferences of the anophelines present. It was soon apparent that adequate specimens could not be collected, either by resting or light trap collections, to run such tests. It is most likely that humans compose 90% plus of the hosts for Anopheles maculatus. No cattle or other larger animals are present within the flight range of the mosquito. The only mammals present besides man are dogs, cats a few wild rats and civet cats.

Data were collected on the parity rates of Anopheles maculatus during the period of investigation. Supplementary collectors were used from 1900-2300 hours to increase the total catch of Anopheles maculatus for determining parity rates. Table 4 gives parity rates for specimens collected. There seems to be little variation in the rate from month to month. Approximately one-third of the An. maculatus examined had at least one blood meal prior to collection.

Larval bionomics: Seasonal abundance of larvae is undoubtedly linked to the availability of oviposition sites at Paroi Rubber Estate. Figure 2 relates larval abundance to rainfall during the period of investigation. Larval populations seem to be inversely proportional to the amount of rainfall. This manifestation is most likely due to continual flooding of oviposition and larval development sites during periods of heavy rainfall such as occurred in November and April. Prior to November, surveys were made in specific sites and numbers of larvae per dipper were recorded. This flooding phenomenon made this typical survey method unworkable. Beginning in November 1973, large areas (at least 10 acres) were designated as individual sites and larval collections were made on a time basis (30 minutes per site) rather than a per dip basis. (See Figure 3) A summary of larval collections, by site, is given in Table 5.

Anopheles maculatus was the predominant anopheline collected during these surveys. No other potential anopheline vector was found. Non-vector anophelines collected included, An. kawari, An. vagus, An. barbirostris and An. nigerrumus. Culicine species found were Culex alienus, C. annulus, C. bitaeniorhynchus, C. fuscocephalus, C. Lutzia sp, C. malyi, C. mumulus, C. pseudovishnui, and C. tritaeniorhynchus.

7. Malaria vector and general mosquito survey, North Sumatra, Indonesia.

Introduction

An entomological survey was made at various locations in North Sumatra. The principal area surveyed was Seruwai, a Coconut Plantation near Belawan, North Sumatra. This area had been surveyed for malaria prevalence by Dondero et al in April and May 1973 and found suitable for a chloroquine resistant Plasmodium falciparum study. An

Table 4. Parity rates of Anopheles maculatus collected at Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia, November 1973 - April 1974.

Month	Parous		Nulliparous	
	No.	%	No.	%
Nov	34	34	67	67
Dec	9	25	27	75
Jan	14	28	37	72
Feb	51	45	62	55
Mar	123	34	236	66
Apr	28	38	46	62
		Average 34		Average 66

Figure 2

Monthly larval collections and rainfall from Paroi Rubber Estate Negri Sembilan, Peninsular Malaysia, November 1973 - April 1974.

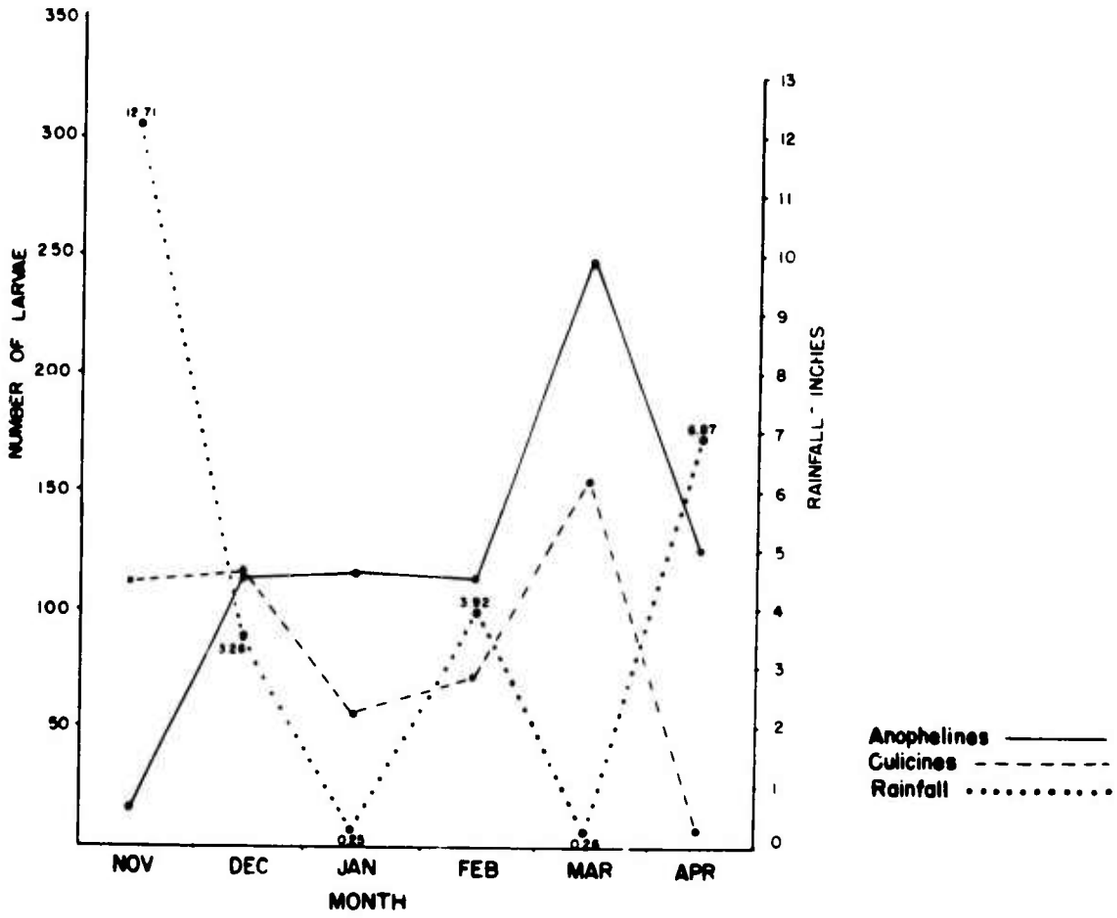
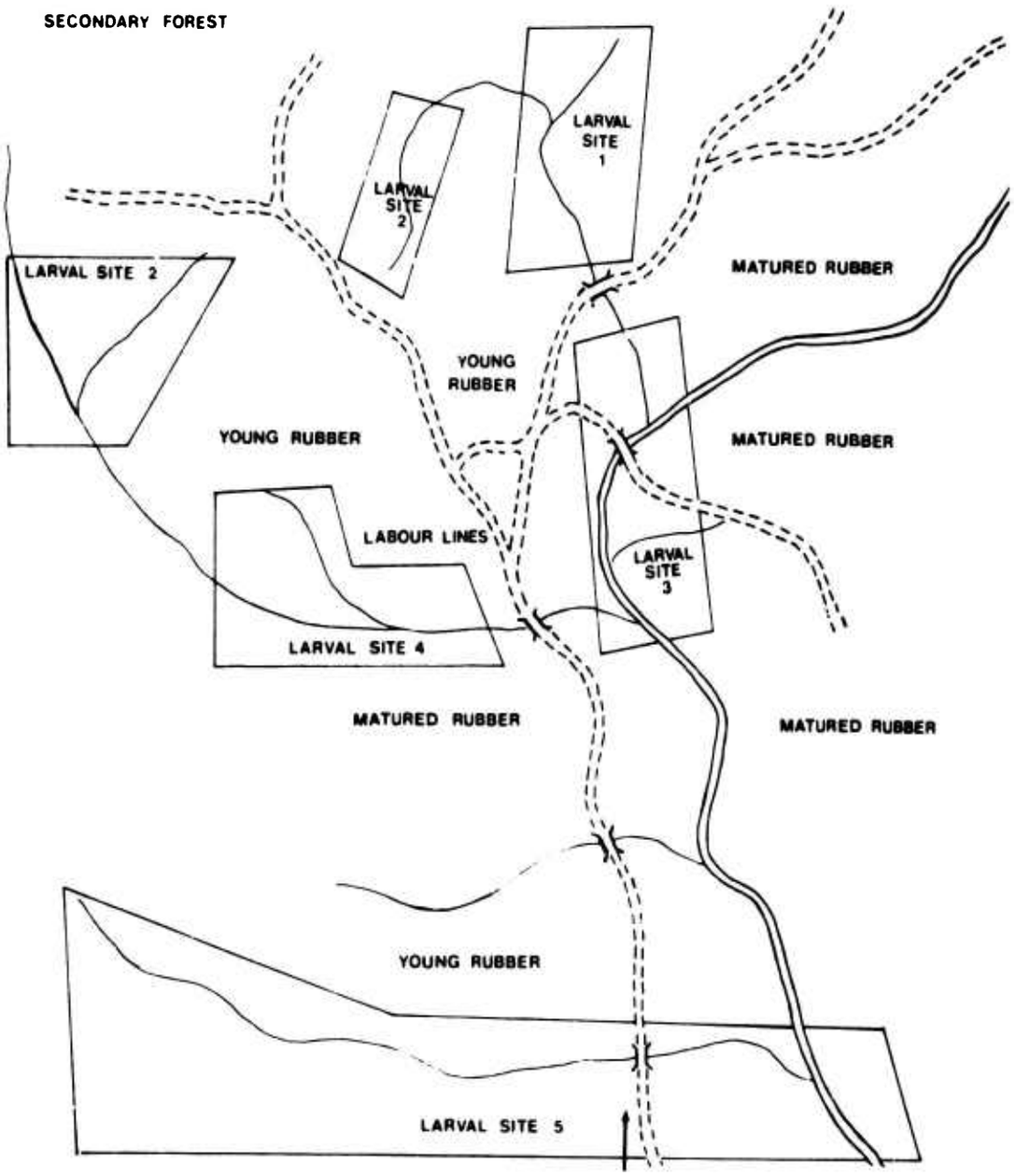


Figure 3 Larval Sites at Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia.*



* Not drawn to scale

Table 5. Monthly larval collections from Paroi Rubber Estate, Negri Sembilan, Peninsular Malaysia, November 1973 - April 1974.

Month	Collection Sites															Total/Month*					
	I			II			III			IV			V								
	A	C	T	A	C	T	A	C	T	A	C	T	A	C	T	A	A1	C	C1	T	T1
Nov	4	14	18	6	35	41	0	0	0	4	26	30	3	34	37	17	17	109	109	126	126
Dec	2	19	21	40	57	97	0	19	19	156	135	291	27	0	27	225	112.5	230	115	455	227.5
Jan	87	4	91	44	31	75	100	66	166	5	2	7	0	0	0	236	118	103	51.5	339	169.5
Feb	77	35	112	52	76	128	45	20	65	39	6	45	17	22	39	230	115	159	79.5	389	194.5
Mar	25	66	91	84	19	103	115	52	167	99	77	176	157	88	245	480	240	302	151	782	391
Apr	54	11	65	1	0	1	0	0	0	21	0	21	61	0	61	137	137	11	11	148	148
Total	249	149	398	227	218	445	260	157	417	324	246	570	265	144	409	1325	739.5	914	517	2239	1256.5

* During November and April one collection per month was made. Other months represent two collections per month. Total numbers for December through March are divided by 2.

entomological survey during this pre-survey period revealed Anopheles sundaicus as the principal man-biter and probably the primary malaria vector in this coastal area. The present survey was extended to other areas in North Sumatra to determine potential malaria vectors present in various habitats and to provide entomological training to personnel in the Malaria Division of Health Services, Medan, North Sumatra.

Collection Areas and description

Seruwai Plantation: A large coconut plantation in the vicinity of Belawan and approximately 15 miles east of Medan, North Sumatra. This is a brackish water area and supports many oviposition sites of Anopheles sundaicus and various culicine species. The plantation is at sea level and in the past was reclaimed from salt water mangrove swamp by a series of locks and dikes, Figure 4, Number 2.

Bohorok Area: This location is approximately 20 miles west of Bendjai or about 40 miles from Medan. Collections were made in or near the kampungs of Tanjong Tinggang and Sedjagat. The area was approximately 500 feet in elevation, and was mostly secondary forest but some basic crops and rubber trees were present. Wild animals seen in the area included wild boar, silver leaf and long tailed macaque monkeys, tree shrews and numerous bird species, Figure 4, Number 2.

Brastagi Area: Brastagi is a mountainous area with an elevation from 3000-6000 feet. Most of the region is cleared for agriculture but a few mountains retain secondary, or occasionally small patches of primary forest. The temperature is usually rather cool throughout the area, Figure 4, Number 3.

Parapat - Lake Toba - Samosir Area: The elevation in this area varies from 4000-7000 feet and, like Brastagi is relatively cool. Most mountain tops are cleared with a few patches of secondary or primary forest remaining. Early Dutch settlers planted considerable stands of European pine which are still apparent in some locations. The valleys are planted almost entirely with padi (rice fields), Figure 4, Numbers 4, 5.

Asahan Falls: This area was similar to the last region in elevation but had large stands of secondary and primary forest. The Asahan river flows through the area and the Falls will soon be the location of a large power-dam facility. This location is closed to the public. The only agriculture is done by small landowners below the Falls. They raise padi, various fruits and peanuts. Figure 4, Number 6.

Methods

Biting Collections: Biting collections using humans as bait were made at Seruwai Plantation, Bohorok and Asahan Falls. Mosquitoes were collected individually as they would bite or come to rest on the

COLLECTION SITES IN NORTH SUMATRA INDONESIA

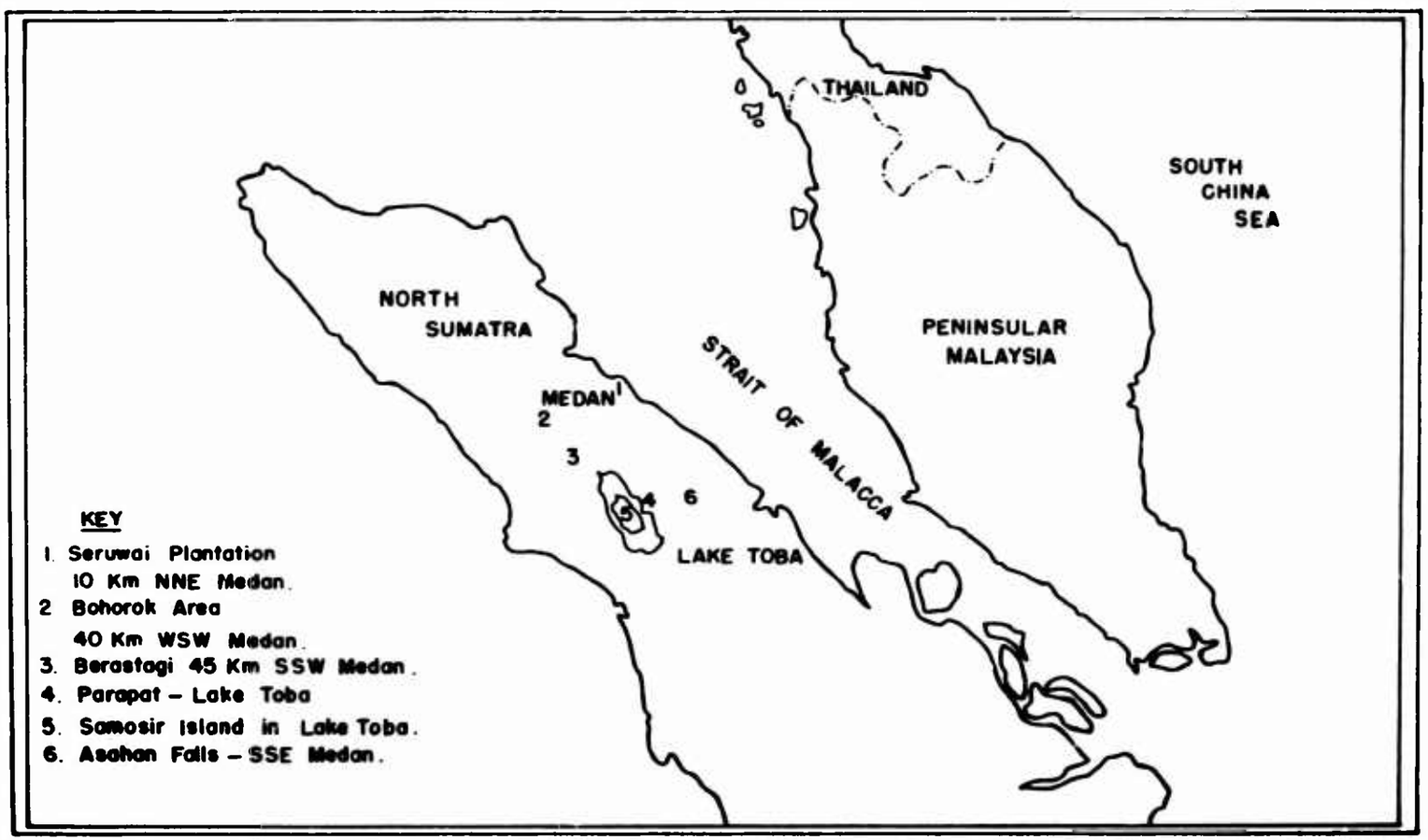


Figure 4

collector. Collection times ranged from 7-11 pm to all night. Anophelines were usually dissected, but selected specimens were saved for taxonomic study. Culicines were all saved and either given to the Health Services Division, Medan, or retained for reference collections.

Larval Collections: Emphasis was placed on larval collections during this survey. Collections were made in all possible habitats throughout the survey areas. The larvae collected were returned to a central laboratory set up in Medan. Here, specimens were reared, larval and pupal skins saved and associated adults pinned. After the first 10 days of collections, specimens that were still in larval or pupal form were returned alive to Kuala Lumpur, Malaysia. Here the rearing was continued by Dr. Ramalingam of the University of Malaya and his staff. Specimens that died were placed in 70 per cent alcohol and later mounted on slides.

Results

Biting Collections: Results of biting collections are summarized in Tables 6, 7, and 8. Biting collections were made at the following locations: Seruwai Coconut Plantations, Tanjong Lenggang (Bohorok Area) and Asahan Falls. Potential anopheline vectors collected in each area included: Seruwai Coconut Plantation - Anopheles sundaicus; Tanjong Lenggang - An. aconitus, An. leucosphyrus and An. maculatus; Asahan Falls - An. maculatus. Dissections were made of guts and glands from the latter two areas. No positives were found.

Larval Collections: A list of the anopheline larvae that have been reared and identified to date is given in Table 9. A complete list of all species will be given in a final report. Two new distribution records for Sumatra have been recorded from these surveys. Anopheles sulawesi recorded previously from the Celebes and An. saungi found in Sabah. Both of these species were collected in the Asahan Falls area at elevations of approximately 5000 feet.

8. Geographical studies of chloroquine resistant falciparum malaria, Peninsular Malaysia.

Introduction

The studies described in the FY 73 Annual Research Progress Report, USAMRU, were continued and nearly completed during FY 74. Six new surveys were undertaken, searching for appropriate study areas, and three in vivo chloroquine resistance tests were performed. Final analysis of these tests is in progress and a report will be prepared for publication. A summary of the work to date is presented below.

Table 6. Human biting collections made at Seruwai Coconut Plantation, North Sumatra, Indonesia, January - February 1974.

Species	Number	Collection Times
Anopheles sundaicus	193	7-10 PM (2 nights)
Culex annulus	38	
C. gelidus	1	
C. sitiens	22	
C. tritaeniorhynchus	6	
Mansonia annulifera	2	
M. dives	9	
M. uniformis	55	
Total:	326	

Table 7. Human biting collections made in Tanjong Lenggang (Bohorok Area) North Sumatra, Indonesia, January - February 1974.

Species	Number	Collection Time
Anopheles aconitus	1	all night (1 night)
An. barbirostris	2	
An. crawfordi	2	
An. indiensis	9	
An. karwari	4	
An. leucosphyrus	6	
An. maculatus	9	
Aedes albopictus	2	
Ae. vexans	1	
Culex bitaeniorhynchus	2	
C. pseudovishnui	79	
Mansonia annulifera	1	
M. crassipes	1	
M. dives	148	
M. uniformis	7	
Total:	274	

Table 8. Human biting collections made at Asahan Falls, North Sumatra, Indonesia, January - February 1974.

Species	Number	Collection Times
Anopheles maculatus	11	7-11 PM (2 nights)
An. peditaeniatus	4	
Culex annulus	4	
C. bitaeniorhynchus	3	
C. fatigans	10	
C. fuscocephalus	20	
C. pseudovishnui	7	
C. tritaeniorhynchus	6	
Mansonia uniformis	3	
Total:	68	

Table 9. Larval collections made at various locations in North Sumatra, Indonesia, January - February 1974.*

Species	Location
Anopheles crawfordi	1
An. donaldi	1
An. hackeri	2
An. indiensis	1
An. karwari	1
An. kochi	1, 3, 4, 5
An. leucosphyrus	1
An. maculatus	5, 6
An. peditaeniatus	1
An. pujutensis	1
An. saungi	2
An. sinensis	2, 3
An. sulawesi	2
An. sundaicus	3
An. vagus	1, 2, 5, 6

1 - Tanjong Lenggang (Bohorok Area)
2 - Asahan Falls
3 - Seruwai Coconut Plantation
4 - Brastagi
5 - Samosir Island
6 - Parapat

* Includes only anophelines identified to date. Additional anophelines and all culicines are still being processed and identified.

Method

The only new variation has been the comparative testing of the efficacy of chloroquine alone versus chloroquine combined with pyrimethamine, in an area where about half the infections were resistant at the R-1 level to chloroquine alone. This combination ("Darachlor": 150 mg chloroquine base to 15 mg pyrimethamine) is recommended for use in Malaysia by the World Health Organization, and is used extensively for presumptive treatment and/or prophylaxis by the National Malaria Eradication Project and the Malaysian Armed Forces.

Results

From a randomly divided group of infected persons on one rubber estate 88 were treated as per routine with chloroquine (25 mg/kg) while 75 received the same dosage of chloroquine plus 2.5 mg pyrimethamine per kilogram. The resistance rate among the chloroquine-pyrimethamine group was no different from that in the chloroquine only group - 45% and 46% respectively.

Summary of Chloroquine Resistance Studies FY 73-74.

Although field studies in Southern Thailand showed over 50% of the P. falciparum to be chloroquine resistant, comparable past studies in geographically nearby northeastern Malaysia had found only a few percent. Quantitative surveys in Malaysia have been few and blood was examined only up to 7 days post-treatment. Several small hospital studies on subjects from northwest and southern Malaysia and Singapore revealed primarily late recrudescing R-1 resistance.

A field-expedient in vivo method was instituted to detect late R-1 type resistance. P. falciparum infected subjects were treated with 25 mg/kg of chloroquine, their urine tested before and after, and their blood examined at weekly intervals for 28 days. A control group comprising subjects either with malaria other than P. falciparum or with no infection was similarly treated and followed. Thus at the same time a search was made for chloroquine resistant P. vivax. Concurrent entomological investigations were conducted.

Studies of this type have been completed in 3 areas of Peninsular Malaysia: one among Malay school children and villagers in Trengganu, on the East Coast 100 miles south of the Thai border; 3 studies among rubber estate workers in Pahang in central Peninsular Malaysia; and 3 studies on similar workers in Negri Sembilan about 50 miles south of Kuala Lumpur.

In Trengganu 440 people were fully studied, including 70 cases of P. falciparum. The overall resistance rate was 5% after deducting the expected reinfection rate of 0.8% (based on the acquisition of new P. falciparum among the control subjects.)

In Pahang 634 people were studied in 3 areas, including 390 with asexual P. falciparum. The resistance rates were estimated at 46%, 50% and 54%. An additional 75 cases of P. falciparum were treated with chloroquine plus pyrimethamine and at least 45% of these proved resistant.

In Negri Sembilan the resistance rate ranged from 11% to 23%, although in several small studies higher rates were found.

Nearly all the resistant P. falciparum encountered was of the R-1 type, mostly late recrudescing, but including early recrudescing R-1 as well.

About 240 P. vivax and 35 P. malariae infections, including mixed infections, were studied. None appeared resistant to the drug.

A summary of findings to date are presented in the map.

9. Preliminary survey for chloroquine resistant malaria in North Sumatra, Indonesia.

Introduction

There is little published information on the status of chloroquine resistant malaria in Indonesia, particularly on the island of Sumatra. Plasmodium falciparum resistant to chloroquine occurs in the neighboring countries of Thailand (Bourke et al, 1966; Cadigan et al, 1968), Peninsular Malaysia (Sandosham et al, 1967; McKelvey et al, 1971; Andre et al, 1972; Dondero et al, in preparation), Sabah, North Borneo (C.M. Han, and Y.S. Huang personal communication; Clyde et al, 1973) and Singapore (Colbourne et al, 1970). It therefore seemed reasonable to expect that some such resistance may occur in Indonesia.

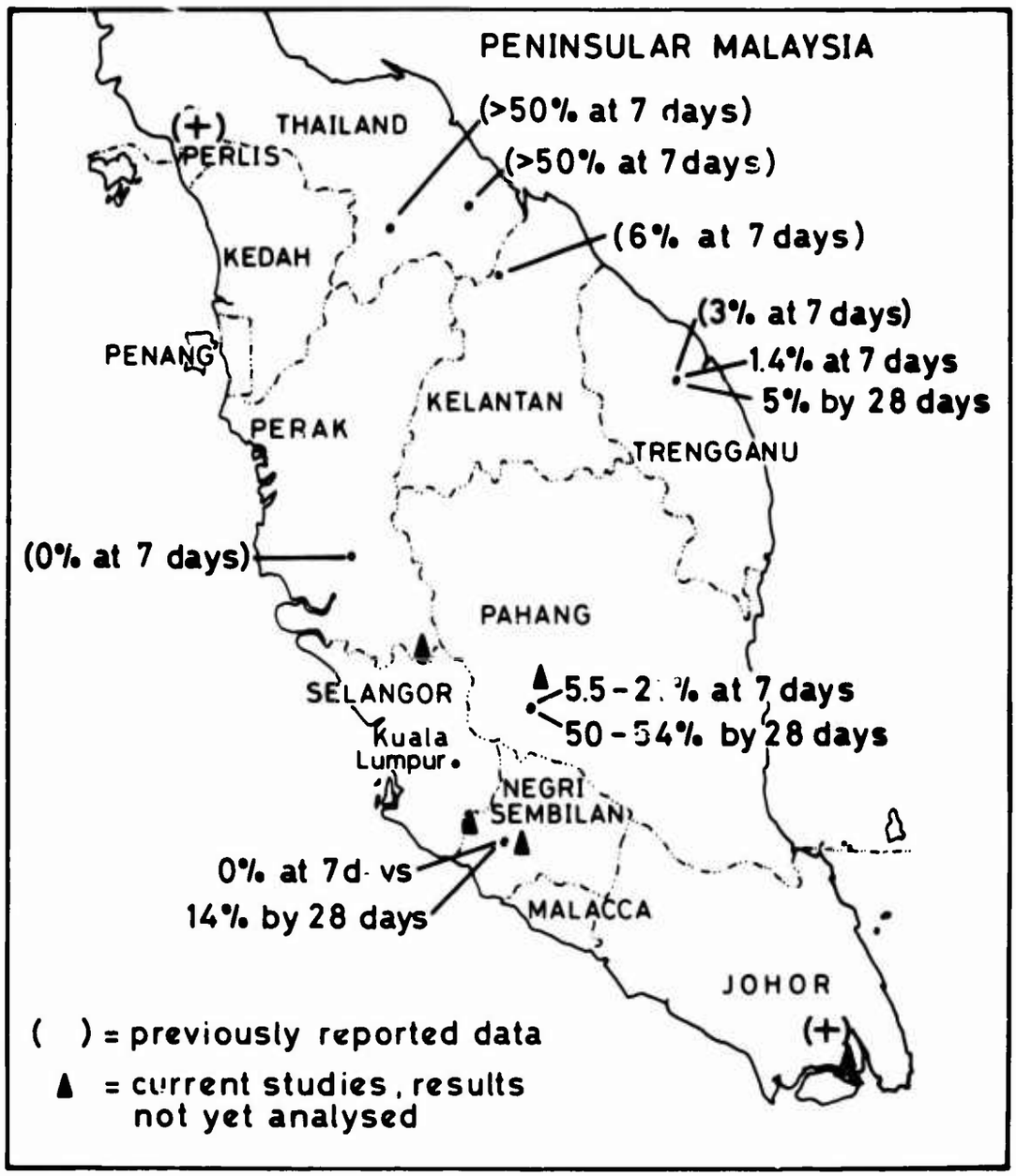
An unpublished, and as yet unconfirmed report (Wolfensberger to Prof. J. Sulianti Saroso, National Institute for Medical Research, Jakarta, Indonesia) suggested that about half of the tested vivax malaria was resistant to chloroquine in such widely separated areas as Nias Island off the west coast of North Sumatra, East Java, and Timor Island far to the east.

Because of the lack of information on P. falciparum, and because of the report which alleged resistant P. vivax, a preliminary survey for chloroquine resistant malaria of both species was undertaken in North Sumatra.

Materials and Methods

An initial survey for appropriate sites for a 28-day in vivo chloroquine resistance study was made in April-May 1973. Requirements were: an appreciably high prevalence of Plasmodium falciparum infection; accessibility, to allow numerous repeat visits necessary over 28 days; and a cooperative population sufficiently nonmobile to allow follow-up examinations.

MAP SHOWING COMPILED RESULTS OF COMPLETED STUDIES PLUS SITES OF CURRENT STUDIES



For preliminary surveys, finger-stick blood smears were obtained, stained with Giemsa and microscopically examined under oil immersion for 100 high powered fields. Personal data were recorded. Children aged 2 through 9 years old were examined for splenomegally.

Aside from the initial screening test during the preliminary malaria survey, the proposed test was to have been an in vivo, in situ study, with follow-up for 28 days and observation of control subjects for evidence of new infection. However, this was precluded by an unanticipated low malaria prevalence in the selected study sites at the time scheduled for the study. The only method used, therefore, was a 7 day screening test, the W.H.O. Standard Test (W.H.O., 1967; 1973), slightly modified as follows. Subjects found positive for malaria were weighed and personally administered a 3 day, 25 mg/kg course of chloroquine (Avloclor, Imperial Chemical Industries). Repeat duplicate thick blood smears were made just as treatment was initiated. Urine was collected before and on the last day of treatment and tested for chloroquine (Wilson & Edeson, 1954). Follow-up blood examination was made on day 7 in the preliminary survey, and on days 6 and 7 in the later survey. Asexual parasitemia on day 7 in a subject who had been given full treatment, and whose urine became positive for chloroquine, was considered to indicate chloroquine resistance.

Results

Initial Malaria Survey: Although P. vivax was considered common in North Sumatra, little P. falciparum had been previously documented except on Nias Island, (Public Health Institute, personal communication). In April-May 1973, with the support of the provincial Health Department, we surveyed 4 areas accessible from Medan. (See Map).

Seruwai Plantation in Labuan Deli, near the port of Belawan, 30 km north of Medan is a flat, low-lying coconut plantation of about 600 hectares, bordered on three sides by coastal mangrove swamp. Virtually the entire labor force is of Javanese origin. Larval mosquito collections, reported elsewhere (Parsons et al, in preparation), revealed numerous Anopheles sundaicus breeding in many areas of the plantation including near the labor lines (houses).

The second site, Kg. Seg. Baru, in Hamparan Perak, about 35 km north west of Medan, is a riverine kampung inhabited largely by Sumatran Malays raising rice and fruits. Kg. Bandar Kalipah, in Deli Serdang, near Tebing Tinggi, about 90 km south east of Medan is a rice growing kampung inhabited by a variety of North Sumatran peoples. Punahan Seruwai is a large rubber plantation at Pemetang Siantar, approximately 200 m south of Medan. The population there is largely of Javanese origin.

As shown in Table 1, only Seruwai Plantation had a moderate prevalence of malaria: 7.9% of the group had P. falciparum, 2.6% P. vivax, and there was an 8.2% spleen rate.

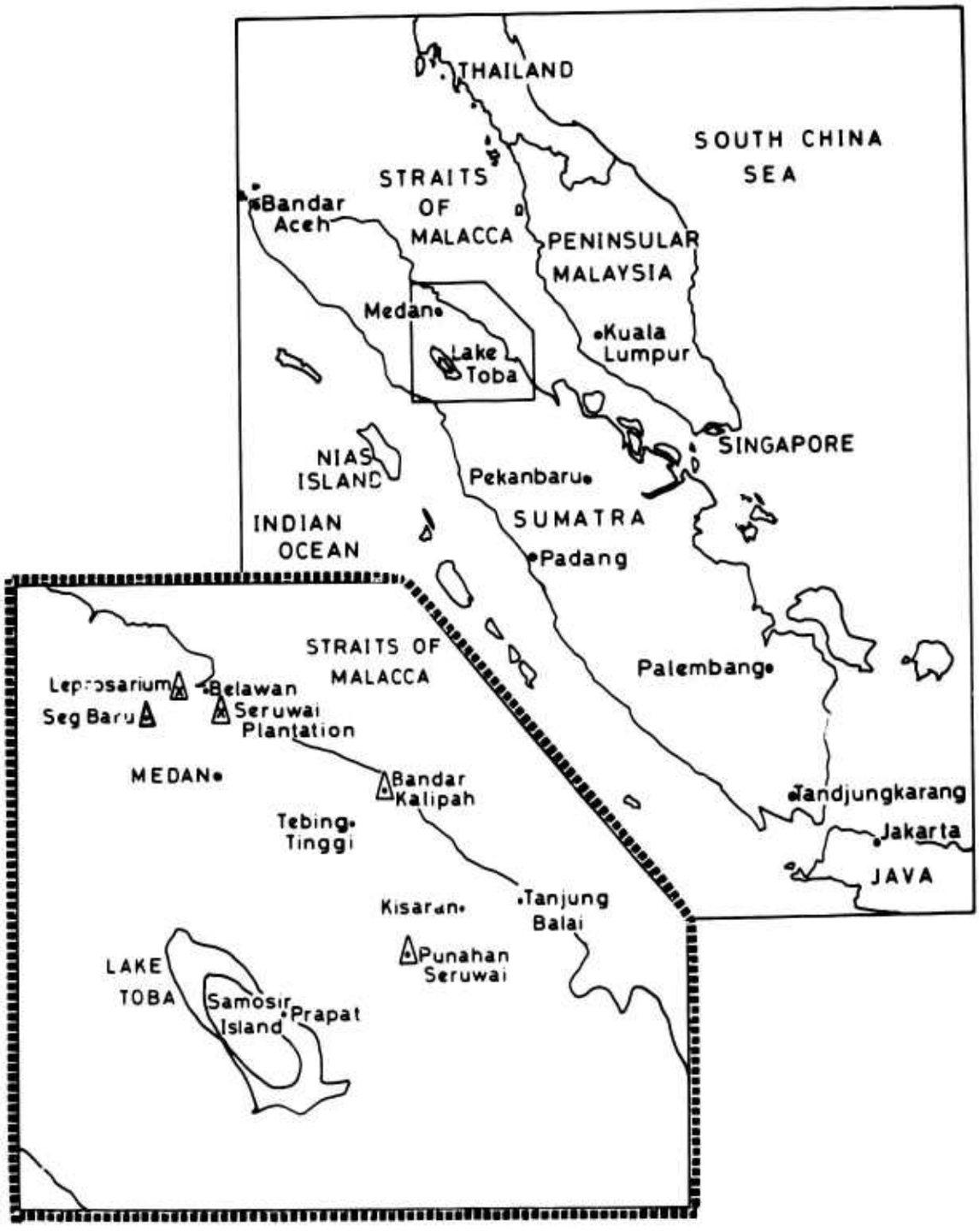

THAILAND
SOUTH CHINA SEA
STRAITS OF MALACCA
Bandar Aceh
PENINSULAR MALAYSIA
Medan
Kuala Lumpur
Lake Toba
SINGAPORE
NIAS ISLAND
Pekanbaru
INDIAN OCEAN
SUMATRA
Padang
STRAITS OF MALACCA
Palembang
Belawan
Seruwai Plantation
MEDAN
Bandar Kalipah
Tandjungkarang
Jakarta
Tebing Tinggi
JAVA
Tanjung Balai
Punahan Seruwai
LAKE TOBA
Samosir Island
Prapat

Table 1. Results of malaria blood surveys, North Sumatra, Indonesia.

Place	Date	Group composition	No. examined	Malaria pos.	No. falcip.	No. vivax	Spleen rate
Seruwai Plantation, Labuan Deli	24, 25 & 29 April 1973	50 adult plus all non-working children	380	40 (10.5%)	30	10	8.2%
Kg. Seg. Baru, Hamparan Perak	26 April 1973	villagers of all ages	145	0 (%)	0	0	0%
Kg. Bandar Kalipah Deli Serdang	28 April 1973	villagers of all ages	252*	7 (2.8%)	3	4	1.5%
Punahan Seruwai Rubber Estate, near Kisaran	3 May 1973	children	240	5 (2.1%)	0	5	not examined
Seruwai Plantation, Labuan Deli	17-19 Jan 1974	entire estate population	584	14 (2.4%)	8	6	8.4%
Leprosarium, Belawan	21 January 1974	children	58	2 (3.5%)	0	2	2.7%

* 2 night bloods found positive for Brugia malayi.

This plantation, with a population of about 600 was selected for further study, as was the nearby government leprosarium with about 200 residents. One of the authors (G.T.) had noted enlarged spleens among dependent children at the leprosarium about 2 years earlier, although no actual malaria survey had been performed.

First Chloroquine Resistance Test: In April-May, 1973, subjects found to have malaria in the survey at Seruwai Plantation, Labuan Deli, were screened for chloroquine resistance.

Twenty-nine cases of P. falciparum with asexual parasitemia and one case with gametocytes only were fully treated with chloroquine. The post-treatment urine of one 7 year old subject, who was observed taking the pills and who denied vomitting, was found negative for chloroquine. He was followed up but excluded from the study. Thus, 28 asexual parasitemias met the test criteria. Nine cases of P. vivax were satisfactorily treated.

All bloods were negative for asexual parasites on the 7th day.

Scheduled for a time estimated locally to be the malaria "season", this study was hindered by an unexpectedly low malaria prevalence.

Among the 608 people at Seruwai Plantation, 584 (96%) were examined for malaria, with only 14 individuals with positive smears (2.4%), 7 with P. falciparum trophozoites (1.2%), 1 with P. falciparum gametocytes only (0.2%), and 6 with P. vivax (1.0%). All except 2 of the P. falciparum infections had scanty parasitemias. The spleen rate was 8.4%.

At the leprosarium no P. falciparum was found. The P. vivax rate was 3.4%.

The small number of cases, mostly with low density parasitemias, made the more complex 28-day follow-up study impractical. Therefore only the brief field test was conducted with post-treatment blood examinations on days 6 and 7.

Following treatment, all 7 asexual P. falciparum, 1 mixed and 6 P. vivax infections, plus the 1 P. falciparum gametocytemia, cleared and were blood negative for asexual parasites on days 6 and 7.

Discussion

The unexpectedly low prevalence of malaria at the time of our proposed 28-day study prevented a more definitive search for chloroquine resistance. However, a total of 36 patients with asexual P. falciparum and 16 P. vivax infections were treated and observed for asexual parasites after 7 days, with no evidence of chloroquine resistance in either species.

While a 7 day follow-up period can be expected to demonstrate the more serious R-2 and R-3 type resistance, it is inadequate to detect late recrudescing R-1 resistance, which is by far the type most commonly encountered in Peninsular Malaysia (Dondero et al, in preparation). Moreover, old infections, as in this study - low density parasitemia in asymptomatic hosts - are not ideal for demonstrating resistance in vivo, since such infections respond to lower doses of drug than do acute ones, particularly in non-immune hosts.

Nonetheless, this study suggests that the more serious R-2 and R-3 resistance is unlikely in the area studied. Based on experience in nearby Malaysia, it would even seem unlikely that greater than 25% R-1 resistance occurred in the study area, since at least a few cases of early recrudescing R-1 resistance, detectable on day 7, would have been expected.

We found no evidence of resistance to chloroquine among our 16 treated cases of P. vivax, despite the unpublished report cited above, stating that as much as half of the infections with this species tested on Nias Island (North Sumatra), East Java, and Timor Island appeared resistant to chloroquine at 7 days. Since the start of the North Sumatra survey, a short-term follow-up study on Timor, in the same area where Dr. Wolfensberger worked, also failed to confirm the existence of chloroquine resistance (B. Gundelfinger, personal communication).

According to informal information, chloroquine seems not to have been long or heavily used in North Sumatra, quinine being a local product and more economical. Therefore we suggest that little "chloroquine pressure" has been exerted, which might have encouraged the appearance of, or increased the frequency of, chloroquine resistance. Indonesian physicians have had personal experience locally with chloroquine therapy; they have not been impressed with frequent treatment failures or early (2-3 weeks) "relapses". Recurrent malaria several months after treatment, consistent with both ordinary relapsing P. vivax and reinfection, has, however, been encountered. If and as chloroquine becomes more widely used in treatment, or particularly in prophylaxis, the emergence of chloroquine resistant strains may become more apparent, as they have in many other parts of Southeast Asia.

10. Malaria epidemiology.

Human Behavior and its Relation to Acquiring Malaria on a Rubber Estate in Peninsular Malaysia.

In the course of long-term malaria parasite and mosquito studies on Paroi Rubber Estate (USAMRU Annual Research Progress Report, FY 73), we were struck by the fact that some individuals, and particularly some families, were constantly and repeatedly getting malaria, while others had little or none at all. The following is a

preliminary description of our investigation into factors which might have affected whether or not a person in this malarious area got malaria.

We have been checking each of the approximately 180 people for malaria every four weeks over the past 18 months. During that time new infections averaged about 7 percent per month for P. falciparum and slightly higher for P. vivax, although some of these, no doubt, include relapses several months after treatment. The actual incidence by month is illustrated in Figure 1. There was year-round transmission of malaria, with only minor seasonal variation. There were 346 new cases of malaria observed in 143 people. Yet 49 people, or 26 percent, had no malaria at all during the observation period. The group consisted almost exactly of two thirds Indians and one third Malays. There was about 33 percent annual turnover in subjects. 192 people were examined for at least six months.

The workers and dependents reside in a cluster of virtually identical houses on about 2 cleared areas in the central part of the estate. There was no obvious correlation between the location of the house within the compound, and whether the family inside got a lot of malaria. The differences in malaria rates apparently could not be explained in terms of differences in housing.

Efforts were therefore made to study people's individual behavior which might influence their exposure to the vector mosquitoes.

We interviewed all the subjects in their homes enquiring into and observing such aspects as occupation, age, sex, what animals they kept, where their evening hours were spent, what type of clothes they wore, whether and how often they used insect repellent, whether they slept under a mosquito net or burned a pyrethin coil, what clothes they wore to bed, what bedcovers were used, and what time they got up. None used chemoprophylaxis. These data plus the malaria figures were coded for punching on I.B.M. cards and electronic sorting.

We were then able to attempt correlation of various behavioral parameters with frequency and type of malaria, and draw up behavioral profiles on those getting or not getting malaria. Monthly blood examination is still in progress. The following results are based only on the first 16 four-week observation periods.

Indians got malaria about 60 percent more often than Malays. And males one third more often than females.

Not all age groups got malaria at the same rate. As shown in Table 2 the more frequently infected people are those under 20 years old, who, as a group, got two thirds more malaria than those over 20. The question still remains, why did the males, the Indians, and the younger people get more malaria?

Figure 1. PATTERN OF MONTHLY MALARIA ATTACK RATES OVER TIME, PAROI ESTATE, NEGRI SAMBILAN, PENINSULAR MALAYSIA.

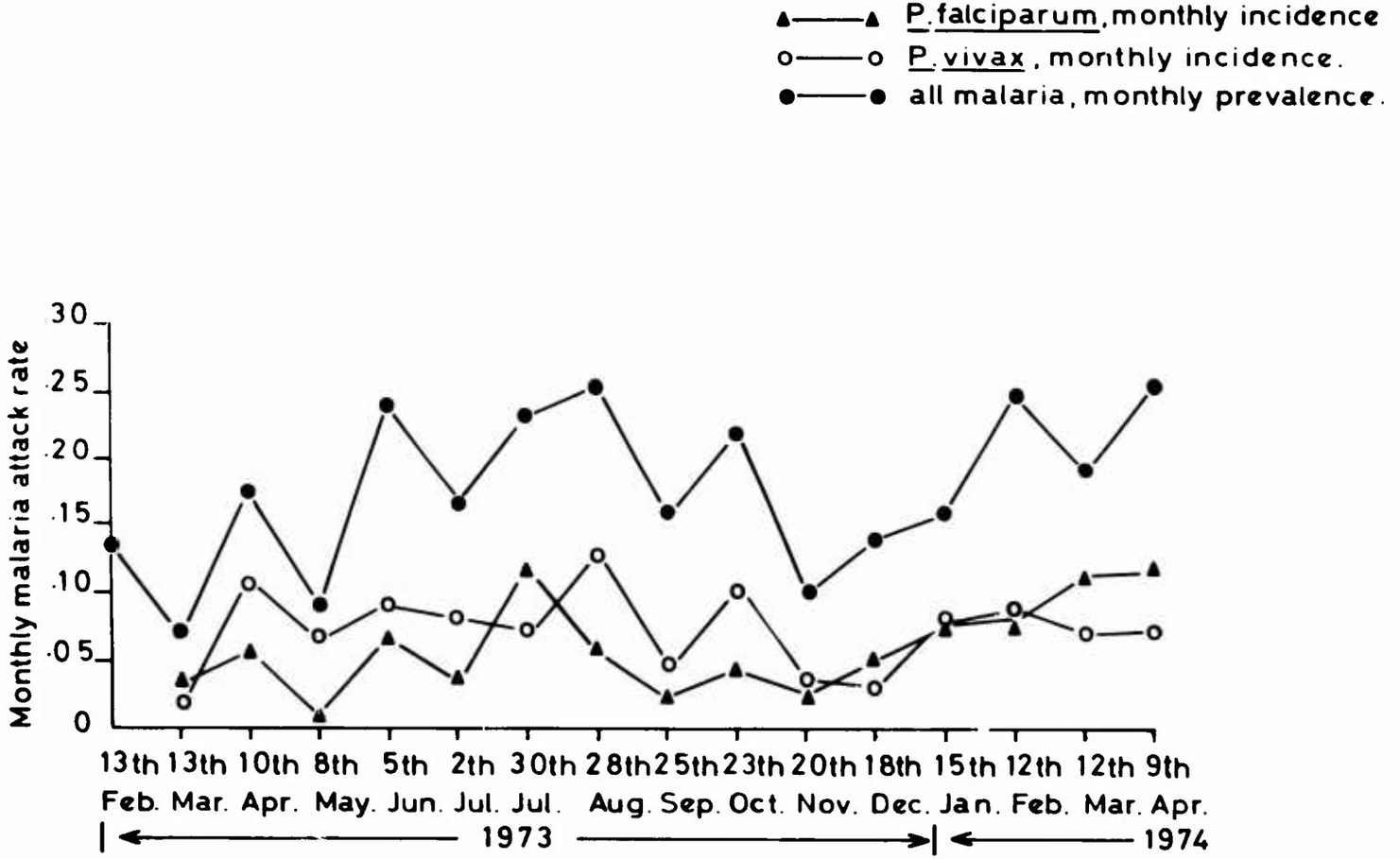


Table 2. Distribution of malaria attack rates and percentage infected by age group.

Age group	Number	Mean attack rate per man month	Percent infected
0-4	38	0.14	66%
5-9	28	0.17	82%
10-14	27	0.19	81%
15-19	24	0.19	83%
20-29	34	0.10	71%
30-39	18	0.08	78%
40-49	11	0.13	64%
50-59	8	0.09	88%
60 +	4	0.04	25%
All ages combined	192	0.14	74%

Occupation: Rubber tappers had the least malaria while the weeders and casual field workers, the school children, and the stay-at-homes all had 60 to 70 percent more. However the anopheline mosquito biting on this estate takes place at night and virtually all the people, whatever their occupation, were in the housing area at that time. The occupational differences appear simply to reflect age differences. The rubber tappers, with less malaria, are mostly men and women over 20, and are the skilled workers. Virtually all the rest, workers and non workers, are under 20. Probably occupational exposure had little to do with who got malaria.

Individual behavior when the vector mosquitoes are biting: Figure 2 shows the biting pattern for Anopheles maculatus as found on this estate, in relation to certain important times. This was the only vector found, and preferred biting outdoors 4:1 over indoors. Field work begins at 5:30 in the morning, at which time there is little biting activity. A large amount of biting occurs before the electricity goes off, which, from our questionaire, is the commonest bedtime. Usually, however, the majority of heavy biting occurs after people have gone to bed. There is the exception that about once a week the generator is kept on till about 11:30 to allow watching special programs on the television. The TV, which is popular on all nights, is located at the shop-canteen in a roofed but sideless structure, completely open to mosquitoes. An. maculatus was common there. Prime television time, drawing large crowds, coincided with prime mosquito time. In addition, some people sat outside at night in front of their houses. No one used insect repellent, which is rather expensive.

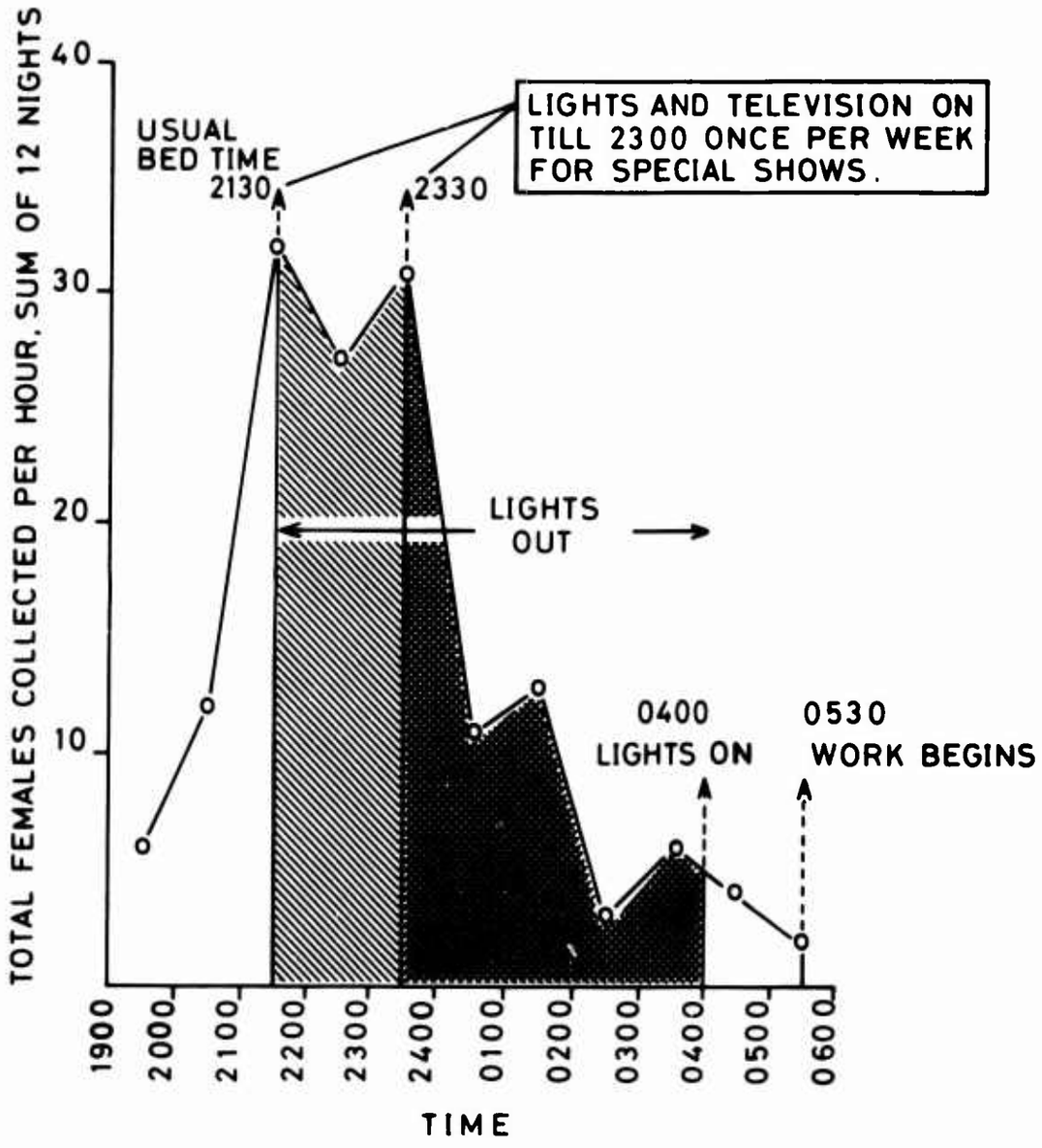
Evening clothing and the amount of time spent outdoors or at the TV area, seemed related to the frequency of mosquito bites and resultant malaria. In the overall group, those who wore short pants or short skirts after dark got 70% more malaria than those who wore long pants or a sarong. There was no apparent difference according to the type of clothes worn on the upper part of the body.

In analysing the results with regard to TV watching, an attempt was made at controlling for age and certain other important variables. Those who watched TV nearly every night (over 40 percent of the population), got one half to two-thirds more malaria than the infrequent TV watchers, when matched for age and other variables.

Other Factors: As Figure 2 shows, well over half of the prime mosquito time occurs after bedtime, even though, given a choice, these vectors apparently prefer to bite outdoors. All the subjects slept indoors with the windows closed, but there are large unscreened ventilation areas under the roofs of all the houses. About one third of the group slept under a mosquito net at all times. They had relatively little malaria. Another one third did not use a net, but burned a pyrethrin coil every night. They got malaria almost twice as frequently as those under nets. Those without a net, who did not burn

Figure 2

COMPILED MONTHLY NOCTURNAL BITING BY ANOPHELES MACULATUS, PAROI RUBBER ESTATE, NEGRI SEMBILAN, PENINSULAR MALAYSIA. NOVEMBER 1973-APRIL 1974.



a coil or who used one only occasionally, fared even worse - they got two and one third times as much malaria as those with a net.

Clearly, a lot of malaria was transmitted to people while they slept. Bed covers and sleeping clothes may have influenced this. Except for a very few, people covered either with a thin cotton sarong worn like a sleeping bag, often with feet sticking out, or with a long blanket of thick cotton reaching from head to toe. When a mosquito net was not used, those covering only with a sarong got over one third more malaria than those using a blanket. This was even more dramatic in relation to the length of skirt or pants worn to bed. Those wearing short pants or skirts and covering only with a sarong developed more than twice as much malaria as those wearing long pants or skirt and covering with a blanket. This most frequently infected group got an average of one new malaria infection per person every four months, or four new infections per year.

We have not yet studied personal differences in attractiveness to the vector mosquitoes. This may reveal another important difference in malaria transmission.

From the preliminary analysis we can suggest certain human behavioral differences which appeared in our study to be related to an increased frequency of malaria. They were: wearing short pants or skirts after dark and to bed; gathering in the crowded, open television area at night; sleeping without the use of a mosquito bed net; and covering with only a thin sarong or nothing in bed.

In review of the ethnic, sexual, and age differences in acquiring malaria, several behavioral explanations suggest themselves. The Malays got considerably less malaria than the Indians. They had proportionately fewer young people living on the estate, but perhaps more importantly, they used a mosquito net more often: 7 families regularly with nets, 3 without, compared to 9 with, 15 without for the Indians. Women got less malaria than the men. The women and the female teenagers tended to wear long skirts, and spend less time in public, particularly at the TV center. The younger children and male teenagers, who as a group had more malaria, wore shorter clothes and spent proportionately more time in front of the TV.

Social-behavioral differences among the individuals may well have influenced who got bitten by mosquitoes and who got malaria. Other factors, such as immunological state and genetic susceptibility, may also take part, but human behavior is probably a very important element. This would suggest that time-honored malaria or mosquito "discipline", such as proper clothing, personal insect repellent, and mosquito netting, to say nothing of chemoprophylaxis, would be useful in preventing malaria under these circumstances.

11. Vertical zonation of mammals and their pathogens in a mature rainforest.

Introduction

Studies of vertical zonation of mammals, their pathogens and associated vectors in a mature rainforest have been continuing since 1969 (SCIENCE, 1970, Vol.169: 788-789). To date, over 2000 mammals have been trapped, marked and released in this area. A portion of these has been repeatedly recaptured, yielding information on growth rates, longevity, serological conversions, persistence of rickettsemia, etc. Each mammal is examined for ectoparasites and blood parasites, such as malarial parasites, microfilaria, trypanosomes. Feces of some have been examined for eggs and cysts of helminths and coccidia. Age and reproductive condition of each mammal is estimated.

The objectives were: 1. to determine the species diversity and relative abundance of mammals in the canopy and on the ground in two areas of the forest. On the basis of recapture data determine the growth rates, longevity, survival, reproductive age, duration of reproductive activity, frequency of parturitions, gestation periods, and movements of various species; 2. to determine the ecological distribution patterns of ectoparasite infestations (including scrub typhus vectors) and infections with blood parasites, microfilaria, rickettsia and viruses; 3. to determine bait preferences of different species of mammals and how this influences their trapping rates; 4. to determine the phenology of trees in the area to find out whether fruiting or flowering influences the trapping results; 5. to determine the edibility of fruits of various trees for various mammals. This is done by direct observation of mammals feeding on the fruit in the field and feeding trials with captive mammals; 6. to determine differences in host species regarding length of rickettsemia, recurrence of infections, fluctuations in antibody, "chigger profiles" (search for forest vectors); 7. to compare infection rates, antibody levels, and vectors (including potential ones) in this and adjacent habitats.

Methods

Out of a total of 400 traps, 200 are operated each week for 5 days alternately in the two areas. Animals captured are brought back to the laboratory, processed, examined, marked and released the next day at the point of capture. Trial baits are placed in alternate traps and are tested against banana. Fruit samples are taken back to the lab for preference testing. Once a week phenological condition of trees is recorded on a map of the area on the record sheets.

Results

Some of the results have been reported already in previous USAMRU-M Annual Reports (1969-73). Figures 1 and 2 show the results

Figure 1. The collection of mammals in traps placed in the canopy at the Bukit Lanjan study area. The collection is divided up into 1969-1971 and 1972-1974 year groups and according to which area the animals were captured (Hill or Valley). The total numbers collected are given below. Species collected less than 5 times in any time/locality category, were not included (*Petaurista petaurista, Iomys horsfieldii, Hylopetes lepidus, Ptilocercus lowii, Tupaia minor, Arctogalidia trivirgatta*).

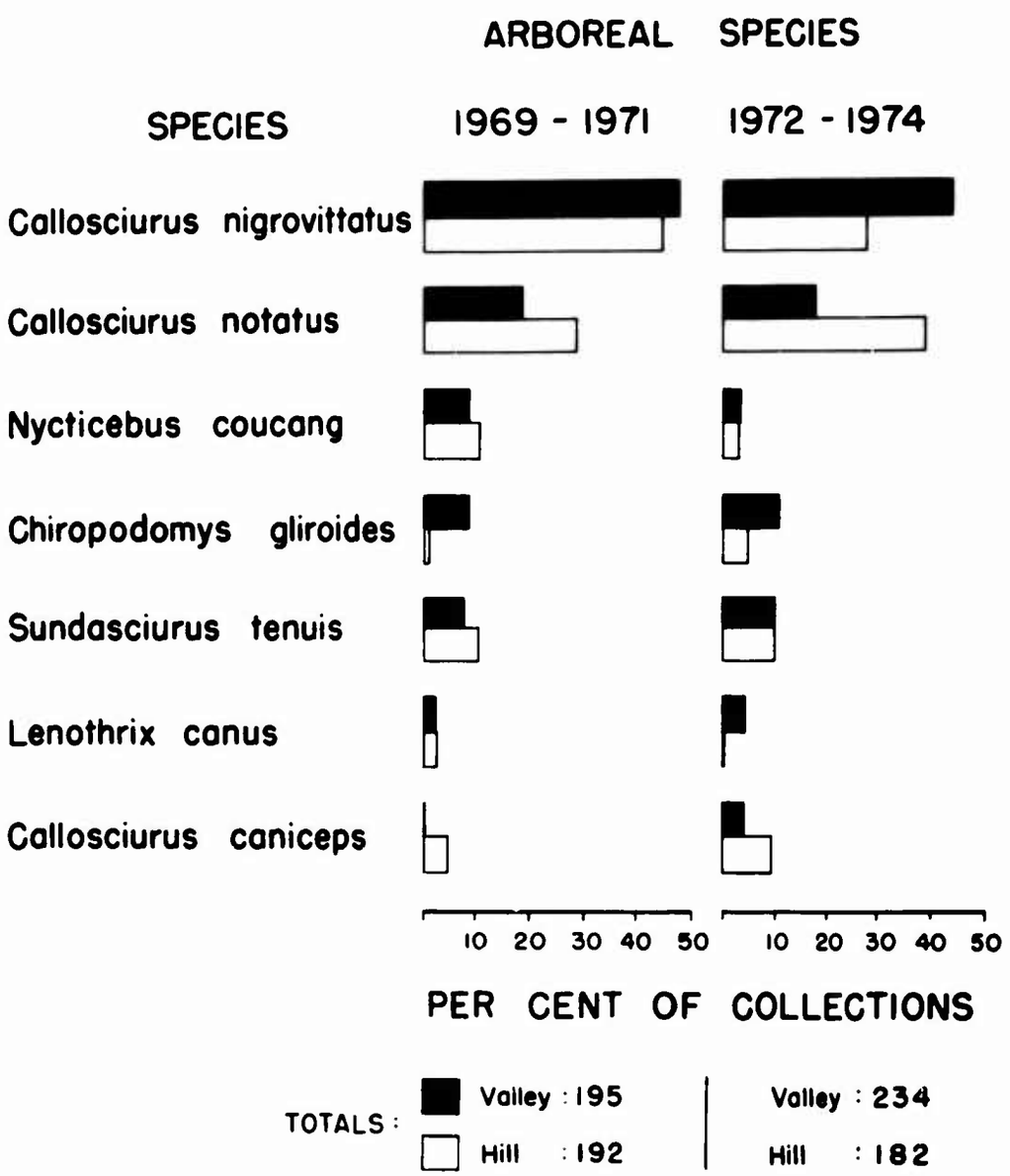
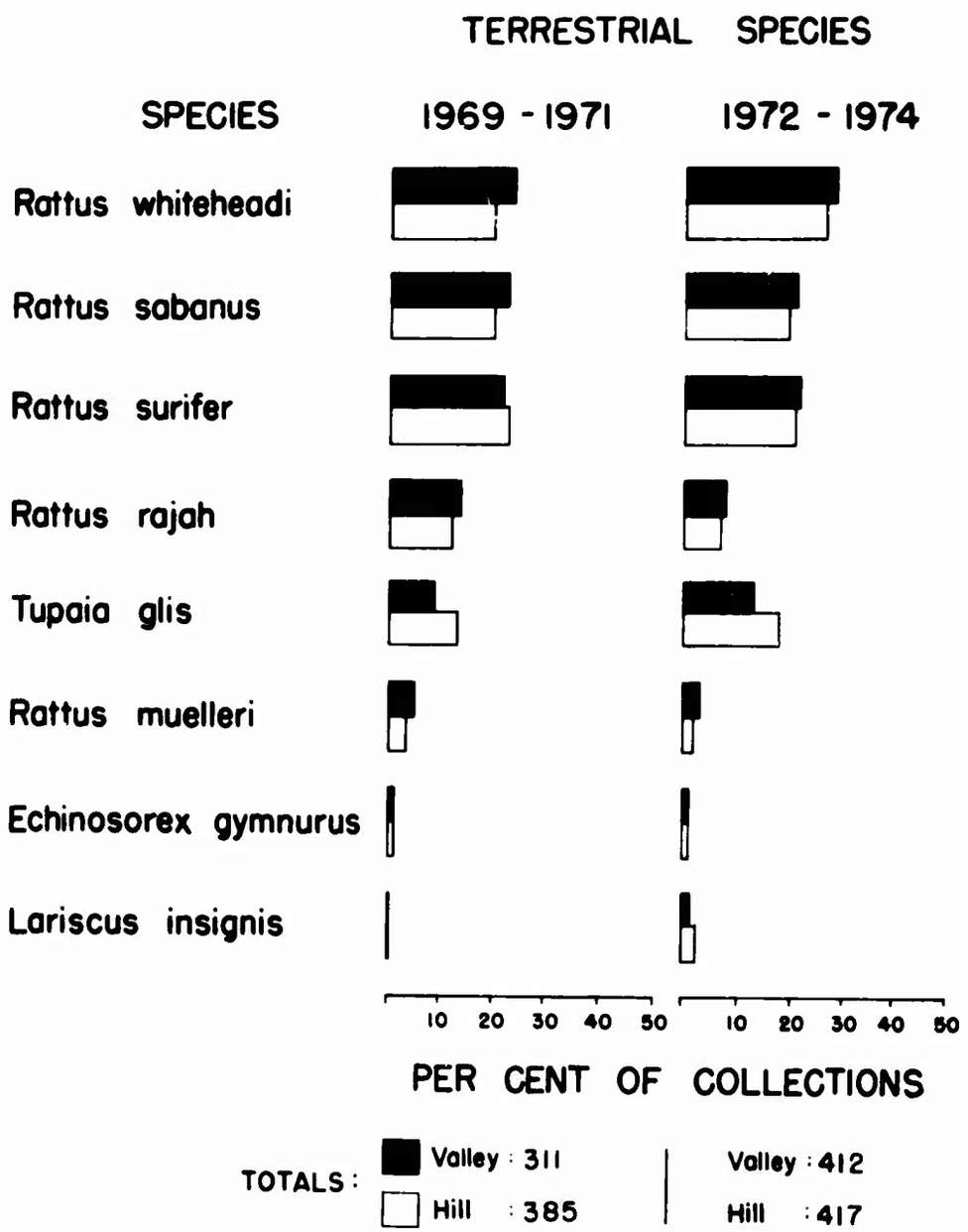


Figure 2. The collection of mammals in traps placed on the ground at the Bukit Lanjan study area (see legend in Fig. 1).



of studies of population structure (as indicated by trapping data) for 15 predominant species. There was little or no difference in the species diversity and relative abundance of terrestrial species in the two parts of the forest (hill and valley) located 500 meters apart. The relative abundance of terrestrial species also did not change much through the years. The arboreal species, however, seemed to be more sensitive to the differences in vegetation and physiography of the two areas sampled, as well as, to changes through time. From 1969 through 1971 Callosciurus nigrovittatus was the most commonly caught canopy species in both areas, although C. notatus was more prevalent in the hill area than in the valley. From 1972 through April 1974 the relative density relationship between these two squirrels remained the same as in the earlier period in the valley. In the hill area C. notatus became more prevalent than C. nigrovittatus. These data, along with smaller scale differences in other species, demonstrate that species composition in the forest canopy is influenced by changes in the habitat occurring through time. The hill area, being closer to the edge of the forest than the valley, seemed to be more responsive to changes, such as cutting of a nearby forest area in early 1972. The data accumulated on growth rates, longevity, etc. have not yet been analyzed. Blood parasites and Group B arbovirus antibodies have been shown to be more prevalent in the arboreal species than in the terrestrial mammals. Rickettsia tsutsugamushi and Coccidial parasites, on the other hand, were more prevalent in the terrestrial mammals than in the arboreal forms. Bait preference, phenological data, and data on chigger profiles, durations of rickettsemia and antibody have not yet been analyzed.

12. Ecological studies of scrub typhus (Rickettsia tsutsugamushi) in differing but adjacent habitats.

Introduction

In the second phase of our scrub typhus studies indicator species of rats were trapped in four habitats at Bukit Lanjan, Selangor. Rickettsial isolation and other data have been summarized for about six months (USAMRU-M Annual Report, 1972). Several refinements were required to eliminate the variables which obscured some of the results of the previous study. Nevertheless, the existing data seem to indicate that rickettsial transmission was occurring at a high rate in the forest and in lalang, and at a lower rate in the village. Since rats seemed to move in and out of the fourth habitat: edge (or "scrub"), it was difficult to determine actual rates of transmission there. Moreover, the four indicator species used were not observed to be entirely habitat specific and although species differences were observed in several parameters concerning infections with Rickettsia, it was not possible to determine reliably in all cases whether these differences were attributable to habitat, or species specific responses to the infections.

The objective was to detect and follow linearly the activity of Rickettsia tsutsugamushi in two different adjacent habitats, lalang and edge ("scrub"), by determining monthly antibody conversion rates in and isolation rates from a single indicator species, Rattus annandalei, rickettsial isolations from chiggers infesting the indicator species, and the strains of Rickettsia present in each of the habitats and through time.

Methods

One species, R. annandalei, is used in the two habitats. The animals are laboratory raised to be sure of susceptibility and to eliminate the problem of distinguishing between cases of reinfections, primary infections, and recrudescence of sequestered infections. 20 meter diameter enclosures were constructed in both habitats to confine movements of the indicator species to the specific habitats and to determine the primary habitat source(s) of chigger infestations and specificity of strains of R. tsutsugamushi, if any. In previous studies (Phase I) Rattus annandalei was found infected with Rickettsia at a rate higher than the average among rats in nature. It was trapped in mature secondary forest, kampong rubber plantations (mixed age, irregularly planted, infrequently cleared of undergrowth) and sometimes in edge habitats ("scrub"). Linear laboratory studies of infections in this rat have been conducted. After pre-bleedings rats are released into the enclosures and kept there for 3-7 days. Feeding is done in open traps to facilitate recapture by accommodating the rats to the traps. While in the laboratory before release, the rats are also entrained to enter traps for food. During the remaining 3 weeks each month the enclosures are made accessible to wild rats by means of sticks leaned against the walls on both sides so that chiggers are continued to be brought into the area by the wild rats, thus approximating events as they would occur normally. Otherwise, the sole source of the rickettsia would be the transovarial route (which may or may not be the only source) in the resident Trombiculid populations. After being in the field for the predetermined period of time, the indicator rats are brought back into the laboratory for rickettsial isolation and serological studies and they are examined for chiggers. Infection in chiggers is detected either by inoculation into mice or by microscopic examination for fluorescence (Department of Acarology). Bleeding of rats is done every 8 days for the first 40 days and once a month thereafter.

Results

To date the results indicate that more transmission is occurring in the lalang habitat (67%, N=33) than in the "scrub" (30%, N=40). Also, the course of infection and antibody response is very different in the field infected rats than in those infected by inoculation in the laboratory. In previous laboratory studies with the same species, R. annandalei antibody appeared 8-16 days post-inoculation; depending on the dosage of the inoculum, rickettsemia

began as early as 4 days post-inoculation. Field exposed rats yielded rickettsial isolations as early as 4 days after release into the enclosures, but antibody did not appear until 23-78 days after exposure. Rickettsemia in the laboratory inoculated rats lasted 1-8 days (mode 4 days), but in the field exposed rats infections lasted from 8 to over 41 days, the mode being 20 days. These differences could be attributable to dissimilarities of rickettsial strains involved.

Continued efforts have resulted in additional data from Phase II studies. Last year we reported on the microdistribution of rats and isolations of Rickettsia tsutsugamushi from the rats captured in the lalang habitat at Bukit Lanjan. Figure 3 shows the initial capture records of Rattus argentiventer and R. tiomanicus at the edge of the forest in the four habitat study. 100 traps were set, each 5 meters apart, at the boundary between the forest and the field. The predominant species captured was R. tiomanicus. R. argentiventer, the lalang rat, was quite abundant in the area encompassed by traps 29-50, less so elsewhere. The rickettsial isolation rate was higher in R. argentiventer than in R. tiomanicus. The distribution of the infected rats (both species) correlated with the frequency of captures, indicating that infections occurred wherever there were rats. Together with the data from the lalang habitat, these results indicate that in this area the movements of infected rats are not restricted, and by inference, it appears that the distribution of the infected chiggers also may be fairly evenly dispersed. If there are "scrub typhus islands" in this area they are probably abundant. The area included in the lalang trapping grid and the line of traps at the edge habitat extended for nearly 700 meters. According to Harrison (1958, J. Mammalogy, 39: 190-206) individual R. tiomanicus range over quite a large area, but nearly 100% of their activity would be confined to an area less than about 250 meters in diameter. Although home ranges of individual rats overlap, those of the individuals at the extremes of an area 700 meters in diameter would not. Indeed such an area would include nearly three sets of mutually exclusive ranges, if one calculates according to Harrison's maximal values. It is likely, however, that there would be even less overlap.

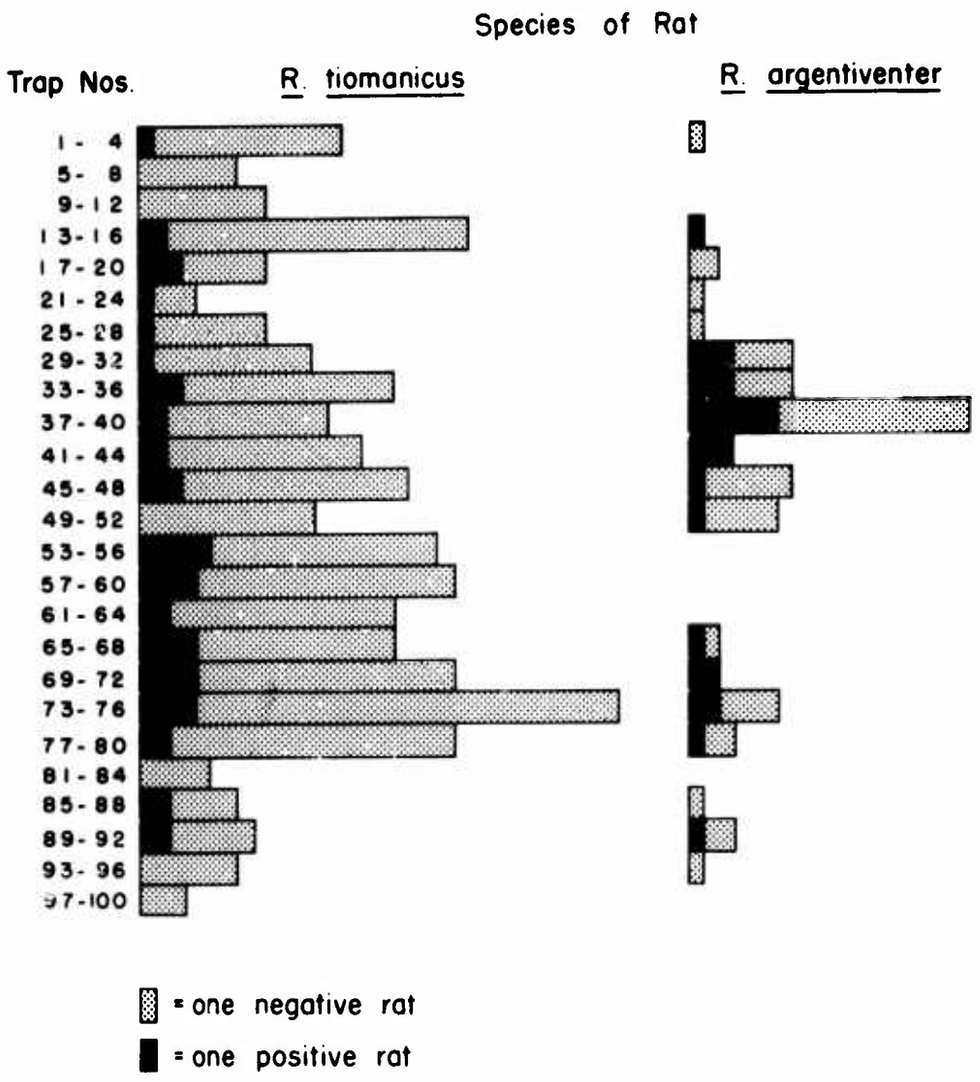
13. Ecological, taxonomic, and zoogeographical studies of mammals.

Introduction

These studies have been conducted to provide a general framework for the understanding of the role of mammals, the major hosts of zoonoses, in the various interactions within the ecosystems in which zoonotic diseases are transmitted. Various ways have been used to measure population densities to reduce biases inherent in the use of trapping alone. Mark-and-release methods have been used in several habitats to determine whether differences in population structure are present. Also, the effects of human disturbances have been studied.

We have attempted to determine densities of tree cavities suitable for nesting for arboreal mammals, the species diversity and relative

Figure 3. The pattern of initial captures of two species of rats (hatched bars) in a trap-line placed along the edge of the forest which bordered a cleared area grown over with lalang, rhododendron, shrubs, wild giner, etc. The dark bars represent rats which were infected with *Rickettsia tsutsugamushi*.



abundance of species occupying such tree cavities, the overlap of ecological niches (use of space) of various species, and other pertinent ecological and parasitological information. We have also studied populations of mammals in differing habitats, including transitional ones.

Method

In addition to standard capture-mark-release-recapture techniques we have employed Orang Asli (Aborigines) to search for arboreal nests. These nests have been mapped in a 200 ha area in a primary forest near Segamat Kechil, Johore, and have been examined periodically for occupants. We have taken advantage of recent and long-term human alterations of habitats elsewhere and have studied the mammals before and after, as well as, during the course of such alterations.

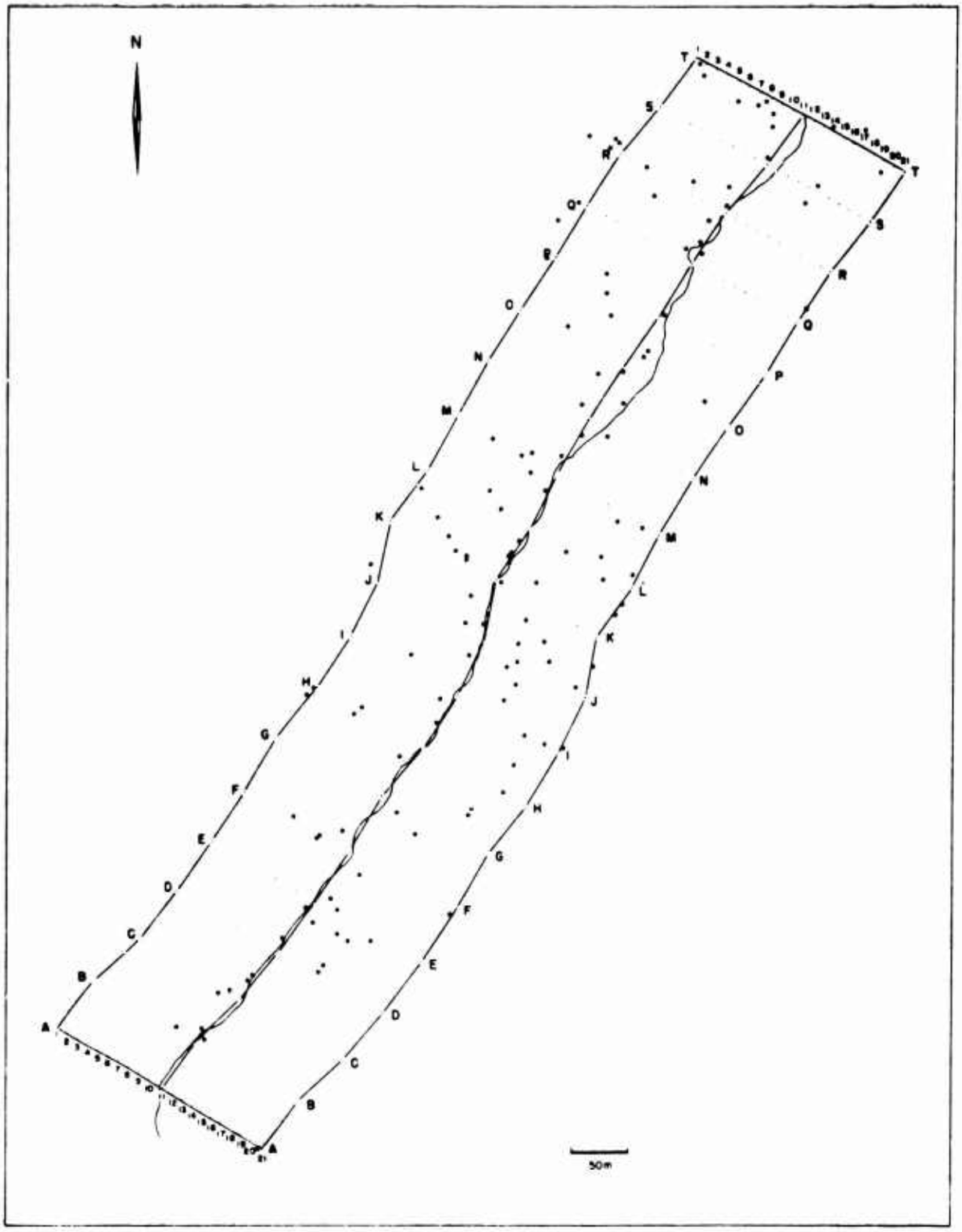
Results

Figure 4 shows one of the study areas, 200 ha in a primary forest near Segamat Kechil in Johore. Open circles represent tree cavities suitable for nests, closed circles indicate occupied cavities. The density of nest cavities was about .5 per hectare. Thirteen of these were occupied on initial examination, approximately 10%. The tree cavities were used primarily by Hylopetes spadiceus, and less by Pteromyscus pulverulentus and Petinomys setosus. Only a few Hylopetes and Petinomys have been trapped in such forests and no Pteromyscus. From these results it is clear that although great efforts are expended by trapping (the IMR efforts have resulted in over 10,000 mammals trapped over the last 26 years), some species would appear to be extremely rare, but are actually not uncommon in such habitats. Studies of man-altered habitats at Bukit Mandol and Bukit Lagong in Selangor, indicate that the species diversities of terrestrial and especially arboreal mammals differ greatly from those in primary forests or less disturbed areas. Some species which appear to be uncommon in the less disturbed areas often occur as the dominant species in disturbed areas. The significance of this, from the standpoint of epidemiology, is that if certain species are favorable, but uncommon hosts of zoonotic diseases, alterations of habitats may encourage their proliferation. Conversely, infection rates with certain parasites may decrease in disturbed habitats (e.g. Plasmodium infections in Petaurista, Mut Yap and Lim 1973, Southeast Asian J. Trop. Med. Pub. Hlth., 4: 377-

14. Management of a tropical laboratory animal resources facility. The R. annandalei production has continued to supply animals for scrub typhus investigations; however, the numbers produced are still too small to allow experimentation with their husbandry.

The average litter size during the last year (56 litters) was 3.71 (S.D. 1.38), still small compared to the common laboratory rat. Six pairs of R. annandalei have been housed in standard plastic rat

Figure 4. Study area near Segamat kechil Johore. Open circles represent nest holes in trees similar to those from which flying squirrels have been collected. Closed circles represent nests occupied (mostly arboreal animals).



cages ($10\frac{1}{2}$ x 8 x 19) and have produced litters, but not enough to determine if there is a real difference between standard cages and those which provide a nest box.

The lesser mouse deer colony began to produce second generation laboratory-bred young this year. However, survival of the young has been poorer this year with about one-third of the young dying before one month of age. Gross post-mortem examinations, negative bacteriological findings, and examinations of the dams indicate that inadequate milk production is responsible for the deaths. Changes have been made in the diet and method of water supply to try to increase milk production.

A disease of unknown etiology occurred in the 66 P. cristatus and 3 gibbons in the USAMRU colony. One gibbon and 6 P. cristatus died during the nine weeks the disease was seen in the colony. It was characterized by signs of inappetance, copious mucopurulent nasal discharge, diarrhea, and depression. Four animals showed an erythematous rash on the chest and abdomen. Gross lesions in the animals that died were limited to varying degress of pulmonary congestion. All clinically sick animals were treated symptomatically with a kaolin-pectin suspension and oxymetazoline nose drops. Five monkeys of the first 10 that became ill were also treated with 15 mg t.i.d. of doxycycline, but the antibiotic did not appear to alter the course of the disease. Blood, lungs, heart, liver, spleen, and lymph nodes were negative on bacteriological examination and for viruses detectable by mouse or chick embryo inoculation.

21 Macaca mulatta were housed with the affected animals for several months prior to, and during this disease occurrence. None showed any clinical signs of disease during this period. None of the animal caretakers reported or were observed to have any respiratory disease during this period; however, because of deficiencies in the construction of the animal houses the animals could have been infected by persons using a near-by side walk.

Mycoptes musculinus, probably endemic in our colony, began to cause noticeable hair-loss in many weanling mice in February. All mice were dipped twice in 2% Malathion solution at a two weeks interval. This treatment has been successful in eliminating any overt signs of this mite.

Reovirus III has been found in suckling mice from our colony during attempts to isolate Dengue virus from patients and mosquitoes. Although some investigators report that 10-15% of suckling mice issued to them have clinically apparent Reo III infection, only one affected litter has been seen in the breeding colony. It is postulated that the single passage of the virus through suckling mice which is done in attempted isolation of Dengue may increase the virulence of this strain of Reo III.

Although efforts to improve the management of the colony and reduce the incidence of Reo III infection are being made, it is doubtful that Reo III-free mice can be provided from the present inadequate animal house.

Sufficient new cages should soon arrive to provide adequate caging for the mouse colony; however, the same properties of the new cages that allow sufficient air movement for a tropical climate also increase the exposure of the mice to wild rats which infest the animal house. No really effective solution for the wild rat problem is seen until the completion of the new animal facility.

Construction of the new animal facility to be shared by the IMR, the USAMRU, and the University Kebangsaan was begun in May 1974 and should be completed in May 1976. The USAMRU veterinarian has been able to offer many suggestions on the design of this facility to the architect, and most of these suggestions have been implemented. If the necessary equipment is purchased, and proper training and motivation of personnel is done, most of the current animal disease and availability problems will be solved by this facility.

In anticipation of further continued use of this species by the USAMRU, newly captured monkeys housed in individual cages on an exporter's premises are examined for enteropathogens and reaction to intrapalpebral tuberculin. Currently, of 105 animals TB tested, none have been positive. Of 151 monkeys examined for enteropathogens by culture of rectal swabs, 27 have been positive, 21 of those positive have been *Shigella sp*. Interestingly, all of the *Shigella sp*. isolated are resistant on *in vitro* testing to chloramphenicol, and almost all are resistant to ampicillin, tetracycline, and neomycin. Furazolidone and trimethoprim-sulfamethoxazole seem to be the two most effective and practical drugs for treatment of this organism.

An investigation of the efficacy of subcutaneously injected levamisole is also being done in these monkeys, but too few animals have been treated at this time to make an evaluation of its antihelmenthic efficiency.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 072 Ecological Surveys of Tropical Diseases

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PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DAOB 652[illegible]	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORG'N INSTR'N	8b. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM
73 11 01	D.CHG	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	073
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	Cards 114F			CH FR 059

11. TITLE (Precede with Security Classification Code)

(U) Disease Transmission in Tropical Populations

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology [illegible] Biology [illegible] 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 7[illegible]	3	194
FISCAL YEAR CURRENT 75	5	273

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC [illegible]

RESPONSIBLE INDIVIDUAL

NAME: Buescher, E. L. COL

TELEPHONE 202-[illegible]-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Research Unit Belem (Transamazon)

ADDRESS: APO [illegible]

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Llewellyn, C. H. LTC

TELEPHONE

SOCIAL SECURITY ACCOUNT NUMBER [redacted]

21. GENERAL USE

Foreign intelligence not considered

ASSOCIATE INVESTIGATORS

NAME Blumberg, L. CPT, MSC

NAME Rogers, F. CPT, MSC

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Epidemiology; (U) Disease Transmission; (U) Hepatitis; (U) Yellow Fever; (U) Influenza; (U) Measles; (U) Brazil

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

23. (U) Conduct epidemiologic studies of infectious diseases transmitted among populations along the Trans-Amazon Highway in collaboration with the Instituto Evandro Chagas. Information regarding the health hazards are of importance to military personnel transiting or stationed in this geographic area.

24. (U) Conventional procedures and modifications will be utilized.

25. (U) 73 11 - 74 [illegible] This research is complementary to studies being conducted under Work Unit, entitled Epidemiologic Studies of Infectious Disease Transmission Along the Trans-Amazon and Cuiaba-Santarem Highway.) Pilot studies to obtain hospital disease data and define study populations were carried out in Maraba and Altamira. A water-borne outbreak of sylvatic yellow fever was identified and investigated in Garupa. Evidence of significant Mucabo virus transmission and disease was also identified. An outbreak of influenza beginning in a Brazilian military unit in Roiraima was investigated. Virus was isolated and serologic surveys were made. Pilot sutdies were conducted in an onchocerciasis focus in Roiraima territory. This research is complimentary to studies being conducted under DAOB 752b, work unit 095, entitled Epidemiologic Studies of Infectious Disease along Trans-Amazon and Cuiaba-Santarem Highway.

*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 68 AND 1498 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 OC 6465	74 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
73 07 01	D. CHG	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	074
b. CONTRIBUTING				CH FR 002
c. XXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Tropical and Subtropical Disease 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)
PRECEDING FISCAL YEAR 74	10.1	1390
CURRENT FISCAL YEAR 75	10.0	1150

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: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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TELEPHONE: 948-4523
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Bancroft, LTC W.H.
NAME: Davidson, LTC D.E.

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; (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 the ecology and basic biology of causal agents of tropical diseases, and to study variables of host environment 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) 73 07--74 06 The epidemiology of hepatitis, dengue, and Japanese encephalitis in Thailand were studied, with emphasis on improved methods of virus isolation and serological detection. A 4% net annual rate of acquisition of hepatitis was observed in Bangkok children, with adult prevalence reaching 70%. Four viruses, one a possible human pathogen, have been isolated from ticks and are being characterized. Fluorescent antibody stained corneal impression smears are being evaluated in the diagnosis of human and canine rabies. Resistance to chloramphenicol has been observed in a high percentage of typhoid cases. Clinical and epidemiological aspects of Vibrio parahemolyticus diarrhea have been studied. 98% of gonococcal isolates in Bangkok were partially resistant to penicillin, but 97% were fully sensitive to ampicillin. Epidemiological studies of bancroftian filariasis, with initial emphasis on vector identification, have been initiated in an endemic rural focus; 8% of the human population were microfilaremic. Psychological factors leading to drug abuse are being studied, and a questionnaire for predicting drug abuse behavior is being field tested. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 — PREVIOUS EDITIONS ARE OBSOLETE AND FORMS 1498A, 1 NOV 65 ARE OBSOLETE — PPC-Japan

Project 3A762759A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 074 Tropical and subtropical diseases in military medicine

Investigators

Principal: LTC Phillip E. Winter, MC; LTC William H. Bancroft, MC; LTC David E. Davidson, Jr., VC; LTC Franklin H. Top, Jr., MC; Douglas J. Gould, Ph.D.; Joe T. Marshall, Jr., Ph.D.; MAJ Michael W. Benenson, MC; MAJ Richard M. Lampe, MC; MAJ Eliot J. Pearlman, MC; MAJ Harry Rozmiarek, VC; MAJ Robert M. Scott, MC; MAJ Douglas Stutz, MSC; CPT Charles L. Bailey, MSC; CPT Garrett S. Dill, Jr., VC; CPT John S. Jewell, MSC; CPT Robert J. Schneider, MSC; CPT Rowland N. Wilkinson, MSC; MAJ Dumrong Chiewsilp, MC, RTA; Svasti Daengsvang, Med.D.; Chiraphun Duangmanee, M.D.; Kwanyuen Lawhaswasdi, DVM; Udom Lexomboon, M.D., Ph.D.; Phon Sangsingkeo, M.D.; Rapin Snitbhan, M.D.; Prayot Tanticharoenyos, DVM; Markpol Tingpalapong, DVM

Associate: Pongsom Atthasampunna, M.D.; Prakorb Boonthai, M.D., D.P.H., D.T.M.&H.; Tawee Chottipitayasunodh, M.D.; SP4 Ernest T. Comer, Jr.; Richard A. Grossman, M.D.; Pairatana Gunakasem, M.D.; CPT Bruce A. Harrison, MSC; Siriwan Honorasethkul, M.D.; Y.M. Huang, Ph.D.; Suchart Jatanasen, M.D.; SP5 Jerome J. Karwacki, Jr.; Sukachard Kirdpol, M.D.; George Kojak, M.D.; Karoon Mansuwan, M.D.; Pethai Mansuwan, M.D.; Sumitda Narupiti, B.Sc.; Pacharee Nawarat, M.Sc.; Suchitra Nimmannitya, M.D.; Wichiyan Paniom; Pisipong Patamasukon, M.D.; E.L. Peyton; SFC Frank B. Poyas; Serin Punaitanont, Ph.D.; Rampa Rattanarithikul; MAJ John F. Reinert, MSC; CPT Horst Ressdorf, MSC; Chatchaval Rochananond, M.D.; SSG Elisandro Rodriguez; Joseph Rothberg, Ph.D.; CPT John W. Sagartz, VC; Nongnard Sahasakmontri, B.Sc.; Chumneun Satayaphantha, DVM; SFC Charles W. Shaw; Phinit Simasathein, M.D.; Stitaya Sirisinha, DMD, Ph.D.; Sunthorn Sirithanakarn; MAJ Michael R. Spence, MC; Fred D. Stone, M.Sc.; Pramuan Sunakorn, M.D.; Pravit Sunthornsrima, M.D.; Panita Tanskul, M.Sc.; Banchang Thanangkul, M.D.; Chaufah Thongthai, Ph.D.; Sobhon Tirasitara, M.D.; Suwana Vaitanomsat, B.Sc.; Santisook Vibulbuditkij, M.D.; Kitti Watanasirmkit, B.Sc.; SFC Robert E. Weaver

I. VIRUS DISEASES OF MAN AND ANIMALS

A. DENGUE HEMORRHAGIC FEVER

1. Surveillance of Dengue Hemorrhagic Fever Cases in Thailand 1973

The purpose of this study is to establish a system for serologic confirmation of dengue hemorrhagic fever cases reported to the Ministry of Health and to assist in the planning of public health control measures.

Dengue hemorrhagic fever (DHF), a principal infectious disease problem in Thailand, occurs in yearly epidemics throughout Thailand. The disappearance of Chikungunya infection in the past few years leaves dengue viruses as the sole causative agents of mosquito-borne hemorrhagic fever in Thailand. A previous report described a method of blood collection from finger pricks onto small paper discs, which has allowed transportation of blood specimens over long distances in a warm climate (1). A Dengue 2 antigen provides an economic means for testing for secondary infections by microtiter hemagglutination inhibition (HI). The use of only Dengue 2 antigen for serologic confirmation of DHF is only a little less efficient than using antigen of all four types of dengue. The results suggest that it is an acceptable means for confirmation of dengue infections in a DHF surveillance program.

The localities submitting specimens are shown in Figure 1. Information on each reported case was provided on individual patient forms. The forms included instructions for blood collection and for the recording of the patient's history, physician's diagnosis and the laboratory diagnosis. Two small filter paper discs (12.7 mm diameter, No. 740-E, Schleicher and Schuell) were clipped on the positions for acute and convalescent blood samples. The patients were bled from the finger tip on the day of admission and on the last day of hospitalization. The patient forms with the dried blood samples were mailed to the Division of Epidemiology, Ministry of Public Health and recorded; then transported to the Department of Microbiology, Faculty of Public Health for HI testing. After analysis and tabulation, the results of the laboratory tests were returned to the Division of Epidemiology and to the physicians in charge of the patients.

Dried blood samples were eluted with borate saline pH 9.0, and treated with kaolin and goose red cells. The eluates from paired

sera were tested with a microtiter HI test (2). Dengue 2 antigen was used to test all sera; however in cases of encephalitis, Japanese Encephalitis Virus (JEV) antigen was also used.

Cases were considered positive for dengue or JEV infection if the paired sera showed either 1) a four-fold rise in antibody titer regardless of the titer of the initial serum, or 2) a titer greater or equal to 1:640 in both sera. Paired sera that had antibody titers that did not meet these criteria came from cases considered to be unconfirmed (negative). HI tests were not done on some sera due to miscellaneous technical factors, such as inadequate blood, short stay in the hospital and variation of the units of antigen used in each test. The cases with untested sera were considered undetermined.

Tables 1 to 4 present the results of HI testing with dengue 2 antigen for each province and town. Specimens were collected from one hospital in each location. Sera were received from 1236 patients. The 1110 cases of dengue sampled represented 13.4% of the 8275 total cases reported for all of Thailand. Some locations provided too few specimens to come from all of the patients; but the number of specimens from 4 regions can be considered representative of the reported cases of DHF in Thailand. The ratio of correct diagnosis of DHF cases for each region is 1:2.4 (Central), 1:3.0 (North), 1:1.9 (Northeast) and 1:2.5 (South). When compared to the ratio 1:2.4 for all cases, serologic confirmation of cases from the North region is below the national average. The accuracy of physicians making the diagnosis varied greatly between locations.

There were requests for JEV tests from every region. The results of HI tests confirmed JEV infection in 14.7% of all cases of encephalitis (Table 5).

As revealed in Figures 2 to 6, in the Central and North regions the peak numbers of confirmed cases came in July. The peaks of confirmed cases and reported cases coincided for the Central region, but in the North the greatest number of admissions occurred in August. In the Northeast and South there was a shift of confirmed cases to September. The peak incidence of confirmed cases of JEV came in July.

The widespread yearly epidemics of dengue hemorrhagic fever in Thailand create an important public health problem. This surveillance system may become useful for confirming cases of dengue sufficiently early in an epidemic to permit the initiation of control measures, e.g., insecticide spraying. The one month delay

Table 1. Results of HI Tests for Dengue and Japanese Encephalitis Virus Infections in Central Region, 1973

Province	Dengue 2				JEV			
	No. of spec.[1]	HI Pos	HI Neg	Undet.[2]	No. of spec.	HI Pos	HI Neg	Undet.
Bangkok	6	2	4					
Samut Sakhon	32	10	17	5				
Samut Prakan	1		1					
Samut Songkhram	36	5	26	5				
Nonthaburi	61	15	38	8				
Pathum Thani	2	1	1					
Nakhon Pathom	97	23	53	21	2	2		
Ratchaburi	12	6	5	1	1		1	
Petchaburi	4	2		2				
Suphan Buri	7	2	4	1	1	1		
Sing Buri	5	2	2	1				
Ayuthaya	24	14	9	1				
Saraburi	102	39	41	22	3		3	
Lop Buri	15	7	8					
Ang Thong	13	6	3	4				
Chon Buri	8	4	3	1				
Chachoengsao	3	1	1	1				
Rayong	198	119	37	42	1	1		
Chantha Buri	20	14	6					
Prachin Buri	42	16	22	4	9	1	8	
Nakhon Nayok	1			1				
Total	689	288	281	120	17	5	12	0

1 Number of specimens submitted for testing

2 Undetermined

Table 2. Results of HI Tests for Dengue and Japanese Encephalitis Virus Infections in North Region, 1973

Province	Dengue 2				JEV			
	No. of spec.[1]	HI Pos	HI Neg	Undet.[2]	No. of spec.	HI Pos	HI Neg	Undet.
Chiang Rai	7	1	5	1	6	1	5	
Chiang Mai	65	35	27	3	12	4	8	
Mae Hong Son	5	2	3		1			1
Lampang	4		4		2			2
Lamphun	4	1	3					
Nan	93	8	58	27	30	5	21	4
Phrae	9	5	4		9	5	4	
Uttaradit	16	5	8	3	11	5	4	2
Tak	2	1	1					
Sukhothai	9	5	3	1	4	1	3	
Phitsanulok	6	5		1	1			1
Phetchabun	10	5	3	2	1		1	
Phichit	1			1				
Kamphaeng-Phet	9	2	7		7	1	6	
Nakhon Sawan	9	2	6	1				
Total	249	77	132	40	84	22	52	10

1 Number of specimens submitted for testing

2 Undetermined

Table 3. Results of HI Tests for Dengue and Japanese Encephalitis Virus Infections in Northeast Region, 1973

Province	Dengue 2				JEV			
	No. of spec.[1]	HI Pos	HI Neg	Undet.[2]	No. of spec.	HI Pos	HI Neg	Undet.
Nakhon Ratchasima	27	12	13	2	7	2	5	
Khon Kaen	1	1						
Chaiyaphum	7	4	3		3	1	2	
Sakon Nakhon	2	2						
Nakhon Phanom	8	5	1	2	1			1
Kalasin	8	3	3	2				
Nong Khai	4	1	3					
Roi Et	8	5	3					
Buri Ram	5	2	2	1				
Si Sa Ket	3	1	1	1				
Udon Thani	10	5	5					
Yasothon	41	21	19	1				
Ubon Ratchathani	1		1					
Total	125	62	54	9	11	3	7	1

1 Number of specimens submitted for testing

2 Undetermined

Table 4. Results of HI Tests for Dengue and Japanese Encephalitis Virus Infections in South Region, 1973

Province	Dengue 2				JEV			
	No. of spec.[1]	HI Pos	HI Neg	Undet.[2]	No. of spec.	HI Pos	HI Neg	Undet.
Chumphon	1			1				
Ranong	1		1					
Surat Thani	22	8	13	1	3	1	2	
Phang-nga	1			1				
Krabi	4		3	1				
Phattalung	13	5	7	1	1		1	
Nakhon Si Thammarat	38	26	12					
Trang	4	1	3		1		1	
Songkhla	67	16	49	2	6		6	
Satun	2	1	1					
Pattani	18	5	11	2	3		3	
Narathiwat	2	2						
Total	173	64	100	9	14	1	13	0
Total for all of Thailand	1236	491	567	178	126	31	84	11

1 Number of specimens submitted for testing

2 Undetermined

Table 5. Detection of JEV Antibody by HI in Cases of Encephalitis in Thailand

Region	1973									
	Jan-Mar		Apr-Jun		Jul-Sep		Oct-Dec		Combined	
	No.*	JEV+**	No.	JEV+	No.	JEV+	No.	JEV+	No.	JEV+ (%)
North			10	4	70	7	4	1	84	12 (14.3)
Northeast					9	3	2	0	11	3 (27.3)
Central			4	0	8	3	5	2	17	5 (29.4)
South	2	0	14	0	5	0	17	2	38	2 (5.3)
Combined	2	0	28	4	92	13	28	5	150	22 (14.7)

* Number of specimens submitted for testing

** Confirmed positive for JEV antibody

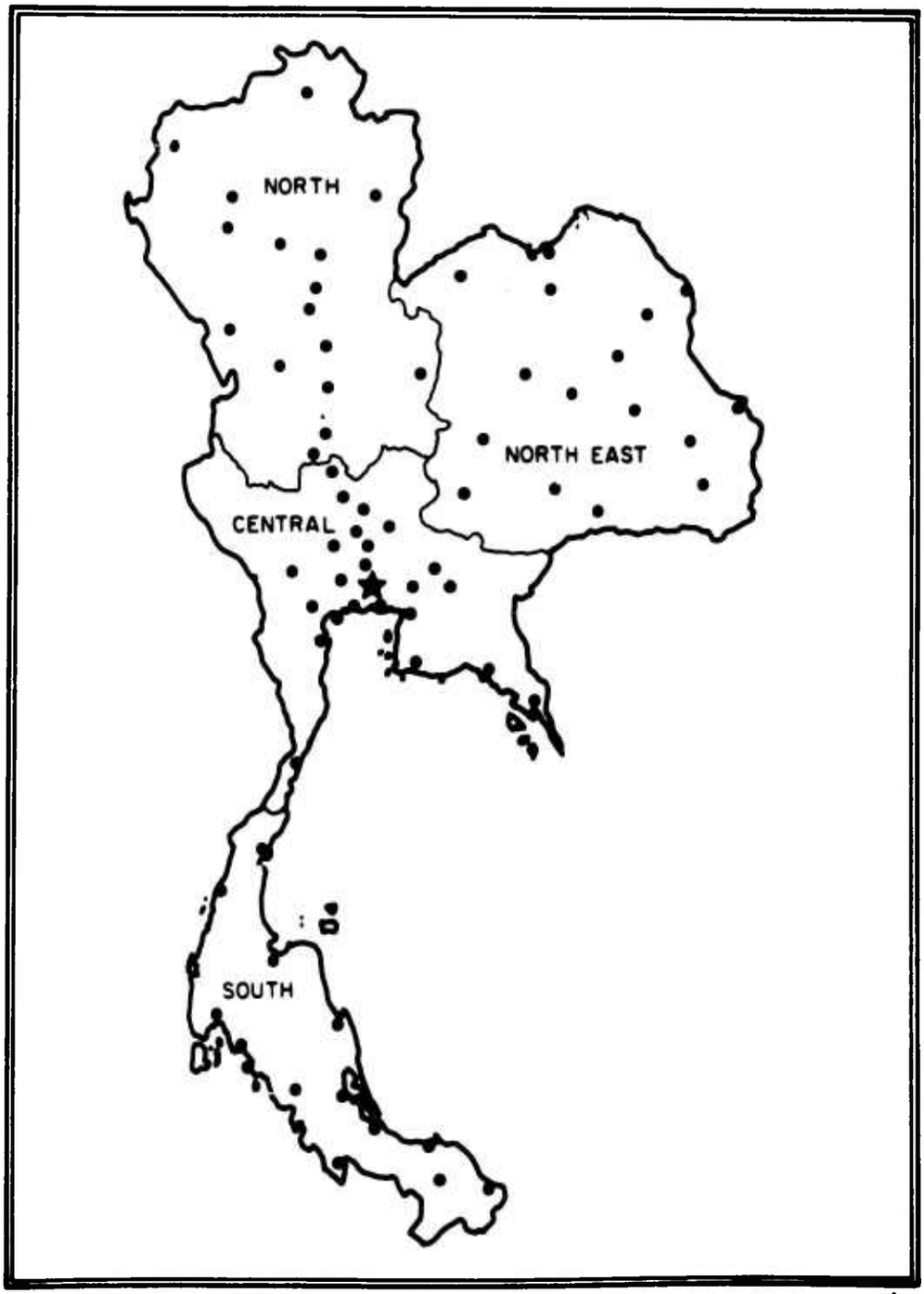


FIGURE 1. MAP DEMONSTRATING PROVINCES OR TOWNS OF STUDY (●), (★).

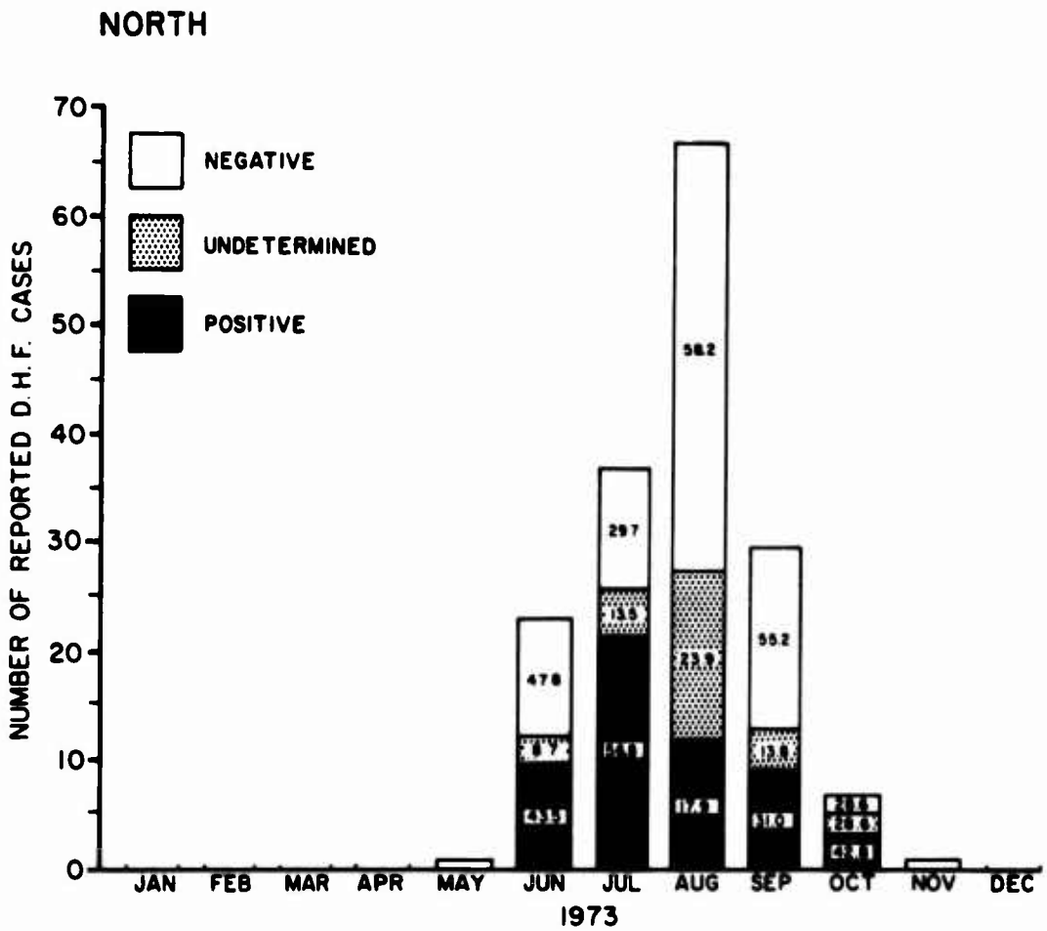


Figure 2. Frequency distribution of cases of hemorrhagic fever from the North Region of Thailand tested by HI for Dengue 2 antibody. Cases were confirmed positive (black), undetermined (dotted) or negative (white). Numbers within the bars indicate the percent of cases.

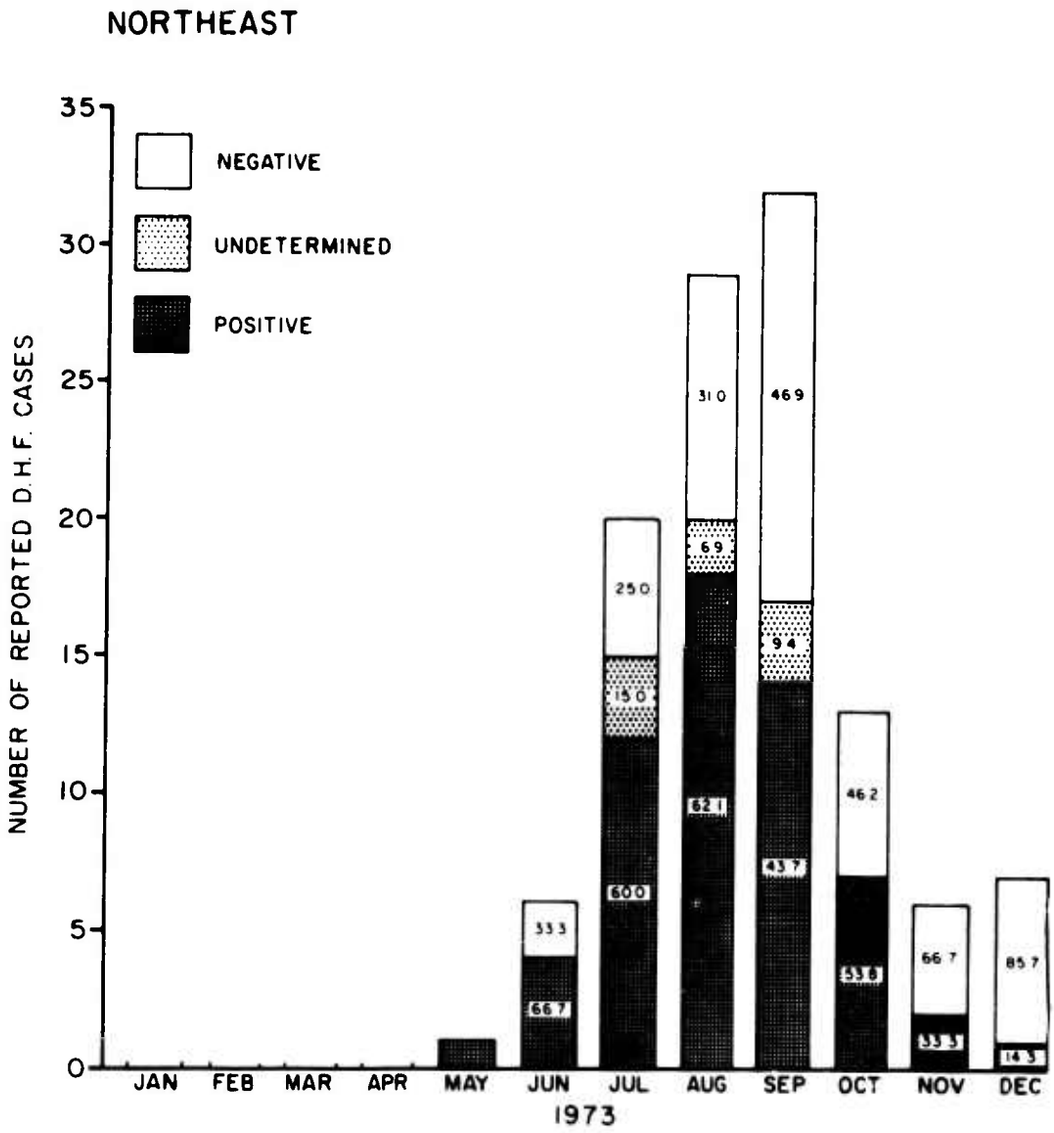


Figure 3. Frequency distribution of cases of hemorrhagic fever from the Northeast Region of Thailand tested by HI for Dengue 2 antibody. Cases were confirmed positive (black), undetermined (dotted) or negative (white) Numbers within the bars indicate the percent of cases.

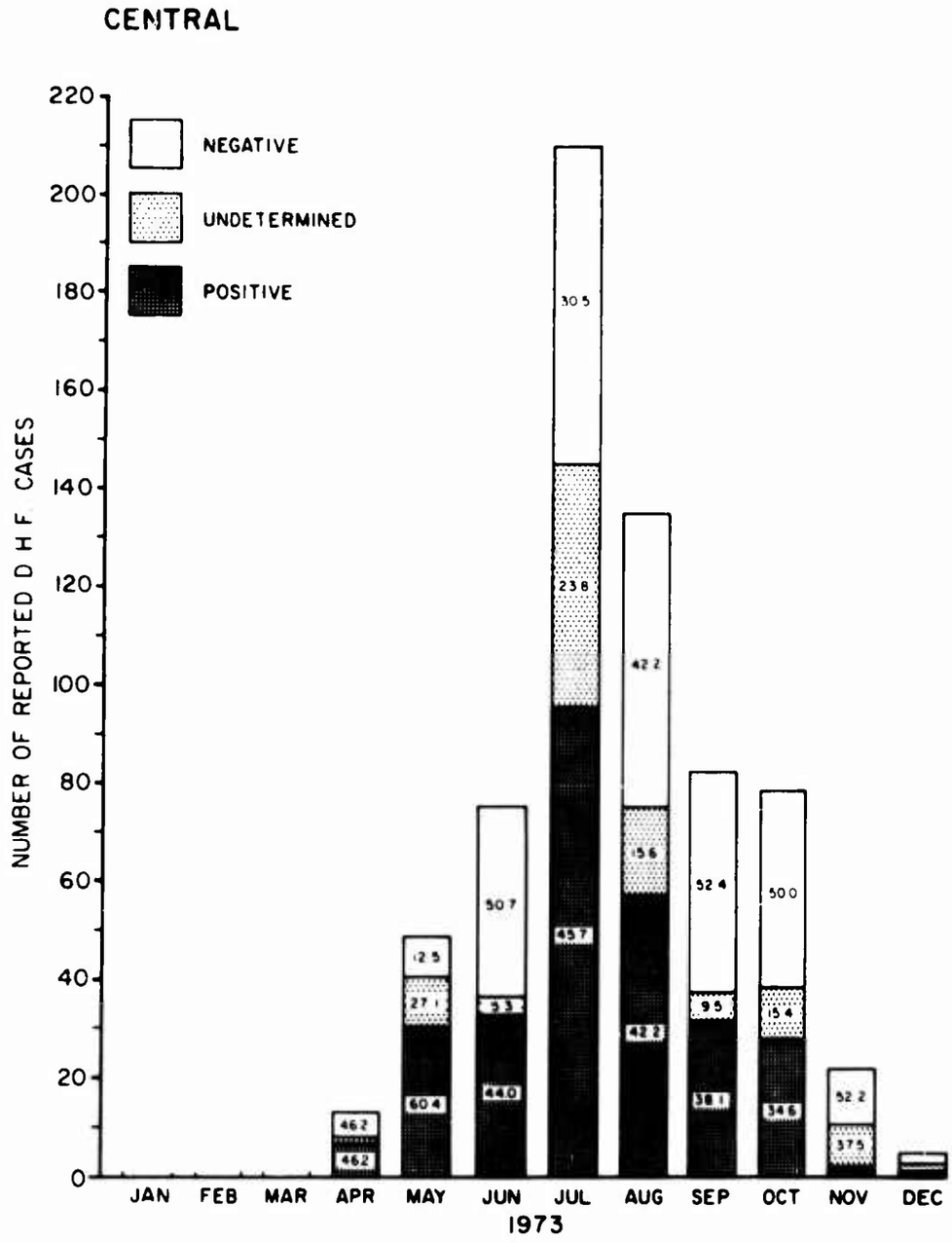


Figure 4 Frequency distribution of cases of hemorrhagic fever from the Central Region of Thailand tested by HI for Dengue 2 antibody. Cases were confirmed positive (black), undetermined (dotted) or negative (white). Numbers within the bars indicate the percent of cases

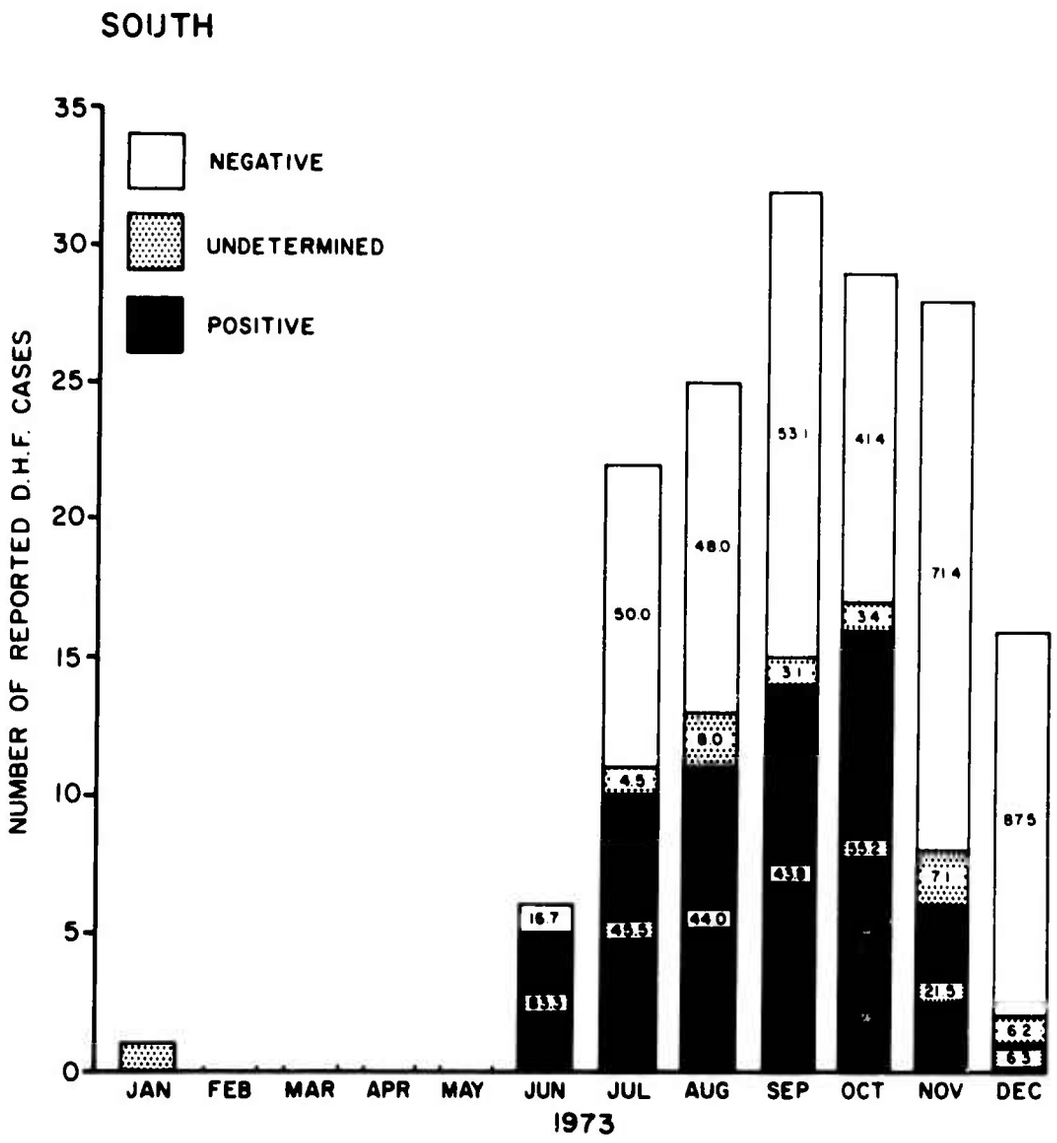


Figure 5. Frequency distribution of cases of hemorrhagic fever from the South Region of Thailand tested by HI for Dengue 2 antibody. Cases were confirmed positive (black), undetermined (dotted) or negative (white). Numbers within the bars indicate the percent of cases.

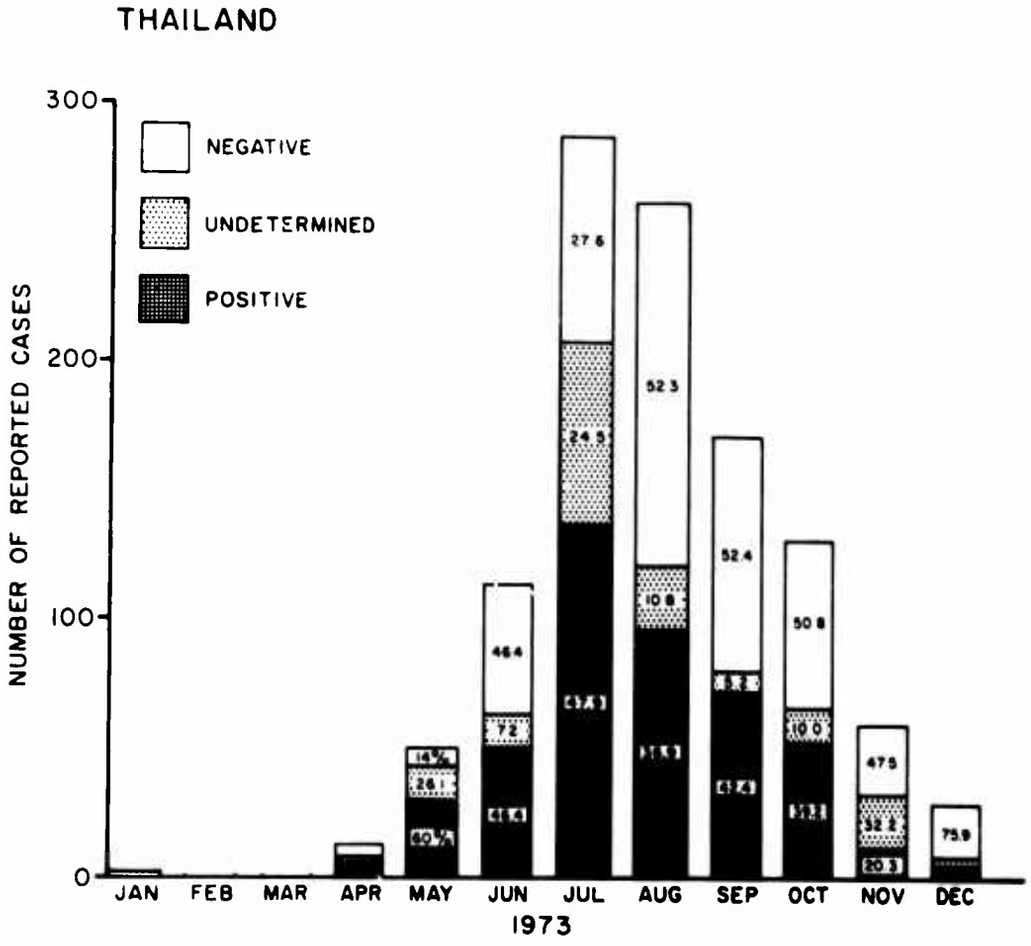


Figure 6 Frequency distribution of cases of hemorrhagic fever from all of Thailand tested by HI for Dengue 2 antibody. Cases were confirmed positive (black), undetermined (dotted) or negative (white). Numbers within the bars indicate the percent of cases.

in the peak number of confirmed cases in the Northeast and South regions compared to the North and Central is of uncertain importance but will be looked for again in the next epidemic. Throughout Thailand only 44% of the sampled cases of dengue were confirmed. Only 15% of the sampled cases of encephalitis were found to have JEV infections. The results suggest that the criteria for the clinical diagnosis of DHF and JEV need to be emphasized more.

2. A Review of Two Systems for Reporting Cases of Hemorrhagic Fever

Two methods of reporting cases of Hemorrhagic Fever (HF) in Thailand have been in use by the Department of Virology, SMRL, for several years. One system tabulates case reports provided by provincial health authorities to the Ministry of Health (MH). The other system reports the numbers of hospital admissions in the Bangkok-Thonburi area and is based on data collected by a Medical Research Technologist of the Department of Virology (DV). Both reporting systems have been functioning for several years; the DV system has been in use since 1958.

The MH system is being supported by a serologic screening program of the Department of Microbiology, School of Public Health, Mahidol University.

This study reviews two systems providing data to the Department of Virology on cases of Hemorrhagic Fever to search for information useful for future studies. Where possible, the results of the systems are compared.

MH System: For purposes of analysis, provinces were grouped into six case-reporting regions with geographic boundaries used by the government for census purposes (Fig 1). Population figures for mid-1973 were obtained from the Ministry of Health.

The numbers of cases and deaths for each province were tabulated each week and kept up-to-date by any additions or deletions reported later. Since reports were unavailable for January to May, this study only reviews the reports from June to December, 1973. Nevertheless it includes nearly all of the "epidemic" period. The data was used to determine disease specific incidence rates, case fatality ratios and the provinces reporting the most cases.

DV System: A medical research technologist visited 18 hospitals in Metropolitan Bangkok at one or two month intervals. At each hospital she received data on every patient hospital admission with a final diagnosis of hemorrhagic fever. The information was provided by the head nurse of each pediatric service and included the number of cases and deaths per month and the age, sex and home town of each HF patient.

Comparison of Reporting Systems: The only areas from which both reporting systems received common data were Bangkok and Thonburi. Since the Ministry of Health undoubtedly had different sources of information than SMRL, the numbers of reported cases would not be expected to be equal. Nevertheless the case-fatality ratios should be similar. In fact, the CFR based on DV data was nearly four times higher than that of the MH system (Table 1). This difference may reflect MH underreporting, DV overreporting or both.

Table 1. Comparison of Reports of Hemorrhagic Fever from Bangkok-Thonburi Provided by Two Survey Systems

Month	Dept of Virology			Ministry of Health		
	Cases	Deaths	CFR*	Cases	Deaths	CFR
June	162	0		148	0	
July	189	0		120	0	
August	252	5		133	0	
September	168	2		146	2	
October	166	0		141	0	
November	123	2		115	0	
December	87	1		53	0	
7 Month Totals	1147	10	0.87	856	2	0.23

* Case fatality ratio.

MH System: Cases and deaths due to HF were reported from all regions of the country in every weekly report. Variations in regional disease specific incidence rates (DSIR) suggested some areas were hit harder than others (Table 2); however, the variability of the case fatality ratio (CFR) suggested case reporting was not uniform.

Table 2. Reports of Hemorrhagic Fever in Thailand (June-Dec 1973) provided by the Ministry of Health

Region	Population Mid 1973 (x 1,000)	Total Cases	Disease Specific Incidence Rate (per 100,000 pop.)	Total Deaths	Case Fatality Ratio (per 100 cases)
North	4502	771	17.1	14	1.82
North Central	3654	194	5.3	16	8.25
Northeast	13351	2584	19.4	149	5.77
South Central	9041	1527	16.9	18	1.18
Southeast	2196	840	38.3	7	0.83
South	4589	352	7.7	18	5.11
Thailand	37333	6268	16.8	222	3.54

In fact, 17 provinces, representing 17% of the population of Thailand, reported less than 10 cases each throughout the 7 month reporting period. Nine of the low reporting provinces were in the South, but some were adjacent to areas reporting a high incidence of disease.

The 10 most populous provinces, representing 35% of the population, reported 49% of all cases and 43% of the deaths; however, the provinces reporting the most cases were not necessarily among the most populated (Table 3).

There were striking differences between regions in the patterns of weekly case reports (Fig 2). The South-Central region including Bangkok-Thonburi reported more than 25 cases per week from early June to December. The Southeast also reported more than 25 cases per week in early June, reached a peak by the end of July and declined by mid-September. The Northeast and North regions showed rising numbers of cases two and four weeks later than the Southeast, which peaked in late July and declined sharply by mid-October. The South region showed no distinct peak period of disease. With the exception of the Northeast region, the province reporting the most cases from each region closely represented all case reports from that region.

DV System: An analysis of the characteristics of the patients in Metropolitan Bangkok showed an approximately equal sex distribution. There were 791 males with 10 deaths and 856 females with 16 deaths. The age of the patients ranged from less than 1 year to 16 years with a mode age of 7 years for both sexes.

There has been a change in the age distribution of reported cases during the past 10 years. A comparison of three reporting eras showed the median age of HF patients has shifted progressively from 3 years 10 months (1962-1965) to 5 years 7 months (1971-1973). The percentage over age 12 has changed very little (Fig 3). The apparent shift in age distribution correlates with a progressive decline in the total number of cases each year and loss of the formerly apparent every-other-year peak numbers of cases (3).

DISCUSSION: Both systems are subject to over and under-reporting of cases and deaths. Nevertheless, the information provided by the DV system should be more consistent from year to year since it is gathered by one person and is based on final diagnoses.

Neither reporting system is adequate by itself for epidemiologic work in the absence of laboratory tests to confirm clinical diagnoses.

Table 3. Provinces Reporting the Most Cases of Hemorrhagic Fever June - December 1973

Area	Rank by Population	No. Cases	Disease Specific Incidence Rate (per 100,000 pop.)	No. Deaths	Case Fatality Ratio (per 100 cases)
Bangkok-Thonburi	1	856	25.0	2	0.23
Khon Kaen	7	446	40.8	22	4.93
Chantaburi	57	438	188.8	3	0.68
Chiang Mai	6	390	35.1	4	1.03
Ubon Ratchatani	5	387	31.2	8	2.07
Nakhon Ratchasima	3	380	22.0	21	5.53
Udorn Thani	4	251	19.7	7	2.79
Nakhon Si Thamarat	8	241	25.2	10	4.15
Chaiyaphum	14	219	32.3	17	7.76
Roi Et	11	218	26.6	19	8.72

Table 4. Frequency of HF Cases by Hospital 1973

Hospital	Cases	Deaths
Children's	624	12
Siriraj	341	3
Prapinklao	189	2
Chulalongkorn	138	3
Wachira	104	1
Bumrasnaradura	57	0
Ramathibodi	31	1
Bangkok Christian	31	1
Bhumipol	28	1
Lertsin	27	2
Phramongkutklao	21	0
Tobacco	16	0
Police	7	0
Mission	5	0
Bangkok General*	3	0
Central	3	0
Kwongsiew	3	0
Sahapayabarn	0	0
TOTAL	1630	25

* Reports from August to December only.

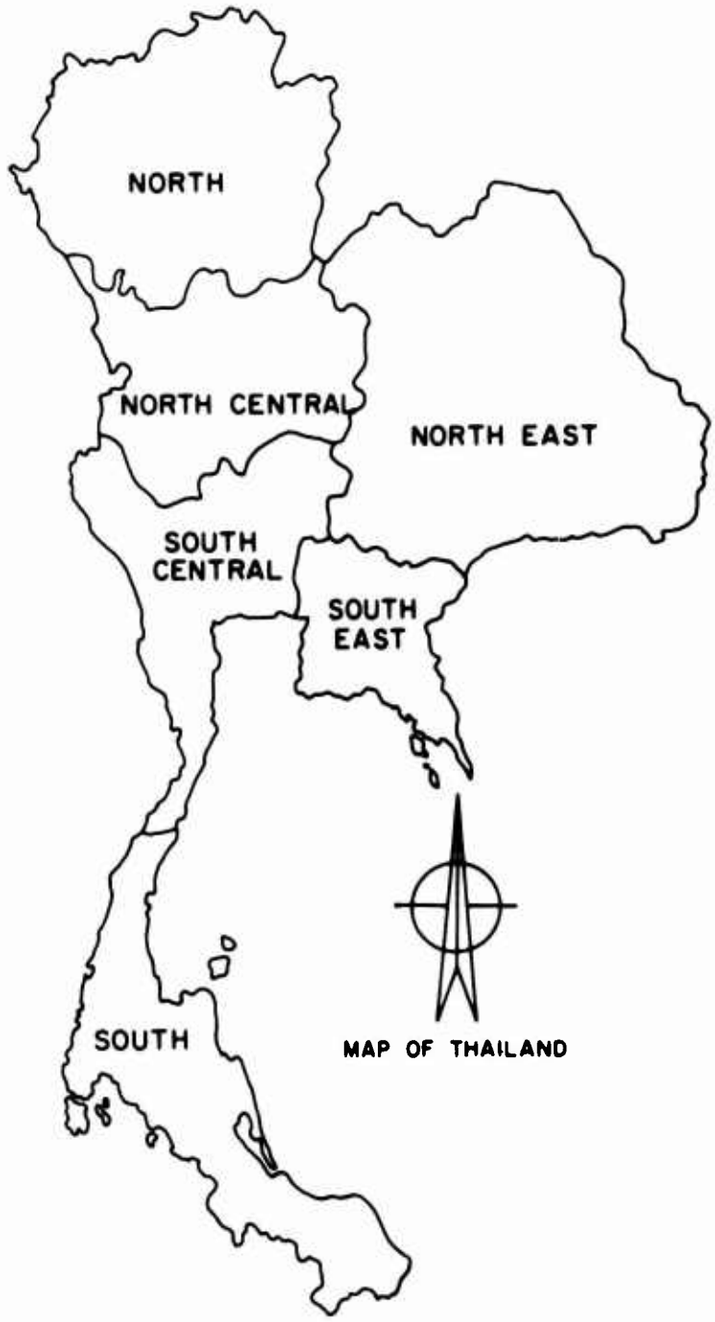


Figure 1. Geographic regions of Thailand reporting cases of Hemorrhagic Fever to the Ministry of Health.

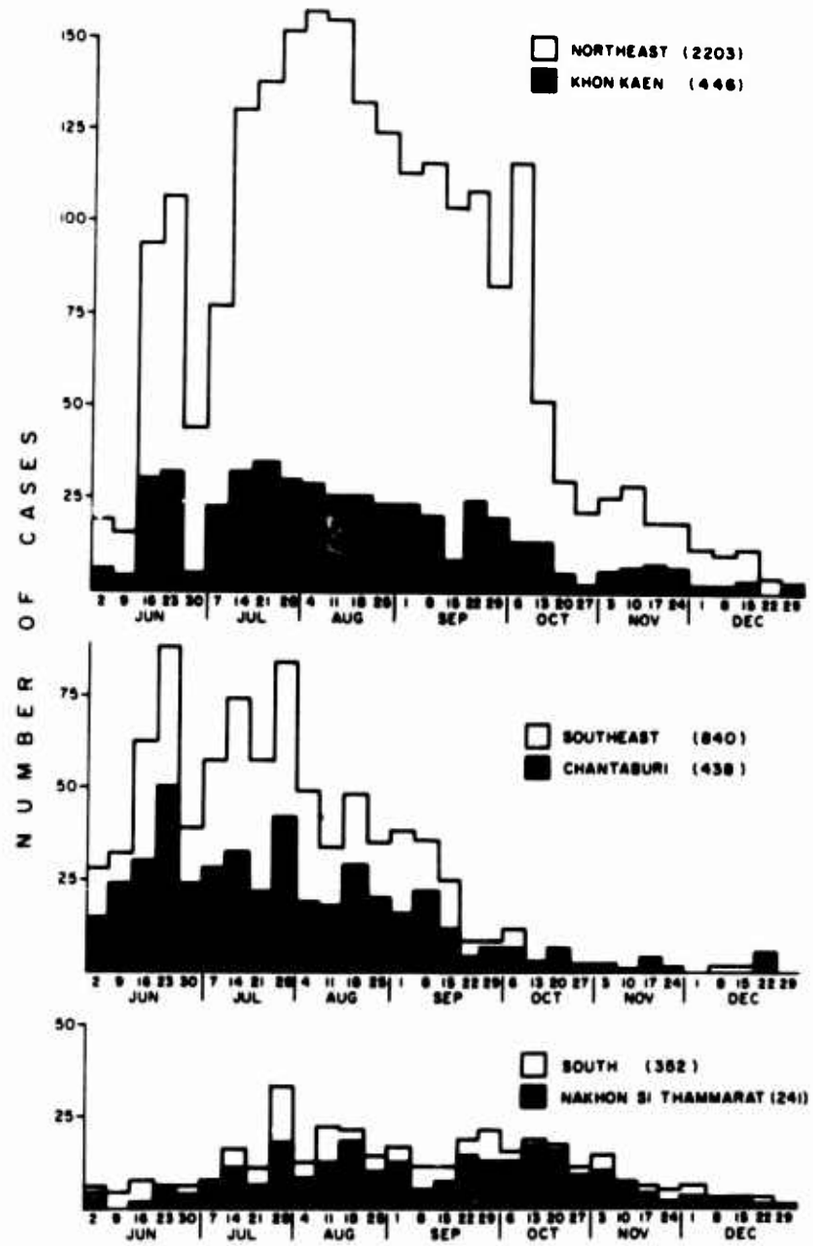
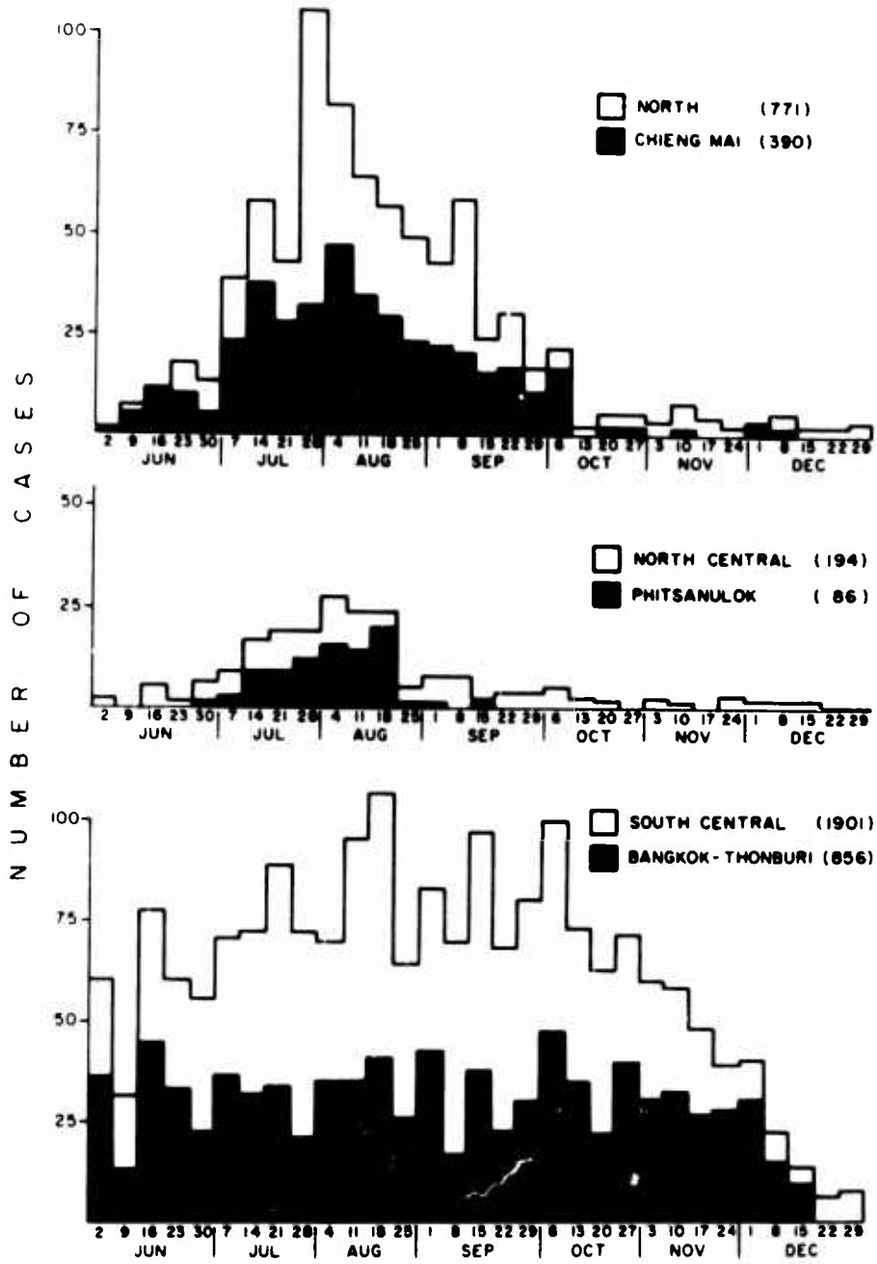


Figure 2. Cases of Hemorrhagic Fever reported to the Ministry of Health each week for seven months of 1973. White bars represent all cases reported from the region. Black bars represent the provinces reporting the most cases for each region.



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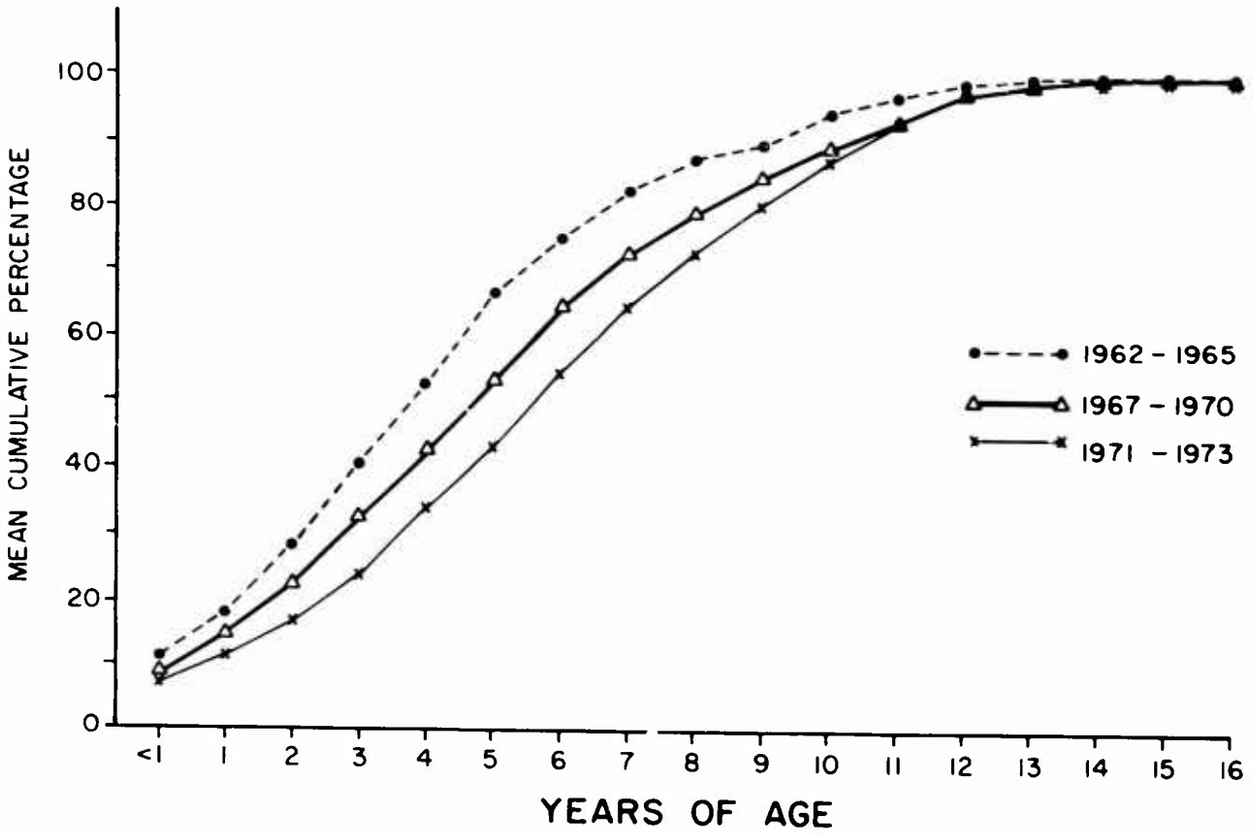


FIGURE 3. CHANGES IN THE AGE DISTRIBUTION OF PATIENTS HOSPITALIZED WITH HEMORRHAGIC FEVER IN BANGKOK-THONBURI FOR THREE TIME PERIODS. THE NUMBER OF CASES FOR EACH TIME PERIOD IS 1962-1965, 13,278; 1967-1970, 7093, AND 1971-1973, 5095. IN RECENT YEARS RELATIVELY FEW CHILDREN UNDER 5 YEARS OF AGE WERE ADMITTED TO A HOSPITAL.

The relatively fewer children under 5 years of age who are now being hospitalized probably reflects the greater diagnostic skill of the physicians who must select those patients for admission who are most apt to develop hemorrhagic complications. It is also possible that as Metropolitan Bangkok has grown, children may go longer periods of time between consecutive dengue infections.

As in former years, Children's Hospital reported the largest number of cases (38.3%) and deaths (48.0%)from the greater Bangkok area (Table 4). These figures reflect the fact that Children's Hospital remains the primary referral center for severe cases of HF.

3. Dengue Infection at Children's Hospital of Bangkok

Dengue virus infections are an increasing public health problem for both military and civilian populations in the tropics and subtropics. Among US military troops stationed in the Pacific Theater it is an annoying and potentially critical problem. In Thailand and elsewhere in the Southeast Asia, dengue hemorrhagic fever (DHF) is a major cause of morbidity and mortality among children.

A need exists to develop vaccines to control this disease. In the past dengue vaccination has been attempted and was possibly effective in modifying a dengue epidemic in Puerto Rico in 1963; however, the vaccine used in this case was manufactured by a method which is unacceptable by present-day standards. Development of virus vaccines presently calls for the original isolation and propagation of candidate viruses on cell lines certified by the United States Department of Health Education and Welfare, Division of Biological Standards. With a view towards isolation of candidate dengue strains, acute plasma was collected from patients presenting with symptoms compatable with dengue hemorrhagic fever at the Children's Hospital of Bangkok.

Recent evidence has suggested that both viral and non-viral antigens may be involved in the host response to viral disease. Technology for isolating, purifying and studying many of these antigens has been developing over the past several years. With dengue, several antigens have been identified and at least one has been purified to the point where study is possible. This antigen is a soluble complement fixing antigen, which develops in dengue infected suckling mice. Preliminary evidence suggests that humans recovering from secondary dengue develop humoral antibody against this antigen late in their convalescence. Sera were collected at specified intervals after diagnosis of DHF for study of the temporal development of antibodies to this and other viral and non-viral antigens.

DESCRIPTION: Patients suspected of having dengue virus infections were selected from the outpatient clinic and the infectious disease wards of Bangkok Children's Hospital. A standardized chart of pertinent signs, symptoms, and laboratory findings was instituted on each patient. Blood was obtained on the day of diagnosis and on days 3, 5, 15, and 30 after the day of selection. Blood drawn on the first day was divided into plasma and serum portions. On subsequent collections blood was allowed to clot and serum was collected.

Plasma was used for isolation of viruses using a direct and delayed plaque technique. Isolates were identified by plaque reduction neutralization test using monkey antisera.

Sera were used for standard 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 were tested simultaneously. Aliquots of plasma and acute and convalescent sera were stored at -70°C for reisolation on certified cells and for specified dengue serologic tests against viral and non-viral antigens. At the conclusion of collection, clinical, isolation and serological data were used to identify individuals infected with dengue and to determine the type of antibody response and the severity of the illness. Patients were considered to have had dengue infection if a four-fold rise in antibody titer to at least two of the group B antigens was found between 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 infected patients have been previously reported. Patients with HI antibody titers of >1:640 to at least two dengue antigens were considered to have secondary infections while those with convalescent antibody of ≤1:640 were considered to have primary infections. Grading of severity of DHF used criteria established by one of us (SN) and used in the past:

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 rapid, weak pulse with narrowing of pulse pressure (<20 mm Hg) or hypotension (systolic pressure <90mm Hg).

Grade IV: Moribund patients with undetectable blood pressure or pulse.

PROGRESS: Specimens were collected from 134 patients. These patients were seen and diagnosed as having illness compatable with dengue infection. Twenty-seven were collected in the outpatient clinic and 107 were obtained on hospital wards. Acute and at least one convalescent sera were obtained from 123 patients and 95 of these were diagnosed as dengue by viral isolation, by serological criteria or both. Of these 95, 91 were hospitalized for their illness. Twelve patients exhibited low level antibody responses characteristic of primary infection, and 8 of these were hospitalized. Sixty-two patients showed a four-fold rise in antibody titer and another 19 had high fixed titers. All of them were considered to have had secondary infections.

A breakdown of clinical and laboratory findings for these 95 patients is shown in Tables 1, 2 and 3. The findings were essentially similar to those of previous clinical studies. Comparison of patients showing primary and secondary responses demonstrates that DHF occurred in both groups. All but one patient with shock had secondary dengue infections. The exception was an 8 month old baby from whom only an acute plasma was obtained. Dengue 2 virus was isolated from this plasma at a time when no antibody was detectable; the mother unfortunately was not tested for antibody to dengue. Since no convalescent serum was collected, this patient could not be classified on the basis of antibody titer. He was considered to be a primary case because the acute plasma taken on the 5th day of illness had a titer of $\leq$ 1:20 to all group B antigens tested.

Four patients were sampled early in the course of illness before developing thrombocytopenia. Figure 1 shows the relationship of platelet counts, antibody titer and hematocrit to the time course of disease for one patient. There appeared to be a temporal relationship between the time of platelet decrease and the development of shock.

Between July and December of 1973, 22 strains of dengue were isolated from the 95 patients with evidence of dengue infection, representing an isolation rate of 23% (see Table 4 and 5). Nine strains were isolated from thirteen patients with serological evidence of primary disease; isolation was successful in 69% of primary cases. At the time plasma was obtained for isolation, only one individual had dengue antibody. In this case the titer for the homologous strain of dengue virus was four-fold lower than that of the other dengue

Table 1. Summary of Clinical and Laboratory Findings in Thirteen (13) Primary Dengue Patients

Findings	UF* (5)**	Dengue Hemorrhagic Fever			
		Gr I (3)	Gr II (4)	Gr III (1)	Gr IV (0)
Fever	5/5 (100)****	3/3 (100)	4/4 (100)	1/1 -*****	0/0 -
Hepatomegaly***	0/5 (0)	1/3 (33)	2/4 (50)	1/1 -	0/0 -
Postive tourniquet test	0/5 (0)	3/3 (100)	4/4 (100)	1/1 -	0/0 -
Petechiae	0/5 (0)	0/3 (0)	4/4 (100)	1/1 -	0/0 -
Other signs of bleeding	1/5 (20)	0/3 (0)	0/4 (0)	0/1 -	0/0 -
Hemoconcentration	0/5 (0)	0/3 (0)	2/3 (67)	1/1 -	0/0 -
Platelet counts ≤50,000	0/5 (0)	0/3 (0)	2/4 (50)	1/1 -	0/0 -

* UF indicates undifferentiated fever
** Number of patients
*** ≥ 2 cm below costal margin
**** Percentage of cases
***** 8 month old infant

Table 2. Summary of Clinical and Laboratory Findings in 82 Secondary Patients

Findings	UF* (2)**	Dengue Hemorrhagic Fever			
		Gr I (14)		Gr III (22)	Gr IV (6)
Fever	2/2 (100)****	14/14 (100)	38/38 (100)	22/22 (100)	6/6 (100)
Hepatomegaly***	1/2 (50)	6/11 (56)	15/30 (50)	16/19 (84)	5/5 (100)
Positive tourniquet test	0/2 (0)	12/13 (92)	36/36 (100)	22/22 (100)	6/6 (100)
Petechiae	0/2 (0)	0/13 (0)	29/36 (81)	16/22 (73)	4/6 (67)
Other signs of bleeding	0/2 (0)	0/13 (0)	14/36 (39)	7/22 (32)	5/6 (83)
Hemoconcentration	0/2 (0)	7/11 (54)	18/36 (50)	18/19 (95)	6/6 (100)
Platelet counts ≤50,000	0/2 (0)	8/14 (57)	24/38 (63)	21/22 (95)	6/6 (100)

* UF indicates undifferentiated fever
** Number of patients
*** ≥ 2 cm below costal margin
**** Percentage of cases

types tested, suggesting antigen-antibody complexes had been formed. The 82 secondary cases yielded 13 (16%) isolates. Dengue antibody was absent from the acute plasma in only three secondary cases, all of which had plasma obtained within the first two days of illness. The remaining ten had initial antibody titers ranging from 1:20 - 1:320 for the homologous virus (see Table 6). In the majority of isolations from secondary cases (11/13) the antibody to the homologous types was lower than or equal to that of other types. Two had four-fold lower antibody titers to the infecting dengue type than to other dengue types tested, again possibly indicating complex formation. Dengue types 1, 2 and 3 were isolated; see Table 4 for a breakdown by antibody response and Table 5 for a breakdown by grade of disease.

Table 3. Hemagglutination Inhibition Antibody Levels in Convalescent Sera from 94 Patients with Dengue

Grade (s) of Disease	Primary Infection (Titer < 1:640)	Secondary Infection (Titer ≥ 1:640)
UF*	5	2
I & II	7	52
III	0	22
IV	0	6
TOTAL	12	82

* UF indicates undifferentiated fever

Of the 22 isolates, plasma containing 4 dengue-1, 4 dengue-3 and 7 dengue-2 were shipped to Walter Reed Army Institute of Research for reisolation as candidate strains on certified cell lines for vaccine development.

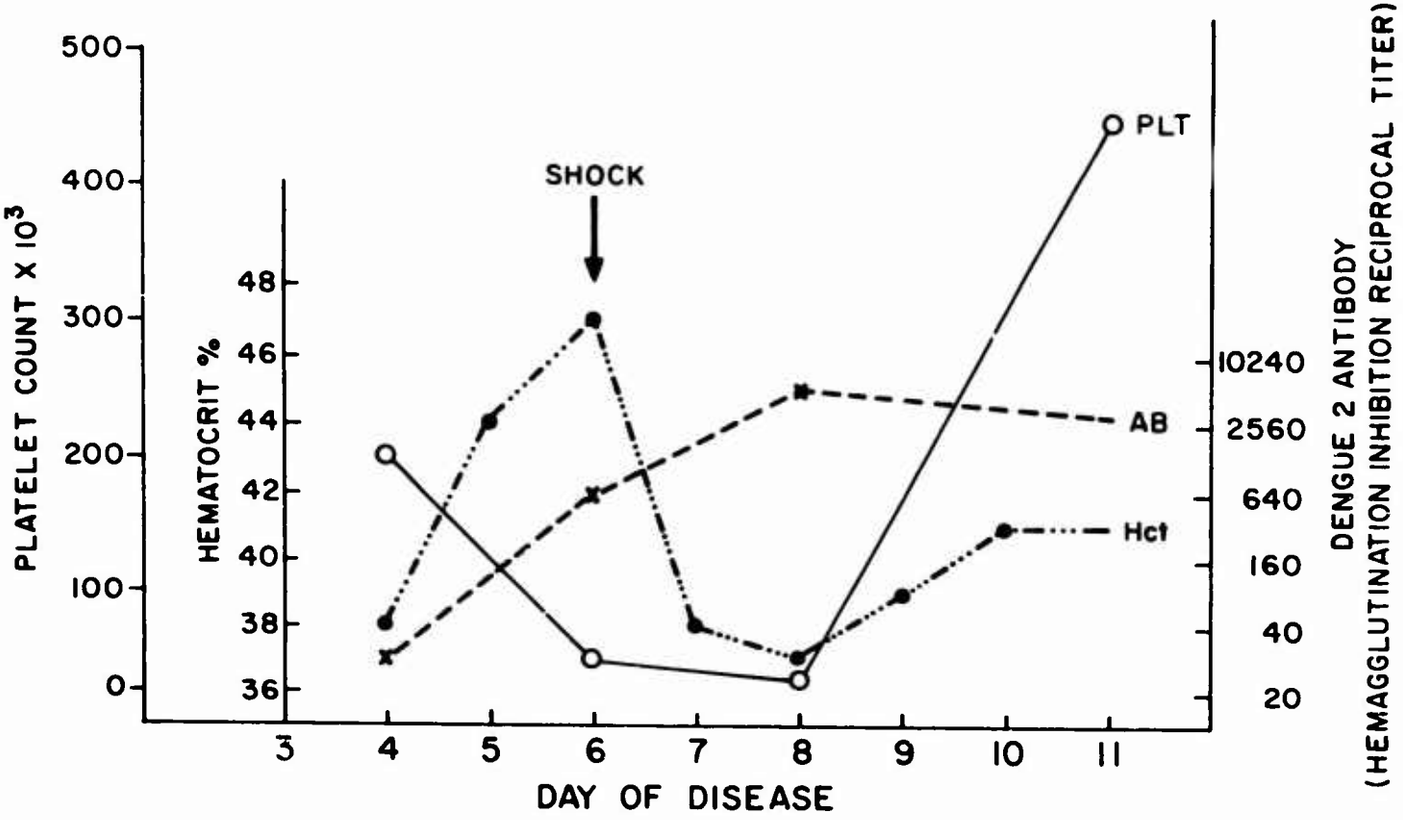


Figure 1. Platelet counts, hematocrits and antibody titers from one patient, showing the relationship of these parameters to the development of shock. PLT indicates platelet counts, AB indicates Dengue-2 antibody titer, Hct indicates hematocrit.

Table 4. Dengue Strains Isolated from Human Infections: Dengue Type and Convalescent Hemagglutination Inhibition Antibody Responses

Antibody Response	Number of Pacients	Total Isolations	% Isolation	Strains Isolated			
				Type 1	Type 2	Type 3	Untyped
Primary (< 1:640)	13	9	69	3	2	3	1
Secondary (≥ 1:640)	82	13	16	1	9	1	2
TOTAL	95	22	23	4	11	4	3

Table 5. Dengue Strains Isolated from Human Infections: Dengue Type and Clinical Syndrome

Clinical Syndrome (No. of cases)	Number of Strains Isolated			
	Type 1	Type 2	Type 3	Untyped
UF* (7)	2	1	2	1
G I (17)	1	0	0	0
G II (42)	1	4	2	2
G III (23)	0	5	0	0
G IV (6)	0	1	0	0
TOTAL	4	11	4	3

* UF indicates undifferentiated fever

Table 6. Distribution of Dengue Isolations by Hemagglutination Inhibition Titers to Dengue 2 or to the Homotypic Virus in the Acute Serum

Reciprocal Titer	No. of Patients	Dengue Isolates	
		No.	%
<20	20	13	65
20	5	4	80
40	2	2	100
80	6	0	0
160	4	2	50
320	14	1	7
≥ 640	44	0	0
TOTAL	95	22	23

Table 7. Patients from whom Convalescent Specimens were Collected on Approximately Day 3, 5, 15 and 30 Following Clinical Diagnosis

Antibody Response	No. of Patients	No. of Isolations	Grade of Disease				
			UF*	I	II	III	IV
Primary (<1:640)	7	3	1	4	2		
Secondary (≥1:640	34	5		4	18	10	2
TOTAL	41	8	1	8	20	10	2

* UF indicates undifferentiated fever

Follow-up specimens were obtained on 41 patients, approximately 3, 5, 15 and 30 days after the diagnosis of DHF (see Table 7). Of these, 34 were secondary patients and 6 were primary patients. In 3 of the primary patients and 5 of the secondary patients dengue virus was isolated. Of the secondary patients, 4 were Grade I, 18 Grade II, 10 Grade III and 2 Grade IV. The primary patients included 1 undifferentiated fever (UF), 4 Grade I and 2 Grade II. These acute and longterm follow-up specimens will be tested for antibody to viral and non-viral antigens.

4. Propagation of Dengue Virus in Inoculated Mosquitoes

The purpose of this study was to establish a method for infecting mosquitoes with dengue virus to use in future studies of viremia in Hemorrhagic Fever patients.

Tissue culture methods of isolating and identifying dengue viruses are exacting, time consuming and expensive. Rosen (4) has developed

a mosquito assay technique which does not require tissue culture and which may be even more sensitive than currently used tissue culture methods. An effort is being made to establish this assay method at SMRL to permit a prospective controlled study of both dengue isolation systems during the next hemorrhagic fever season.

DESCRIPTION: Colony-raised mosquitoes were obtained from the Dept of Entomology, SMRL. All manipulation of live mosquitoes was done within a screened cubicle. Experiments used Aedes aegypti and Armigeres subalbatus species. Whenever possible only male mosquitoes were used.

Seed viruses stored at -70°C were thawed and diluted in Medium 199 with 20% heat-inactivated fetal bovine serum, pH 8.2. Each virus dilution was prepared just before use and held in an ice bath.

The inoculating needle was made from a glass capillary, 0.7-1.0 mm outside diameter, graduated into 1 mm segments, drawn to a sharp tip over an alcohol flame and sterilized in an autoclave. For inoculation, a capillary was held in a stainless steel holder connected to plastic tubing leading to a 3 way stopcock and a 30 ml glass syringe. The capillary needle was filled by submerging the point in a virus solution and carefully drawing on the syringe. The volume in the capillary was precisely controlled with the syringe plunger.

Prior to inoculation, each mosquito was held in an individual cotton-stoppered test tube. The tube was immersed in a beaker of wet ice for 5 to 10 minutes to anesthetize the mosquito. The immobilized mosquito was placed in a 100 x 15 mm petri dish on the stage of a dissecting microscope. The inoculating capillary was inserted into the thorax through the suture just anterior to the sternopleuron and below the first thoracic spiracle. After the needle was introduced, the mosquito was positioned so the fluid level in the needle could be observed through the microscope. The inoculum was the amount of fluid in a 2 mm length of the needle, approximately 0.0013 ml.

Mosquitoes inoculated with the same dilution of the same virus were placed in a gauze-covered paper cup containing 5% sucrose and held for 7 to 10 days at 27°C, 85% R.H. Fresh sugar pads were provided daily. Starting 1-2 hours after inoculation, records were taken daily of the number of surviving mosquitoes.

For virus isolation in tissue culture, the surviving mosquitoes were killed by freezing on day 8. Mosquitoes used for indirect fluorescent

antibody staining were killed on day 10. Virus isolation in LLC-MK2 cells used the direct plaque technique.

Slides for indirect fluorescent antibody (IFA) staining were cleaned with ethyl alcohol. The head was cut from each mosquito to be tested and squashed on a slide under a coverslip precoated with 1% silicon solution (Siliclad). The coverslip was then discarded and the head preparation allowed to dry in air. After removing any excess chitinous material, the remaining tissue was fixed in cold acetone (4°C) for 10 minutes and dried in air. The slide was flooded with dengue hyperimmune mouse ascitic fluid diluted 1:10 in phosphate buffered saline (PBS) and incubated at 37°C for 45 minutes in a moist chamber. The slide was washed twice for 5 minutes with PBS and dried with a cold air blower. One drop of fluorescein conjugated anti-mouse gamma globulin (Antibodies Incorporated) diluted 1:10 in PBS was added and allowed to incubate at 37°C for 45 minutes. The slide was washed twice with PBS and mounted in buffered glycerol (1 part PBS to 9 parts glycerol) before being examined under a fluorescent microscope.

Initial studies were directed at determining the survival of mosquitoes after inoculation, the optimal age of mosquitoes and the susceptibility of a larger species, Armigeres subalbatus. In addition the titers of virus passed through mosquitoes were compared to those of the original seeds.

PROGRESS: Mosquito mortality was evaluated by comparing early and late survivors. Early survivors were the mosquitoes that were alive 24 hours after inoculation. Late survivors were alive 7 days after inoculation. Death during the first 24 hours was assumed to be due to trauma inflicted by inoculation. Later deaths were due to trauma, infection, advanced age or other reasons.

It was found that with experience in inoculation, the percentage of early and late survivors increased. (Table 1)

Mosquitoes that were 3 to 5 days old at the time of inoculation survived somewhat better than those younger or older (Table 2); however, mosquitoes up to 13 days old were used successfully. The age of mosquito seems to have little bearing on the success of inoculation.

An attempt was made to improve mosquito survival by trying to infect a larger species. Two hundred fifty-five Armigeres subalbatus were inoculated with the same volume as the A. aegypti. Survival of

Armigeres was 85% at day 1 and 70% on day 7 compared to 69% and 60% respectively for A. aegypti. Virus was not isolated from any Armigeres, so these mosquitoes cannot be used to detect dengue.

Table 1. Effect of Experience at Inoculating A. aegypti Mosquitoes on the Number of Early and Late Survivors 1973-1974

Month	No. Inoculated	Early Survivors No.	(%)	Late Survivors No.	(%)
September	480	316	(66)	258	(54)
October	332	195	(59)	168	(51)
November	299	232	(78)	187	(63)
January	359	249	(69)	230	(64)
February	389	296	(76)	271	(70)
Combined	1,859	1,288	(69)	1,114	(60)

The direct plaque titers in tissue culture for viruses passed through mosquitoes were usually within one log of the original seed viruses (Table 3). By calculating the amount of virus actually inoculated into the mosquitoes it was found that the virus multiplied 0.1 to 8000 times in the insect host. No conclusions can be drawn on the relative sensitivty of the two methods, but the results do suggest that the mosquito inoculation method of virus isolation may detect low concentrations of virus in human sera that might otherwise be missed.

The IFA test gave excellent fluorescence in infected LLC-MK2 cells. The failure to find fluorescence in the mosquito heads may be due to (1) inadequate removal of auto-fluorescing chitinous debris and/or (2) absence of salivary gland material from the prothorax. A study is being made of these possibilities. In addition, direct fluorescent antibody testing will be tried in an effort to more closely follow the Rosen technique. Plans are being made to determine the relative sensitivity of the two methods of virus isolation for detecting viremia in hemorrhagic fever patients.

Table 2. Survival of A. aegypti Mosquitoes of Different Ages

Age at Inoculation (Days)	No. Inoculated	Early Survivors		Late Survivors	
		No.	(%)	No.	(%)
1	45	25	(56)	24	(53)
2	135	102	(76)	88	(65)
3	233	219	(94)	196	(84)
4	345	244	(71)	193	(56)
5	194	167	(86)	150	(77)
6	216	126	(58)	117	(54)
7	60	39	(65)	29	(48)
8	254	170	(67)	153	(60)
9	75	62	(83)	49	(65)
11	75	59	(79)	55	(73)
13	60	45	(75)	38	(63)
19	48	6	(13)	0	(0)
20	59	24	(41)	22	(37)

Table 3. Multiplication of Viruses in A. aegypti Mosquitoes

Virus Strain	Mosquito Pool		Seed Virus Titer (LLC-MK2)	Virus Multiplication Factor (VMF)*
	No. Mosq.	Titer (LLC-MK2)		
Dengue 1				
CH53432 MK2-5	10	77×10^{-3}	1×10^{-2}	178
CH53432 MK2-5	10	10×10^{-3}	4×10^{-3}	58
CH53432 A. albo.	4	9×10^{-1}	4×10^{-1}	130
Dengue 2				
CH53544 MK2-2	9	40×10^{-4}	4.4×10^{-4}	231
CH53544 MK2-3	10	2×10^{-4}	12×10^{-4}	4
CH53544 MK2-3	4	6×10^{-4}	37×10^{-2}	0.09
CH53544 MK2-3	5	10×10^{-4}	4×10^{-4}	116
CH53974 MK2-3	2	109×10^{-5}	2×10^{-5}	6295
Dengue 3				
CH53489 MK2-3	14	15×10^{-4}	1×10^{-3}	25
CH53489 MK2-3	5	2×10^{-1}	4×10^{-2}	231
CH53875 MK2-1	11	4×10^{-4}	1×10^{-4}	84
CH53875 MK2-1	3	49×10^{-4}	4×10^{-3}	94
Dengue 4				
12M374 MK2-5	1	3×10^{-4}	2×10^{-4}	347
12M374 MK2-6	5	8×10^{-4}	1×10^{-3}	37
Chikungunya				
SM184	7	25×10^{-4}	1×10^{-5}	8247

* $\text{VMF} = \frac{\text{Titer of mosquito pool}}{\text{No. mosquitoes}} \times \frac{1}{\text{Titer of seed virus}} \times \frac{0.3\text{ml}}{0.0013\text{ml}}$

B. JAPANESE ENCEPHALITIS

1. Ecology of Japanese Encephalitis Virus Vectors

The objective of these studies is to investigate the ecology of Japanese encephalitis virus (JEV) in Thailand, with particular emphasis on population dynamics and the development of control methods.

a. CHIANGMAI VALLEY STUDIES:

BACKGROUND: The density of JEV vectors (i.e., Culex fuscocephala, C. gelidus and C. tritaeniorhynchus) in the Chiangmai Valley of Northern Thailand is closely related to the climatic conditions and agricultural practices in that area. As described in previous annual reports the population densities of the three vector species were consistently at their lowest levels during the cool, dry months from December-March each year. In Saraphi and Sanpatong districts where most of these entomologic studies were concentrated during 1973, the usual practice has been to plant a rainy-season rice crop followed by tobacco, vegetables, peanuts and soybeans in the dry season; however, in August, September and October of 1973 a disastrous series of floods destroyed the rice crops in Saraphi district. Because of the loss of their 1973 crops, farmers in that district began planting a winter crop of hybrid rice in December 1973 and January and February 1974. This change in their usual agricultural practice produced large areas of flooded fields in the dry-season, significantly increasing the number of available vector breeding habitats during this time of year. Changes in vector population density and the frequency of human JEV infections were monitored during this period.

DESCRIPTION: Four battery-powered CDC light-traps were operated twice weekly in two rural sites in Saraphi and Sanpatong districts and eight traps were run once a week in Chiangmai City. In addition, three large Magoon traps baited with buffaloes were operated two nights per week in Saraphi district. Monthly indices of vector population densities were based on the number of females of each of the three species collected per trap per night in each study site. Pools of these species were prepared for JEV isolation attempts by the Department of Virology according to established procedures.

PROGRESS: As shown in Fig. 1, CDC light trap indices for both C. fuscocephala and C. tritaeniorhynchus in Saraphi during January 1974 were only slightly higher than in the same month of previous years; however, the density of both species was considerably higher during February 1974 than during the same period in previous years, with approximately a 20 fold increase for C. fuscocephala and a 4 fold

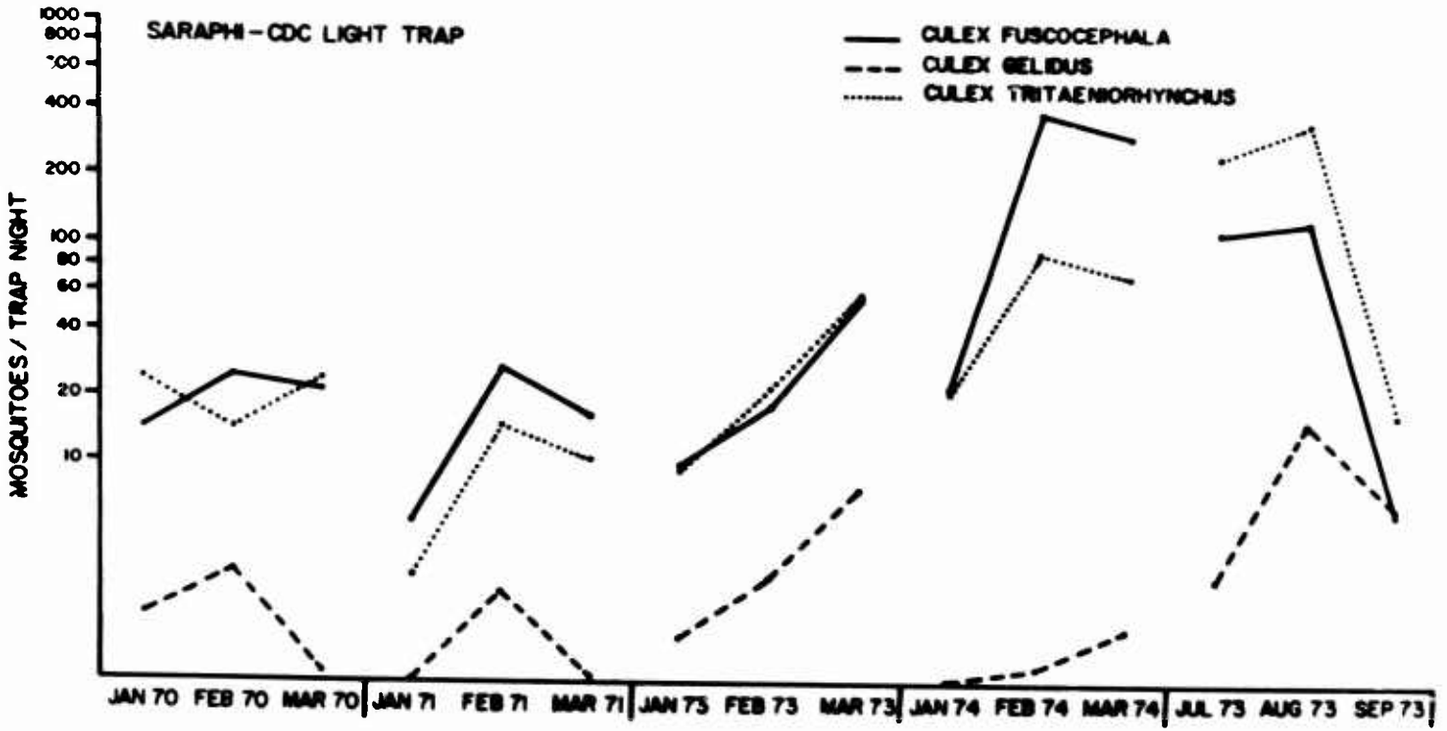


Figure 1. Number of Mosquitoes Collected per CDC Trap Night during Dry Seasons of 1970 – 74 and 1973 Rainy Season – Saraphi District, Chiangmai

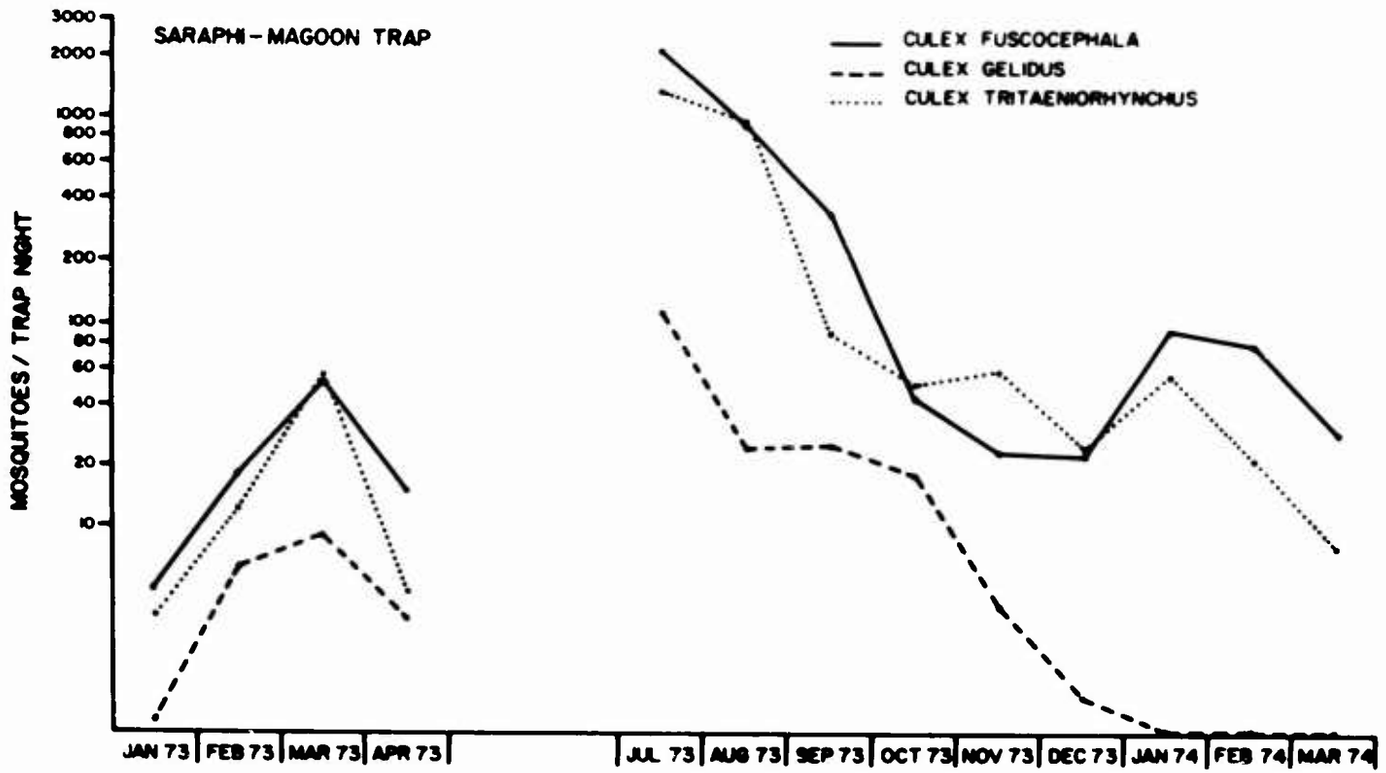


FIGURE 2 NUMBER OF MOSQUITOES COLLECTED PER NIGHT IN MAGOON TRAPS DURING 1973 - 1974 - SARAPHI DISTRICT, CHIANGMAI

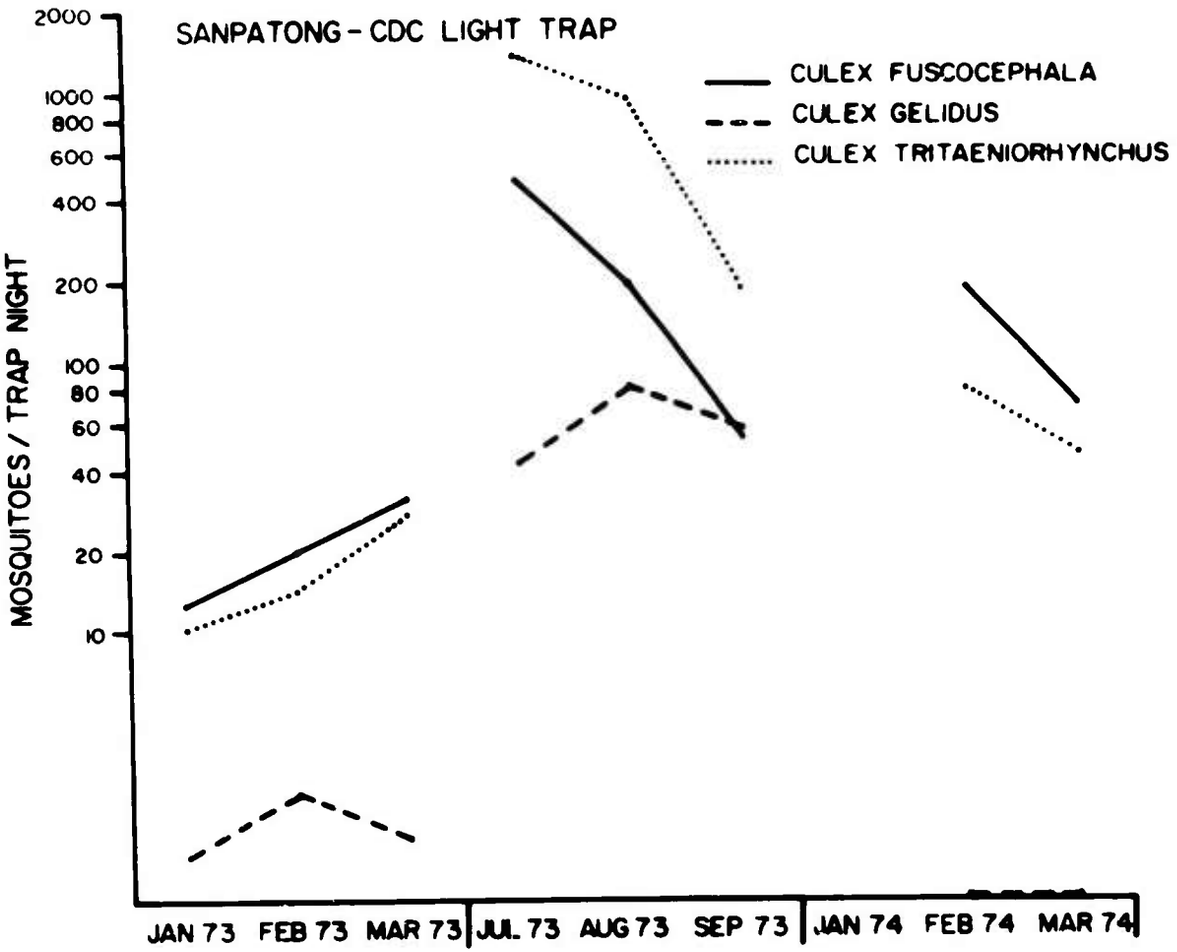


FIGURE 3. NUMBER OF MOSQUITOES COLLECTED PER CDC TRAP NIGHT DURING 1973 AND 1974 – SANPATONG, CHIANGMAI

increase for C. tritaeniorhynchus. C. fuscocephala populations were also considerably higher in February 1974 than they were during the peak of the rainy season (August) of 1973; however, C. tritaeniorhynchus densities were not as high as they were during August of the preceding year. Between February and March 1974 indices of both C. fuscocephala and C. tritaeniorhynchus showed a slight drop. Similar drops were noted during March 1970 and 1971, possible explanations of which are (1) increase in mosquito larvae predator populations, (2) lower humidity during this time of year which shortens life span of mosquitoes and (3) temporary drying of irrigated rice fields causing larval mortality. The C. gelidus indices for February 1974 were slightly lower than they were during this period in previous years.

The indices for JEV vector species collected in Saraphi from the buffalo baited Magoon traps are given in Fig. 2. With this collection method C. fuscocephala and C. tritaeniorhynchus were again significantly higher in January and February 1974 than they were during the same two months of 1973, while C. gelidus collections were smaller during 1974. Unlike the light trap collections, the numbers of mosquitoes of the three species collected in Magoon traps during the winter months of 1974 never reached the levels that were collected in July and August 1973.

The results of CDC light trap collection in Sanpatong during the winters of 1973 and 1974 and the rainy season of 1973 are shown in Fig. 3. No mosquito collections were made in this district during January 1974; however,the C. fuscocephala populations during February 1974 were approximately 10 times greater than the same month of 1973, and close to the same numbers as August 1973. C. tritaeniorhynchus populations were also at about the same levels in February 1974 as they were in August 1973; however, they were about 5 times greater than in February 1973.

b. INSECTICIDE SUSCEPTIBILITY OF JEV VECTORS

BACKGROUND: Insecticide susceptibility tests run during 1970 (5) indicated that adult C. fuscocephala, C. gelidus and C. tritaeniorhynchus were resistant to 4% DDT, the concentration employed for residual spraying of dwellings by the National Malaria Eradication Project in Thailand. The susceptibility of larval stages of these three species to DDT was not determined at that time, but both larvae and adults were found to be susceptible to malathion. The above results are puzzling because DDT is not widely used for agricultural purposes in the Chiangmai Valley, and houses in the area have not been sprayed with this chemical for malaria control for more than 10 years. Thus, there is no apparent DDT pressure on these mosquito populations to account for their state of resistance to this insecticide. On the other hand, organophosphates, including malathion, are commonly used in the Chiangmai Valley to control pests on crops such as tobacco, but insecticides are not used to control pests of rice.

Table 1. Toxicity of DDT and Malathion to Culex fuscocephala and C. tritaeniorhynchus from Saraphi District, Chiangmai Province 1973-74

Species	Stage	Insecticide	LC_{50}*	LC_{90}*	Interpretation
C. tritaeniorhynchus	Adult	DDT	2.72% at 1 hr	> 4.0% at 1 hr	Resistant
"	Larval	DDT	0.0058 ppm at 24 hr	0.012 ppm at 24 hr	Susceptible
C. fuscocephala	Adult	DDT	3.3% at 1 hr	> 4.0% at 1 hr	Resistant
"	Larval	DDT	0.0039 ppm at 24 hr	0.0075 ppm at 24 hr	Susceptible
"	Larval	Malathion	0.034 ppm at 24 hr	0.063 ppm at 24 hr	Susceptible

* LC_{50} and LC_{90}: Concentrations of an insecticide required to kill 50% and 90% respectively, of the exposed population in a specified period of time.

Table 2. Toxicity of DDT and BHC to Culex tritaeniorhynchus from Bang Nam Prieo District, Chachoengsao Province 1973 - 74

Species	Stage	Insecticide	LC_{50}*	LC_{90}*	Interpretation
C. tritaeniorhynchus	Adult	DDT	1.60% at 1 hr	2.22% at 1 hr	Intermediate Resistance
"	Larval	DDT	0.0013 ppm at 24 hr	0.0047 ppm at 24 hr	Susceptible
"	Larval	BHC	0.0124 ppm at 24 hr	0.0327 ppm at 24 hr	Susceptible

*LC_{50} and LC_{90}: Concentrations of an insecticide required to kill 50% and 90%, respectively, of the exposed population in a specified period of time.

DESCRIPTION: To confirm the findings of the 1970 susceptibility tests adults and larvae of C. fuscocephala and C. tritaeniorhynchus were retested. In addition, Culex tritaeniorhynchus were collected from near pig farms at Amphur Bang Nam Prieo, Changwat Chachoengsao, near the eastern edge of the central plain and about 60 kilometers from Bangkok. This area is under cultivation during most of the year with both dry season and rainy season crops. Most of the rice planted in this area is one of the several new hybrid varieties,such as RD-1, which require use of fertilizers and insecticides. In contrast, in the Chiangmai Valley, the majority of the farmers still plant traditional varieties of rice (80 per cent of which is glutinous rice) which do not require fertilization or protection from insect pests. Over half of the farmers in Bang Nam Prieo use granular BHC with fertilizer applications applied at planting time, and more than three-fourths of them employ organophosphates such as parathion, azodrin or endrin against pests on the maturing rice plants. A majority of the farmers in the area report fish are killed following organophosphate applications. Because of the marked differences in rice cultivation as practiced in these two areas of Thailand, the susceptibility of JEV vectors from Chiangmai Valley and Chachoengsao were compared.

PROGRESS: As in the 1970 tests, adults of C. fuscocephala and C. tritaeniorhynchus from Saraphi district of Chiangmai were found to be resistant to DDT. The larvae of both species, however, were susceptible to this insecticide (Table 1). Only C. tritaeniorhynchus from Chachoengsao were tested during this period. While larvae were susceptible to both DDT and BHC, adults exhibited partial resistance to DDT (Table 2). Further tests of C. tritaeniorhynchus, C. gelidus and C. fuscocephala from Chachoengsao against DDT, BHC and organosphosphate insecticides are planned.

2. Japanese Encephalitis Virus Infection in Pregnant Swine.

The purpose of these studies is to establish a relationship between Japanese Encephalitis Virus (JEV) infection and problems with swine production in Thailand.

Evidence from Japan indicates that JEV infection of swine causes problems of infertility, abortion, and the birth of dead or weak piglets. It has become economically advantageous to suspend the breeding of gilts in Japan during the encephalitis season so that economic losses resulting from these problems are reduced.

Work reported in the 1972-73 annual report (6) suggested a reduced reproductive efficiency in a small group of susceptible sows in

Thailand as a result of natural infection with JEV. Infection shortly before breeding reduced the rate of conception, while infection during pregnancy increased the incidence of complications during gestation and parturition.

The Kasetsart University pig farm located in Saraburi Province is being used as the study site. One hundred forty virgin gilts were selected, and serum antibody titers to JEV measured by the hemagglutination inhibition (HI) method initially and every two weeks thereafter until the titers converted to positive. A positive titer is interpreted as an initial titer of 1:80 or greater; conversion is an eight-fold increase in titer over a two week period. The study began before the JE season on 1 Dec 1973 and will continue until all animals have farrowed. The final JEV antibody titer will be determined on all animals at the conclusion of the study. Titers are run as collected, and will also be run in parallel as a group at the conclusion of the study. Complete breeding records are being kept, and the number, health, and appearance of the piglets is being recorded.

The titers of the animals to date are summarized in Table 1. Only 14 animals are 18 months of age, and all of this group show positive or increasing titers. The remainder of the animals are divided equally into a 10 1/2 month old and a 7 month old group. Over 90% of the 10 1/2 month old group show positive or increasing titers, while only 12% of the 7 month old animals show any measureable titer. About 50% of the animals have already been bred, and the remainder will be bred during the next two months.

From the distribution of positive JEV antibody titers among the age groups, it appears that JEV was epizootic in the area less than 10 1/2 but more than 7 months ago. This corresponds to the last encephalitis season in the area. The relatively large number of animals under study and the even distribution between positive and negative titers should give a good indication about the role of JEV infection and resulting reproductive problems in susceptible sows. This study will terminate when all animals have farrowed; in about September 1974.

Table 1. Antibody Titers to JEV

Age	Negative Titer (<1:10)	Intermediate Titer (1:10-1:40)	Positive Titer(≥1:80)
7-8 months (63)	55(87.3%)	4(6.3%)	4(6.3%)
10-10 1/2 months (62)	4(6.5%)	12(19.4%)	46(74.2%)
17-18 months (14)	0	7(50%	7(50%)
Total (139)	59(42.4%)	23(16.5%)	57(41.0%)

C. TICK-BORNE VIRUSES

1. Characterization and Identification of Tick-Borne Viruses in Thailand

This study is designed to study the characteristics of and identify unknown viruses isolated from ticks.

Tick-borne viruses are well known causes of serious and often fatal human infections in many parts of the world. Throughout Asia tick-borne agents have been found which cause fever, hemorrhagic fever and encephalitis. Some, however, are not human pathogens. Two viruses previously found in Thailand, Nyamanini and Pathum Thani,as well as Langat from Malaysia are not known to cause human disease.

The causes of some cases of fever, hemorrhagic fever and encephalitis are not explained. It is possible that some are due to tick-borne viruses not previously identified. For this reason, a study was initiated in 1972 to attempt isolation of viruses from ticks and to identify them (7). Five strains of transmissible agents were recovered. This report describes their characteristics.

DESCRIPTION: Ticks of the same species, collected from the same area at the same time were pooled in groups of 10 to 20. Virus

isolations were made in suckling mice. Each tick pool was ground in a mortar and pestle and suspended in bovine albumin borate saline (2 ml/10 ticks) containing penicillin and streptomycin. The suspension was clarified by centrifugation at 1000 rpm for 30 minutes and the supernate inoculated into 1-2 day old suckling mice in a dosage of 0.02 ml intracerebrally. Two litters of mice were inoculated for each tick pool. The mice were observed daily for signs of illness or death for at least 3 weeks.

Mice that showed signs of neurologic disease were sacrificed and the brains harvested. A 20% suspension of mouse brain was prepared for inoculation of new litters of mice to demonstrate the presence of a transmissible agent and to establish a large supply of infectious seed virus.

Low passage infectious suckling mouse brain was used to attempt infection of a continuous line of LLC-MK2 cells in plaque flasks and glass tubes to determine and compare optimum media, plaque formation and cytopathic effect (CPE). A direct and delayed plaque technique was used which has been highly successful for the isolation of group A and B arboviruses. In an attempt to compare the unknown agents to other tick-borne arboviruses, the mouse brain isolates were tested for size by Millipore filtration, a lipid envelope by ether sensitivity and acid lability at pH 3.0 (8). Agents which grew in tissue cell culture were tested for sensitivity to 5-bromo-2'-deoxyuridine (BUDR), a DNA inhibitor.

Identification of agents was based on neutralization by specific antisera. Each agent was used to prepare CF and HI antigens by a sucrose-acetone extraction technique (9). The antigens were used to make hyperimmune mouse ascitic fluid (HMAF) with Freund's adjuvant and Sarcoma 180 cells in adult mice (10). Plaque reduction neutralization tests were performed using homologous HMAF, a panel of 12 typing antisera prepared by the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, and other reference antisera. Isolates have been sent to reference laboratories to assist in the identification.

PROGRESS: A total of 14,268 ticks were collected from 12 provinces (i.e. Chantaburi, Chon buri, Chumpon, Loei, Mae Hong Sorn, Nakorn Nayok, Nakorn Ratchasima, Phatum Thani, Phangnga, Prachinburi, Ranong and Trang), identified and pooled for virus isolation attempts. Included in the 1267 pools tested were 17 species of ticks belonging to the genera Amblyomma, Aponomma, Argas, Boophilus, Dermacenter, Ixodes, Haemaphysalis, Ornithodoros and Rhipicephalus (Table 1). The largest number of ticks tested belonged to the genera Argas and Haemaphysalis, respectively. A total of 1885 ticks were collected from 815 mammals trapped and/or examined during this period. In addition, 442 ticks were collected from a total of 33 birds trapped

or shot during the same period. The largest number of ticks, however, were collected from vegetation.

Eleven transmissible agents were recovered. Six came from pools of Argas robertsi collected in the vicinity of nests of night herons at Wat Pai Lom in Pathum Thani province. At the present time these agents have not been characterized further.

Five isolates were obtained from Haemaphysalis ticks (Table 2). Four of them came from ticks collected in Khao Yai National Park (KYNP) in Nakhon Ratchsima Province and the other from a single tick removed from a domestic dog in Loei Province. T-867, T-868 and T-870 were obtained from the same species of tick collected at the same time from the same habitat and are considered to be identical. Studies on the identification of isolates have used T-870, T-1642 and T-1674 as prototype strains.

Table 1. Ticks Collected in Thailand and Tested for Viral Agents, 1973-74

SPECIES	No. of Pools	No. of Ticks
Amblyomma testudinarium	11	28
Aponomma lucasi	1	1
Argas robertsi	715	7,437
Boophilus microplus	20	206
Dermacentor sp.	35	61
D. atrosignatus	11	21
D. auratus	28	88
Ixodes granulatus	2	2
Haemaphysalis sp.	22	2,944
H. atherurus	13	53
H. bandicota	6	72
H. cornigera	163	1,191
H. lagrangei	181	1,504
H. obesa	18	42
H. papuana	10	21
Ornithodoros capensis	30	596
Rhipicephalus h. haemaphysaloides	1	1
TOTAL	1,267	14,268

Table 2. Sources of Transmissible Agents from Haemaphysalis Ticks

Tick Isolate	Tick	Region	Habitat
T-867	H. conigera	Khao Yai	Leaf
T-868	H. conigera	Khao Yai	Leaf
T-870	H. conigera	Khao Yai	Leaf
T-1642	H. papuana	Loei	Dog
T-1674	H. papuana	Khao Yai	Leaf

The three isolates showed distinctly different growth characteristics. Although each grew to high titer in suckling mice, T-870 did not produce either CPE or plaques in LLC-MK2 cells regardless of the medium used. Investigators at WRAIR reported they were able to plaque T-870 by adjusting the agar overlay medium. Plaques were not seen when the same medium was used in this department. All subsequent work with this agent was done in suckling mice.

T-1642 did produce CPE and formed homogeneous plaques with clear centers similar in appearance to those of Chikungunya virus (Fig 1). T-1674 produced no CPE but did form plaques of various sizes from small to large without clear centers. Neither of the latter two agents formed plaques similar to our more familiar group B arboviruses, such as dengue or Japanese B Encephalitis virus (JEV). Tissue culture work suggested the three prototype strains were different.

All three isolates were inhibited by exposure to ether and showed acid lability at pH 3.0 (Table 3). Each passed through a 50 nm Millipore filter. T-1642 and T-1674 were not inhibited by BUDR suggesting they are not DNA viruses. T-870 was not tested for BUDR sensitivity since it was not grown in tissue culture. These results give suggestive evidence that all three are viruses and that T-1642 and T-1674 are members of the Togavirus group.

Sucrose-acetone extraction produced strong complement fixing antigens from each virus (Table 4). T-870 formed no hemagglutinin. T-1642 showed weak hemagglutination of goose erythrocytes at pH 6.0. T-1674 formed a strong hemagglutinin with optimum activity at pH 6.7 (range 6.4-7.0) at 22°C.

Table 3. Physical and Chemical Characteristics of Tick-borne Agents

Agent	Nucleic Acid (BUDR)	Envelope (Ether)	Size (Millipore)	Acid Lability (pH 3.0)	Presumptive Classification
Tick Isolate					
T-870	NT*	+**	50 nm	+**	
T-1642	-	+	50 nm	+	Togavirus
T-1674	-	+	50 nm	+	Togavirus
Control Viruses					
Polio I				-	
Chikungunya	-			+	
Herpes	+				

* NT = not tested
** + indicates agent is sensitive to treatment

Table 4. Antigenic Characteristics of Tick-borne Viruses

Tick Isolate	Sucrose-Acetone Antigens		
	SMB Passage	CF	HA
T-870	5	+	0
T-1642	3	+	±
T-1674	4	+	+

Table 5. CF Titers of Suckling Mouse Brain Antigens and Hyperimune Mouse Ascitic Fluid

Sucrose Acetone Antigen	HMAF					
	T-1674	D1	D2	D3	D4	JEV
T-1674	128*	32	32	16	16	32
	64	16	32	64	128	128
D1	8	128				
	4	512				
D2	8		64			
	4		1024			
D3	16			128		
	16			512		
D4	16				128	
	32				512	
JEV	32					512
	8					512

* Reciprocal antigen titer over antibody titer

A B C

Figure 1. Comparison of plaque formation in LLC-MK2 cells of two strains of tick-borne viruses using a direct staining method. A. T-1642, passage SMB-4, TC-1 stained on day 3, formed uniform plaques 1.8-2.4 mm in diameter with clear centers. Photo was taken on day 7. B. & C. T-1674, passage SMB-4, TC-[illegible] ned on day 8, formed plaques without clear centers that ranged from 3-5 mm in diameter. This pass was made by picking a single large plaque. Photo was taken on day 11.

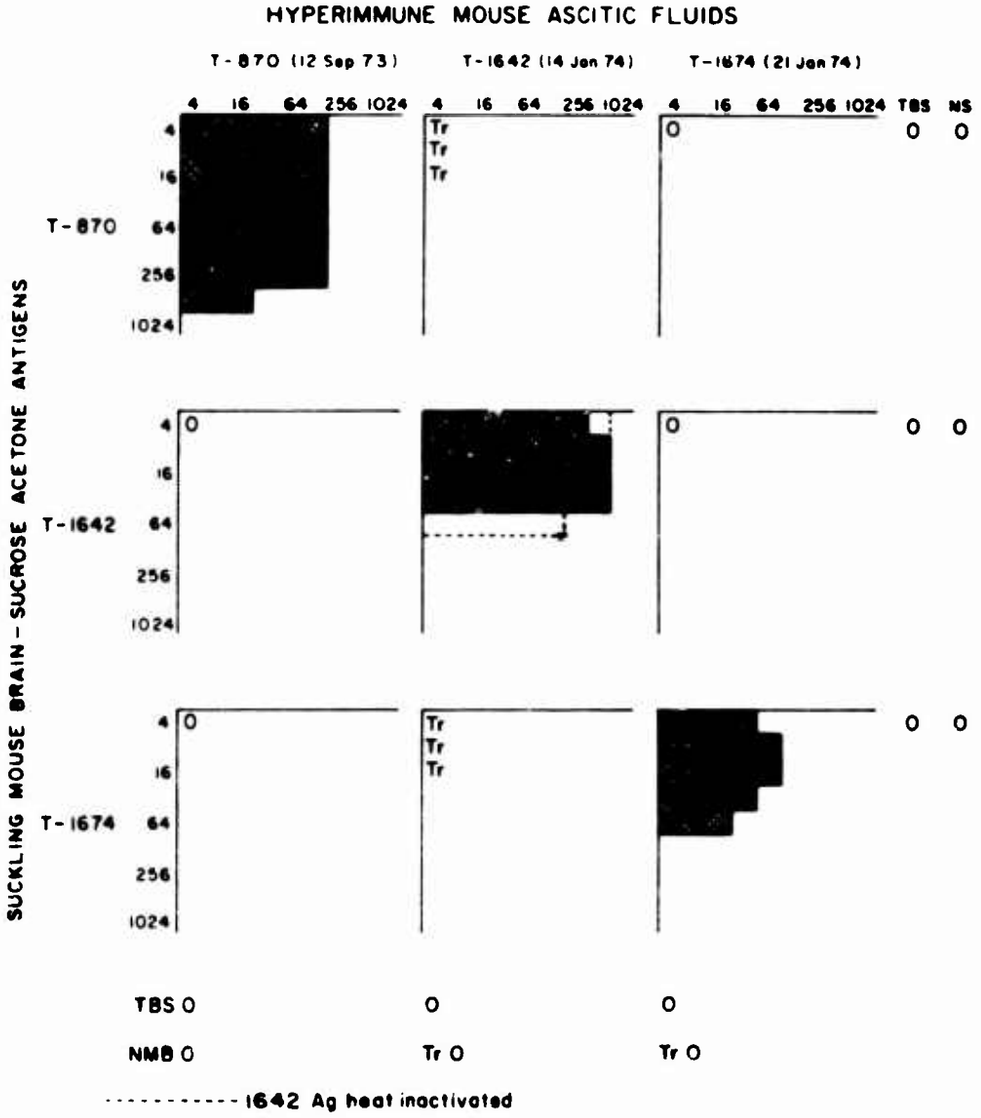


FIGURE 2 COMPLEMENT FIXATION BLOCK TITRATIONS COMPARING THREE VIRUSES ISOLATED FROM TICKS. ANTIGENS WERE PREPARED BY SUCROSE – ACETONE EXTRACTION OF SUCKLING MOUSE BRAINS ANTIBODY WAS PREPARED IN MOUSE ASCITIC FLUID NO CROSS-REACTIVITY BETWEEN THE VIRUSES WAS FOUND.

CF block titrations with sucrose-acetone antigens and hyperimmune mouse ascitic fluid showed that T-870, T-1642 and T-1674 were antigenically unrelated (Fig 2). On the other hand, T-1674 showed considerable cross-reactivity with Dengue 1-4 and Japanese B Encephalitis (Table 5). Plaque reduction neutralization tests showed T-1674 was partially neutralized by a pool of 35 group B arbovirus antisera. It appears, then, that T-1674 is a group B arbovirus closely related antigenically to Dengue 4 and JEV.

T-1642 did not react with any of the 12 different arbovirus grouping antisera. The identity of this virus is totally unknown.

T-870 has been studied by Dr. Robert Shope of the Yale Arbovirus Research Unit (YARU). A preliminary report said he found that in CF tests, T-870 was not closely related to any of over 200 reference arboviruses he tested. It did show a weak cross-reaction with Wad Medanii virus; a tick-borne virus that has been found in Malaysia and Singapore in the past.

Strains of all three viruses and their homologous hyperimmune ascitic fluids have been provided to YARU for further attempts at identification.

DISCUSSION: At least four different viruses have been isolated from ticks during this study. The isolate from the Argas tick in Pathum Thani has not been characterized but is probably related or identical to Pathum Thani virus of birds.

The other three agents have characteristics of Togaviruses and may be pathogenic for mammals. Two viruses, T-870 and T-1642, may be entirely new, since neither show a definite antigenic relationship to any virus included in the pools of grouping antisera.

Incomplete characterization of these agents does not reveal if any of these viruses are pathogenic. Nevertheless since T-1674 is a group B arbovirus it must be considered potentially infectious for man.

2. Antibody to Tick-borne Viruses in Thailand

This study is an attempt to determine if humans and small animals have naturally acquired serum antibody to tick-borne viruses.

Three different viruses were isolated from Haemaphysalis ticks in two provinces of Thailand during the spring and summer of 1973. One tick from Loei Province was removed from a domestic dog. The other

ticks were collected while resting on vegetation in Khao Yai National Park (KYNP), and so gave no information on the potential hosts for the viruses they carried (SMRL Annual Report 1974).

The Haemaphysalis genus of ticks has a wide range of animal hosts including man. There are an estimated 90 species in the genus. The viruses were isolated from H. conigera and H. papuana. Although these species will bite mammals, it is not known if they will bite humans.

A serologic survey of animals and people living in the vicinity of the viruses was conducted to try to determine their natural hosts.

DESCRIPTION: A program for serologic screening of residents of KYNP was initiated with the cooperation of the Chief, National Park Division, Department of Forestry. Surveys were conducted in September, 1973 and February, 1974.

Residents of KYNP include the families of employees of three different administrative agencies working in the park; Department of Forestry (FD), Highway Department (HD) and the Tourist Organization of Thailand (TOT). Each agency has its own work responsibilities and housing areas. TOT families as a group are the highest paid, best educated and enjoy the best living conditions. Members of each group travel widely throughout the park and have access to the areas where the ticks were collected, even though some tick collection sites are long distances from some of the housing areas. (Figure 1).

In each housing area, the study was explained as a survey for malaria and viral diseases. Residents were encouraged to participate on a voluntary basis. Each participant was questioned regarding his or her age, length of residence in KYNP, area of residence, names and relationship of family members, and history of illnesses and hospitalization. Residents were encouraged to provide this information even if they refused to allow blood to be drawn.

Two to 10 ml of whole blood was drawn from each individual, allowed to clot at ambient temperature and centrifuged. Serum was transferred to storage tubes and frozen at -70°C until tested.

Thick and thin blood smears were made for each participant for malaria screening. Malaria smears were stained and interpreted by members of the Department of Microbiology.

Neutralizing antibody was detected by plaque reduction neutralization test (PRNT) for T-1642 and T-1674. Neutralization tests with T-870

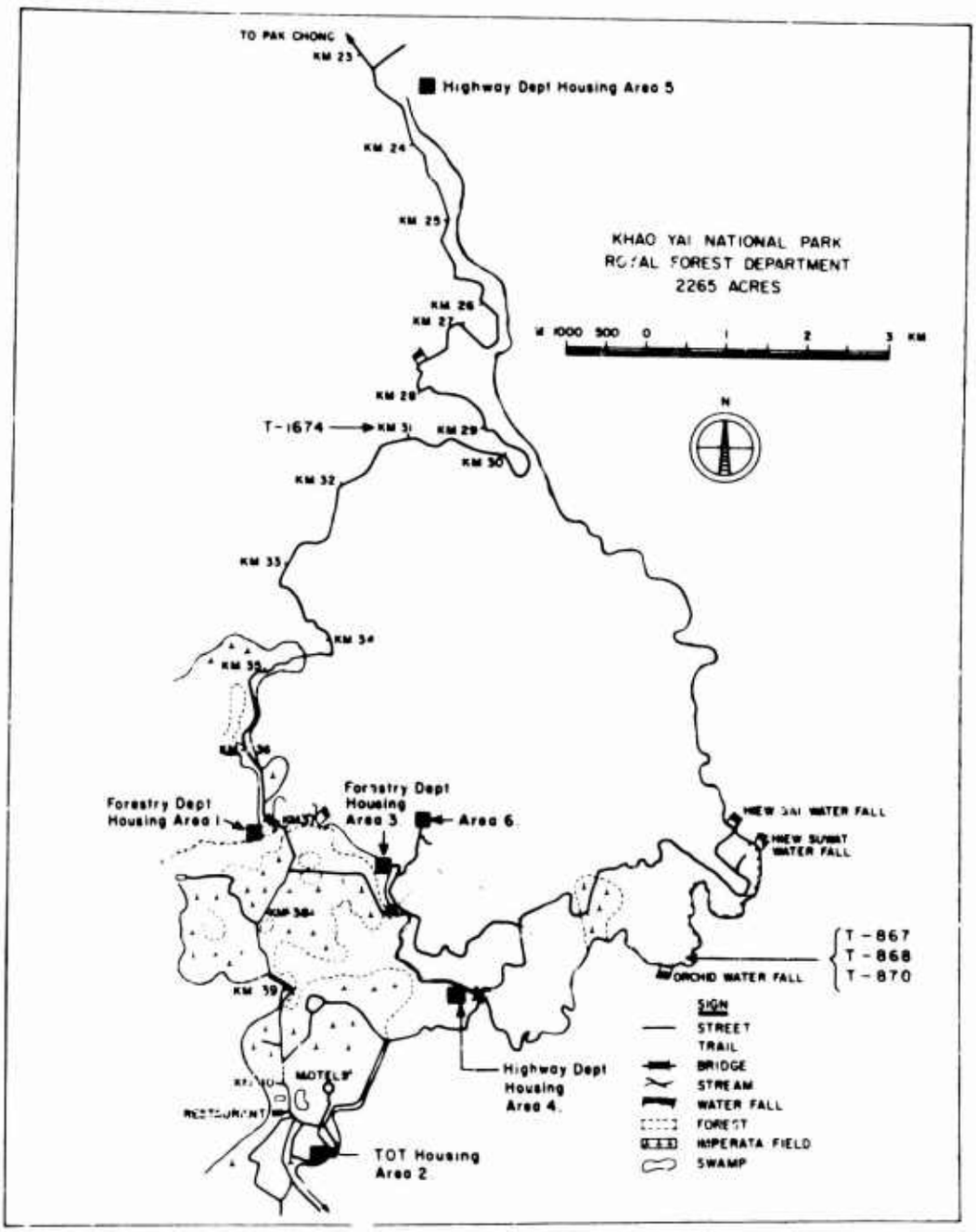


Figure 1 Study area in Khao Yai National Park. Virus isolates were made from ticks collected at Km 31 and Orchid Waterfall. Black squares indicate housing areas of residents

were done in suckling mice. Sera showing at least 50% neutralization at a dilution of 1:10 were considered to be positive and were re-tested in serial dilution.

Microtiter complement fixation (CF) and hemagglutination inhibition (HI) tests were performed by standard techniques using antigens prepared by sucrose-acetone extraction of suckling mouse brains.

Sera were collected from live-caught small animals trapped between Km 30 and 33 and near Km 40 (Figure 1). Each animal was identified by species and location, examined for ticks, bled and released. Some rodent sera were obtained from other areas of Thailand.

No serum samples were obtained from Loei Province, the source of virus strain T-1642. Since this strain was isolated from a tick on a dog, sera were collected from 100 dogs at the Bangkok dog pound. Sera from eight laboratory gibbons were obtained for testing from the Division of Veterinary Medicine.

PROGRESS: Information was gathered by questionnaire on 548 different residents during surveys in September and February. Members of families of Highway Department employees tended to be older and to have lived in the park longer than the other two groups (Table 1). All agencies had a constant turnover in personnel and many people had lived in the park for less than a year.

Table 1. Characteristics of Residents of KYNP. Combined Information from Surveys in September 1973 and February 1974

Agency	No. of Residents	Male: Female	Age in years Mean (Range)	Years of Residence Mean (Range)
Forestry Dept	166	1.81	21.0 (9/12-60)	3.6 (1 day-13 yr)
Highway Dept	134	1.38	26.2 (3-59)	6.9 (15 day-43 yr)
TOT	248	1.05	20.1 (6/12-48)	3.8 (7 day-10 yr)
Total	548	1.32	21.4	4.5

At least one serum specimen was obtained from 77.9% of the 548 residents (Table 2). Paired sera were obtained from 29.4%.

Table 2. Serum Samples Collected from Residents of KYNP

Agency	Number of Sera			Percent of Residents	
	First Serum		Second Serum	At least one Serum	Paired Sera
	Sep	Feb	Feb		
Forestry Dept	100	48	60	89.2	36.1
Highway Dept	34	39	8	54.5	6.0
TOT	156	50	93	83.1	37.5
TOTAL	290	137	161	77.9	29.4

Antibody to Tick-borne Viruses: CF antibody to T-870 was found in only one of 283 sera collected in September (Table 3). The single reactor titered 1:32 to T-870, 1:8 to normal mouse brain and was anticomplementary to 1:4. A second serum from the same individual collected in February titered <1:2. None of 187 sera, including that from the CF reactor, had neutralizing antibody in suckling mice. Thus, there is no conclusive evidence that T-870 can infect people.

A great many sera (44.2%) reacted by HI to T-1674 (Table 3). Even more reacted to other group B arboviruses. Generally, people reactive by HI and CF were over 10 years of age, and represented a fairly constant proportion of each age group. Because of the high degree of cross-reactivity, analysis of T-1674 reactors was confined to the 21 sera with neutralizing antibody (Table 4).

All of the residents with neutralizing antibody to T-1674 came from families of Forestry and TOT employees. The lack of Highway Department personnel may simply be due to underrepresentation of this group in the September survey.

There was no clear correlation of neutralizing antibody with age, sex, duration of residence in the park, area of residence, occupation or HI titer. On the other hand, HI reactivity to T-1674 closely paralleled HI reactivity to other group B arboviruses.

Eleven of 17 (65%) of the PRNT positive residents reported previous tick bites; however, 55.8% of the PRNT negative residents did too. Some residents reported that tick bites caused fever, local swelling

Table 3. Frequency of Antibody in Residents of Khao Yai National Park (Sept, 1973)

Agency	T-870		T-1674			Dengue-2	JEV[1]	Chik[2]
	SMB-NT[3]	CF	PRNT[4]	CF	HI	HI	HI	HI
Forestry Dept.	0/76*	0/98	8/98	7/56	33/97	57/98	57/98	45/98
Highway Dept.	0/24	0/34	0/33	1/7	21/32	32/34	33/34	22/34
TOT	0/87	1/151	13/151	34/94	69/149	101/150	103/150	75/150
Combined	0/187	1/283	21/282	43/157	123/278	190/282	193/282	142/282

1 Japanese Encephalitis Virus
2 Chikungunya
3 Suckling Mouse Brain Neutralization Test
4 Plaque Reduction Neutralization Test
* Number of antibody positive sera over number tested

Table 4. Residents of KYNP with Plaque Reduction Neutralizing Antibody to T-1674

Serum No.	Occupation	Sex	Age (years)	Years in KYNP	Housing Area	HI Titer T-1674	D-2	JEV
54034	FD-Dependent	M	4	4	1	<10	<10	<10
54039	FD-Dependent	M	7	7	1	<10	<10	<10
54040	FD-Dependent	M	4	4	1	<10	<10	<10
54060	Housewife	F	24	2	1	<10	<10	<10
54228	TOT-Dependent	F	4	4	2	<10	<10	<10
54220	TOT-Dependent	M	12	10	2	<10	10	<10
54092	Receptionist	M	22	1/12	2	<10	<10	10
54090	Restaurant worker	F	28	4	2	<10	10	20
54116	TOT-Dependent	F	12	6	2	<10	20	20
54094	Janitor	M	28	1	2	10	20	20
54019	Guest House worker	F	54	9	6	<10	10	40
54077	Janitor	M	22	3/12	2	<10	20	40
54038	Housewife	F	42	11	1	10	40	20
54111	Housewife	F	38	6	2	20	20	40
54107	Guest House worker	M	22	2	2	40	20	40
54029	Guest House worker	M	26	6	1	20	40	40
54091	Carpenter	M	24	4	2	20	80	40
54036	Housewife	F	23	1	1	20	40	80
54076	Receptionist	M	33	5	3	20	80	80
54082	Accountant	M	23	3/12	2	40	80	80
54056	Forest Guard	M	23	2	Waterfall	20	160	160
54078	Restaurant worker	M	22	7/12	2	160	160	160

Table 5. Frequency of Antibody to Tick-borne Viruses

Virus Strain:	T-870		T-1642		T-1674		
Test Used:	SMB-NT	CF	PRNT	CF	PRNT	CF	HI
Serum Dilution:	at 1:10	≥1:4	at 1:10	≥1:4	at 1:10	≥1:2	≥1:10
Human	0/187	1/283			21/282	43/157	123/278
Gibbon							1/8
Tupaia glis (Tree shrew)		3/11					0/19
Hylomys suillus (Pig shrew)							0/2
Dog			1/54	38/100*			
Goat		0/2					
Sheep		0/1					
Melogale personata (Ferret badger)							0/1
Rat		6/103*					0/168

* All 38 reactive dog sera and 4 reactive rat sera reacted with normal mouse brain or were anticomplementary to within one dilution. CF reactive rats were Rattus fulvescens - 1 and R. surifer - 1.

and local pain. None of them had ever been hospitalized. From interviews and examination of skin lesions, it seemed that tick bites were familiar to the residents of KYNP and occurred more frequently in February than September.

One case of Plasmodium falciparum malaria was identified in a Forestry Department employee during these surveys.

Screening tests for antibody to tick-borne viruses in other species is incomplete (Table 5). Nevertheless two rats had CF titers to T-870 of 1:16 and one dog had a titer of PRNT neutralizing antibody to T-1642 of 1:60. CF tests of dog sera were unsatisfactory because 38 percent were anticomplementary.

On the basis of this information it can be concluded (1) ticks commonly bite humans at KYNP (2) tick bites may lead to mild fever and local symptoms in some people (3) T-1674 has infected humans at KYNP in the past (4) T-870 may infect rats naturally and T-1642 may infect dogs. The HI and CF tests are of little value for screening people for antibody to T-1674 because of the high level of cross-reactivity with other group B arbovirus antigens.

D. INGWAVUMA VIRUS

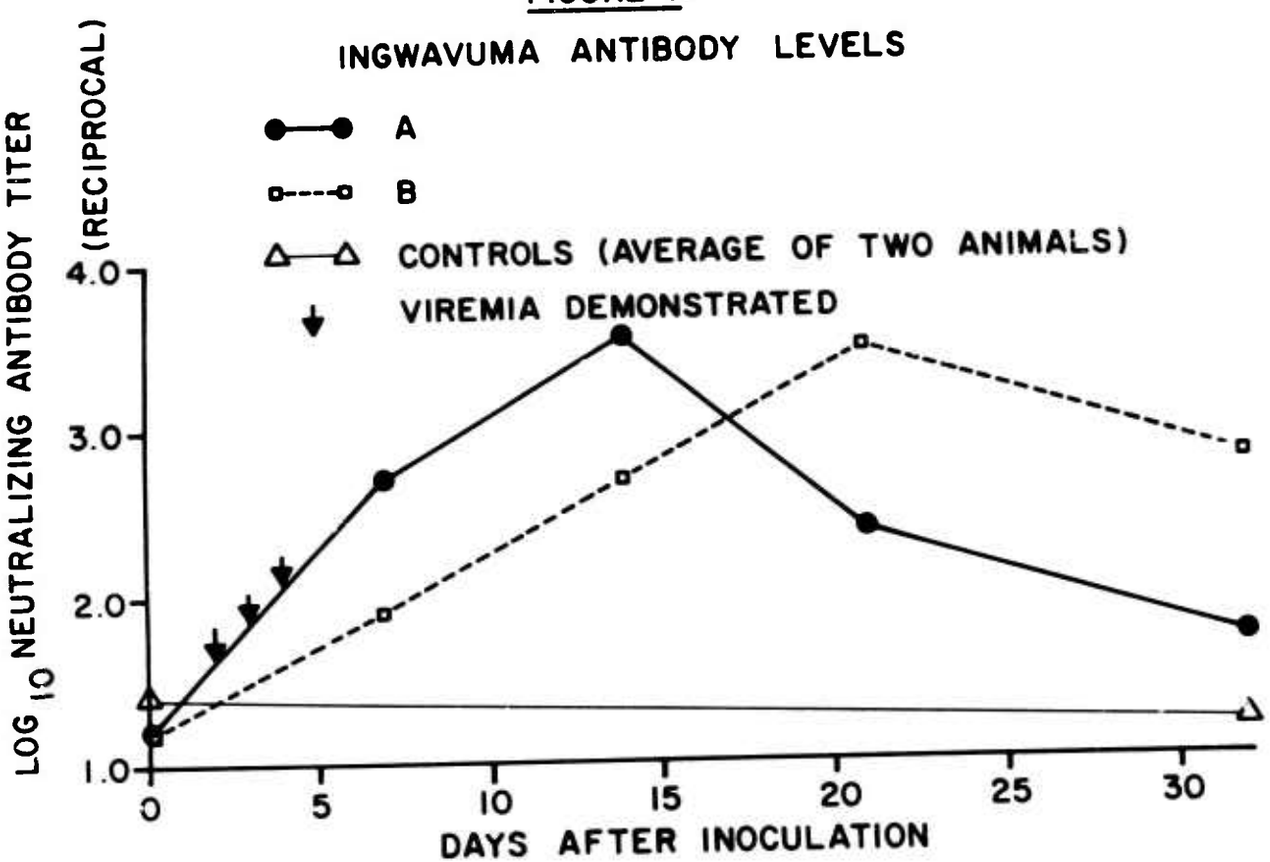
1. Laboratory Infection of Swine (Sus scrofa) with Ingwavuma Virus

Previous studies in this laboratory have shown that a Simbu group arbovirus, Ingwavuma virus, commonly infects pigs in Thailand (11). Although this virus has frequently been isolated from the blood of domestic swine, the duration and magnitude of viremia and the routes by which the virus may be excreted are unknown.

We have performed an experiment to determine the duration and magnitude of viremia, the antibody response, and presence of the virus in nasal secretions and feces of swine infected with Ingwavuma virus.

DESCRIPTION: Four 8 month old landrace pigs lacking Ingwavuma neutralizing (N) antibody at a 1:10 dilution were chosen for this study. These pigs were housed in a mosquito-proof building throughout the study. Two animals were inoculated subcutaneously with 1000 plaque forming units (PFU) of Ingwavuma virus in 0.3 ml of solution. Titration of the amount of virus inoculated in LLC-MK2 cells was performed immediately after inoculation. The animals were closely observed for clinical signs of illness. Rectal temperatures were taken daily for 5 days before and for 7 days after inoculation. Serum was collected at 7 day intervals for 21 days following

FIGURE 1.


INGWAVUMA ANTIBODY LEVELS
A
B
CONTROLS (AVERAGE OF TWO ANIMALS)
VIREMIA DEMONSTRATED
LOG 10 NEUTRALIZING ANTIBODY TITER (RECIPROCAL)
4.0
3.0
2.0
1.0
0
5
10
15
20
25
30
DAYS AFTER INOCULATION

inoculation and tested for Ingwavuma N antibody by standard plaque reduction neutralization test (PRNT). Blood, nasal swabs, and feces were obtained daily from day 0 through day 7. Aliquots of these specimens were tested for the presence and titer of virus by intracranial inoculation into 1-2 day old mice and by direct and delayed plaques in LLC-MK2 cells. Isolates obtained were confirmed as Ingwavuma virus by PRNT tests using mouse hyperimmune ascitic fluid.

PROGRESS: The body temperatures of both infected and control animals remained normal throughout the study. None of the animals showed signs of clinical illness. Ingwavuma virus was isolated from undiluted plasma in one of the infected animals on days 2, 3, and 4 following inoculation. Attempts to isolate Ingwavuma virus from the other infected animal and from both control animals were unsuccessful. Ingwavuma virus was not isolated from the feces or nasal swabs of any of the animals. A primary antibody response to Ingwavuma virus was demonstrated in each infected animal, with serum titers reaching 1:3500 and 1:5120 by day 14 to 21 (Figure 1).

DISCUSSION: Subcutaneous inoculation of 2 previously unexposed pigs with Ingwavuma virus has produced a demonstrable viremia within 48 hours in one pig and a primary antibody response following inoculation in both. Viremia was short lived and low titered, lasting only 3 days, and detectable only in undiluted serum; however, a high titered antibody response was found in both pigs by day 14. Ingwavuma virus was not isolated from the feces or nasal secretions of either pig.

E. HEPATITIS B VIRUS

1. Comparison of Two Methods of Immunoelectroosmophoresis for the Detection of Hepatitis B Antigen and Antibody

Experiments have been conducted to compare the performance of the existing technique for immunoelectroosmophoresis (IEOP) to that used at Walter Reed Army Institute of Research (WRAIR).

BACKGROUND: IEOP is the basic screening test for Hepatitis B antigen and antibody at SMRL. Previous studies have compared the sensitivity of IEOP to that of complement fixation (CF) and solid phase radioimmunoassay (RIA) (12). Although basically the procedures are the same, the results of IEOP tests at SMRL have always revealed more antigen and antibody positives than found at WRAIR. Most of the differences in results between SMRL and WRAIR are probably related to the greater prevalence of Hepatitis B antigen (HBsAg) and antibody (anti-HBsAg) in Thailand. Nevertheless, the two laboratories use somewhat different procedures which might affect the relative sensitivity of the tests.

DESCRIPTION: The differences in the two IEOP procedures are listed in Table 1. In addition, human antiserum is used at SMRL as opposed to high titered rabbit antiserum at WRAIR. Because of insufficient rabbit antiserum, only a human antiserum, designated P.T., was used in this study.

All tests were conducted in standard electrophoresis cells (Buchler and/or Shandon VoKam) with medium grade filter paper wicks (Schleicher & Schuell grade 407W) at room temperature on 7 x 5 cm glass slides overlaid with 10 ml of 1% agarose. Following electrophoresis, the slides were washed for 72 hours in normal saline and stained with 0.025% benzalkonium chloride for 30 minutes. All slides were read with an indirect fluorescent light against a black background.

Test specimens included serial dilutions of serum containing HBsAg/adr (designated 48/0) and anti-HBsAg/adw (P.T.) and a panel of 58 selected clinical sera (25 with HBsAg, 22 with anti-HBsAg and 11 negative).

All preparation of reagents, testing and slide interpretation was done by a person thoroughly experienced in both the SMRL and WRAIR procedures.

PROGRESS: When the two methods were performed as described in Table 1, the results were identical in nearly all respects. This was also true when the SMRL slides were run at constant voltage and the WRAIR slides at constant current. This indicates that the buffer and pH of the slide and the mode of electrophoresis are not significant factors.

The factor that most influenced the performance was well size. The SMRL technique, using pairs of wells 5 mm in diameter, detected antigen and antibody two dilutions higher than the WRAIR procedure with 2 vs 3 mm wells. Yet, when the panel of clinical specimens was tested, the results were the same regardless of well size; only the intensity of the reactions was different.

Staining of the slides with 0.025% benzalkonium chloride increased the number of positive reactions (Table 2). With the clinical sera, however, only the detection of antibody was enhanced. No new antigen reactions were revealed by staining. Unfortunately, staining also accentuated all proteins remaining in the agar giving rise to artifacts.

DISCUSSION: The SMRL procedure is more sensitive than that of WRAIR when the two are used to screen for both Hepatitis B antigen and antibody. When screening sera for anti-HBsAg and when using human

Table 1. Differences in IEOP procedures used at SMRL and WRAIR

Characteristic	SMRL	WRAIR
Chamber buffer	Veronal 0.05M pH 8.6	Veronal 0.05M pH 8.6
Slide buffer	TRIS-EDTA-NaCl 0.01M pH 9.6	Same as chamber
Electrophoresis	Constant current 30 ma/sl for 90 min.	Constant voltage 12 v/sl for 120 min.
Well diameter	5 mm pair	2 vs 3 mm (Ab vs Ag)
Staining	Yes	No
Reference	Alter et al 1971	Prince and Burke 1970

Table 2. Effect of Benzalkonium Chloride Staining on the Titer of anti-HBsAg

HBsAg Dilution	Unstained Reaction			Stained Reaction		
	Intensity		Ab Titer	Intensity		Ab Titer
1:8	4+	at	1:64	4+	at	1:64
1:16	3+	at	1:64	3+	at	1:64
1:32	2+	at	1:8	2+	at	1:64
1:64	0	at	1:4	1+	at	1:64
1:128	0	at	1:4	0	at	1:4

antibody to detect HBsAg, the 5 mm wells and subsequent staining lend greater sensitivity to the test. The other differences listed in Table 1 do not seem to affect the sensitivity in any significant way.

Similiar results were reported by Kissling and Barker (15). They, too, found that variations in buffers, pH, volume of sera employed or amperage applied, did not seem to influence results. They did observe, however, that the selection of antiserum can greatly affect the sensitivity of IEOP procedures.

It can be concluded, then, that the higher rate of IEOP positive reactions obtained at SMRL is influenced by both the prevalence of Hepatitis B antigen and antibody within the population and the sensitivity of the test methods. Future IEOP tests at SMRL will continue to use the existing procedure.

2. The Radioimmune Assay Inhibition Test for the Detection of Antibody to Hepatitis B Antigen

The objective of these studies was to develop methods of detecting, quantifying and identifying the specificity of antibody to hepatitis B antigen (HBsAg).

BACKGROUND: The detection of antibody to hepatitis B surface component (anti-HBs) has been a problem in Thailand as well as elsewhere because of inability of standard serological tests e.g., complement fixation (CF), to detect small amounts of antibody. The application of the immunoelectroosmophoresis (IEOP) test, allowed for the detection of antibody in approximately 10% of urban Thais and was used to determine the development of antibody in recipients of blood containing HBsAg; however, the patterns of antibody acquisition revealed by this detection method pointed to secondary rather than primary antibody responses. Individuals suffering from acute hepatitis B were not found to develop anti-HBs detectable by IEOP following infection. The development of a passive hemagglutination test (PHA) allowed a check on the validity of the IEOP antibody test.

Table 1 demonstrates the increased sensitivity of PHA over IEOP. The lesser sensitivity of the IEOP method and the difficulty of manufacturing and transporting sensitized red blood cells for the PHA method led us to investigate other sensitive methods for antibody determination.

DESCRIPTION: Utilizing a radioimmune assay (RIA) developed and sold by Abbott Laboratories (Ausria kit), and additional step was added to allow for the identification of anti-HBs. This procedure was

Table 1. Comparison of IEOP and PHA for Detecting Anti-HBs in Sera Collected in Huay Kwang 1972

PHA Reciprocal Titer*	No. Sera Tested	IEOP Positive No.	(%)
128	495	0	(0)
256	32	1	(3.1)
512	46	3	(6.5)
1024	30	9	(30.0)
2048	40	30	(75.0)

*PHA tests were performed by the American National Red Cross Blood Research Laboratory, Bethesda, Md.

called radioimmune assay inhibition (RIAI). For each antigen subtype to be used a serum was selected and a RIA extinction curve was performed using normal human serum as a diluent. A dilution of serum containing HBsAg was selected for use in the RIAI. Two-tenths (0.2) ml of the appropriate antigen dilution was incubated for 2 hours at 37°C with 0.2 ml of the serum sample to be tested for antibody activity. One-tenth ml of the mixture was then placed in the bottom of each of two plastic tubes coated with guinea pig anti-HBs. The tubes were stoppered and incubated at room temperature for 16-18 hours overnight. The samples were then removed and the tube rinsed 5 times with 2 ml each of 0.01M Tris-HCL, pH 7.4. One-tenth ml of the ^{125}I-labeled guinea pig anti-HBs solution (approximately 0.1 μCi) was then delivered to the bottom of each tube. The tube was capped and incubated at room temperature for 90 minutes, then aspirated and rinsed 5 times as above.

Seven negative control tubes were prepared using a standard HBsAg negative control provided by Abbott Laboratories. Seven positive control tubes were run for the dilution of HBsAg positive serum used in the RIAI. Each tube was counted in a gamma ray spectrophotometer and the mean counts per minute (CPM) and standard deviation of the positive and negative control tubes were calculated. The percent inhibition of the RIA (% RIAI) of sample sera was computed using the formula:

$$\frac{(MPD - MNC) - ([PD + Sample] - MNC)}{MPD - MNC} \times 100 = \%\ R\ I\ A\ I$$

Where M indicates the mean counts per minute, NC indicates the CMP for the negative control and PD indicates the CPM for the dilution of HBsAg positive serum used in the RIAI.

PROGRESS: Radioimmune assays were run on dilutions of sera containing the three major subtypes of HBsAg; adr, adw and ayw. Figure 1 illustrates dilution extinction curves on three such sera. Dilutions containing approximately 50% of the CPM of the highest counting dilution were used as the antigen for the RIAI, as small changes in antigen concentration should lead to large changes in the amount of ^{125}I-labeled antibody complexed to the antigen. This should be reflected in large changes in the number of counts per minute.

Initial testing of the RIAI method was performed using dilutions of sera known to contain high titers of anti-HBs. These sera were obtained from multiply transfused individuals with complement fixation titers for anti-HBs. Figure 2 shows the percent inhibition of the radioimmune assay of one such serum with a CF titer of 1:8.

RIAI was run on 57 sera obtained from 11 Thai individuals recovering from HbsAg positive hepatitis using an adr antigen, the predominant subtype in Thailand. Of these 11 individuals, antibody capable of inhibiting the radioimmune assay by greater than 50% developed in 10. Thus the RIAI was capable of detecting antibody following acute hepatitis B infection.

A panel of sera, pretested by passive hemagglutination by the American National Red Cross, Blood Research Laboratory (Bethesda, Maryland, USA) were selected for analysis by RIAI. Since the PHA was run using adw antigen coated cells, adw antigen was also used in the experiment pictured (See Figure 3). A scatter diagram of the PHA titers versus the RIAI demonstrates a clear correlation between these two techniques. All of the sera with titers of antibody by PHA of 1:64 or more inhibited the RIA by greater than 50%. Those with titers of less than 1:64 by PHA showed considerable scatter, with the majority of sera inhibiting the RIA by less than 50%. Sera considered to have no antibody by PHA also showed considerable scatter, with RIAI ranging from 80% to 0%. The majority (9/12) of these clustered below 30% RIAI but two were greater than 50%. Repetition of this test using the same antigen and panel of sera showed some lack of reproducibility with those sera having PHA antibody titers less than or equal to 1:64. Similar experiments using other sera tested by PHA also showed variability of RIAI inhibition if the PHA titer was less than 1:64.

With sera showing a high % RIAI it was sometimes possible to demonstrate a difference in the antibody titer to different subtypes.

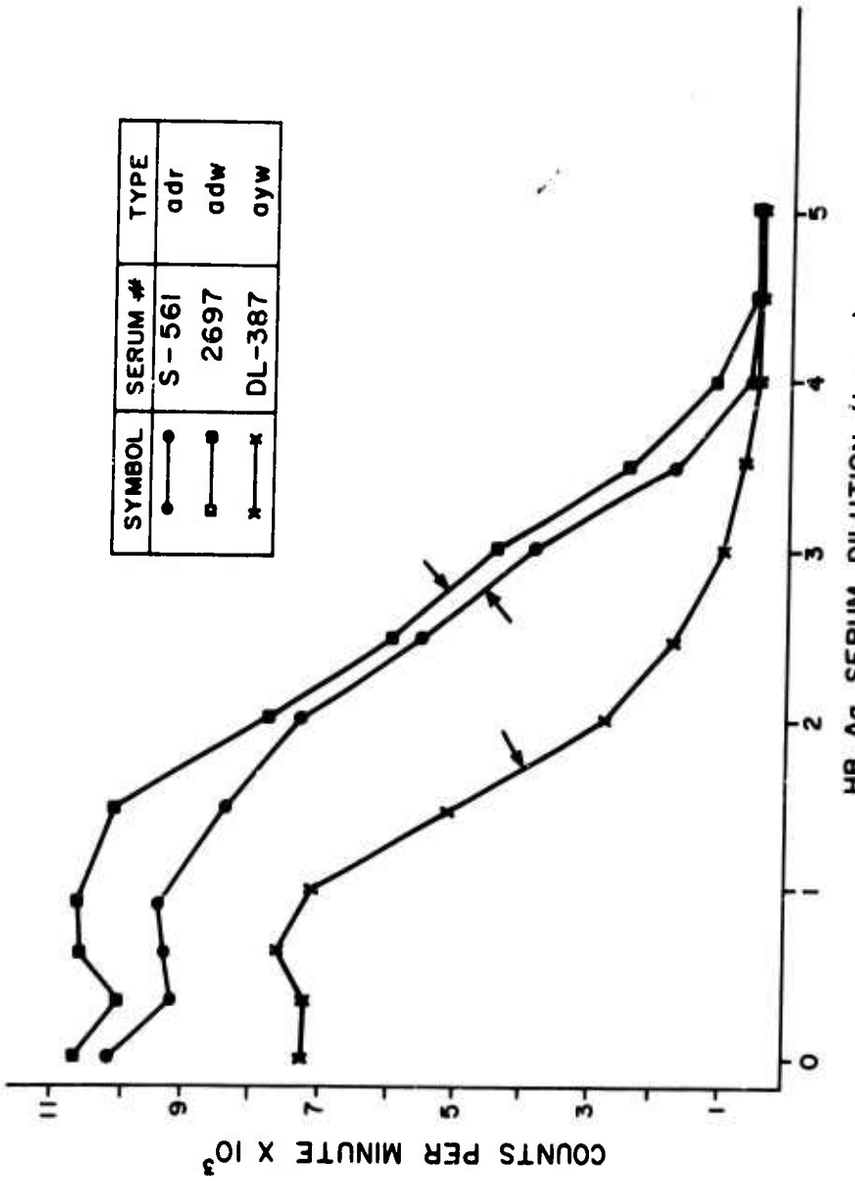


Figure 1. Dilution extinction curves of three sera containing HB_sAg of different subtypes tested by radioimmune assay. Each point represents the mean value of duplicate determinations. Arrows indicate the antigen dilutions used in RIAI tested for detecting HB_sAg; adr (1:512), adw (1:512) and ayw (1:64).

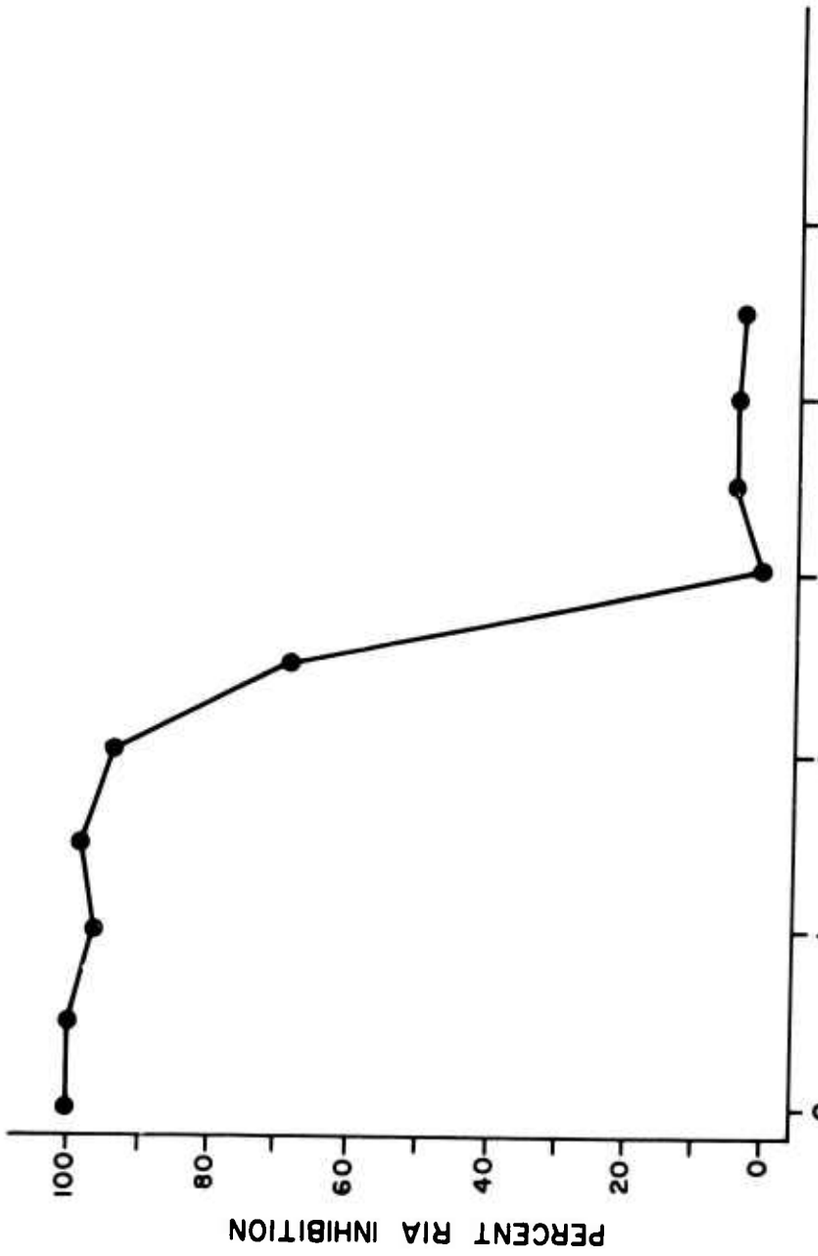


Figure 2. Radioimmune assay inhibition of a constant amount of HB_sAg/adr (S-361) by serial dilutions of a human serum containing anti-HB_s. The serum (P.T.) had antibody titers of 1:8 by CF and 1:300 ($10^{-2.5}$) by RIAI.

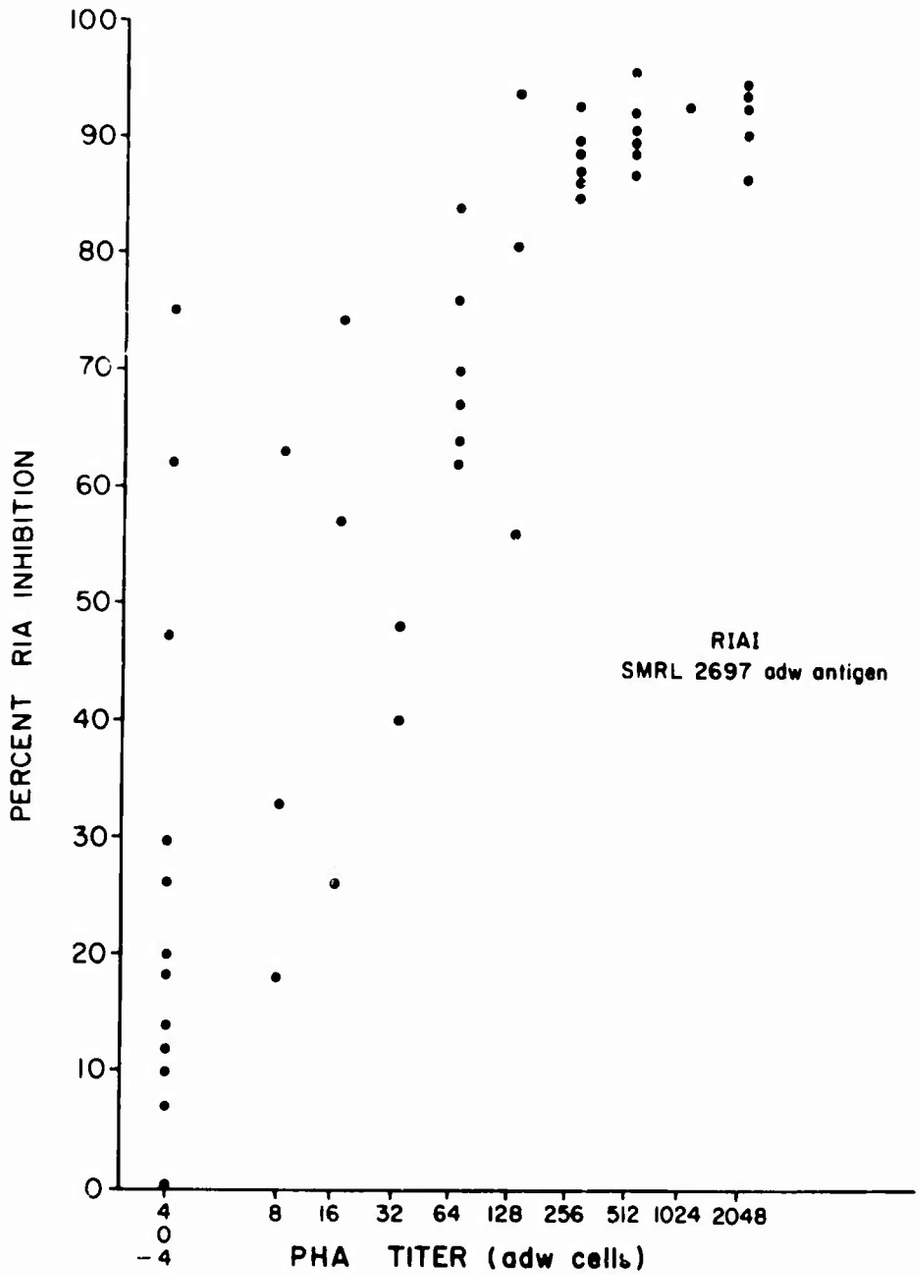


FIGURE 3. PASSIVE HEMAGGLUTINATION TITERS PLOTTED AGAINST PERCENT RADIOIMMUNE ASSAY INHIBITION.

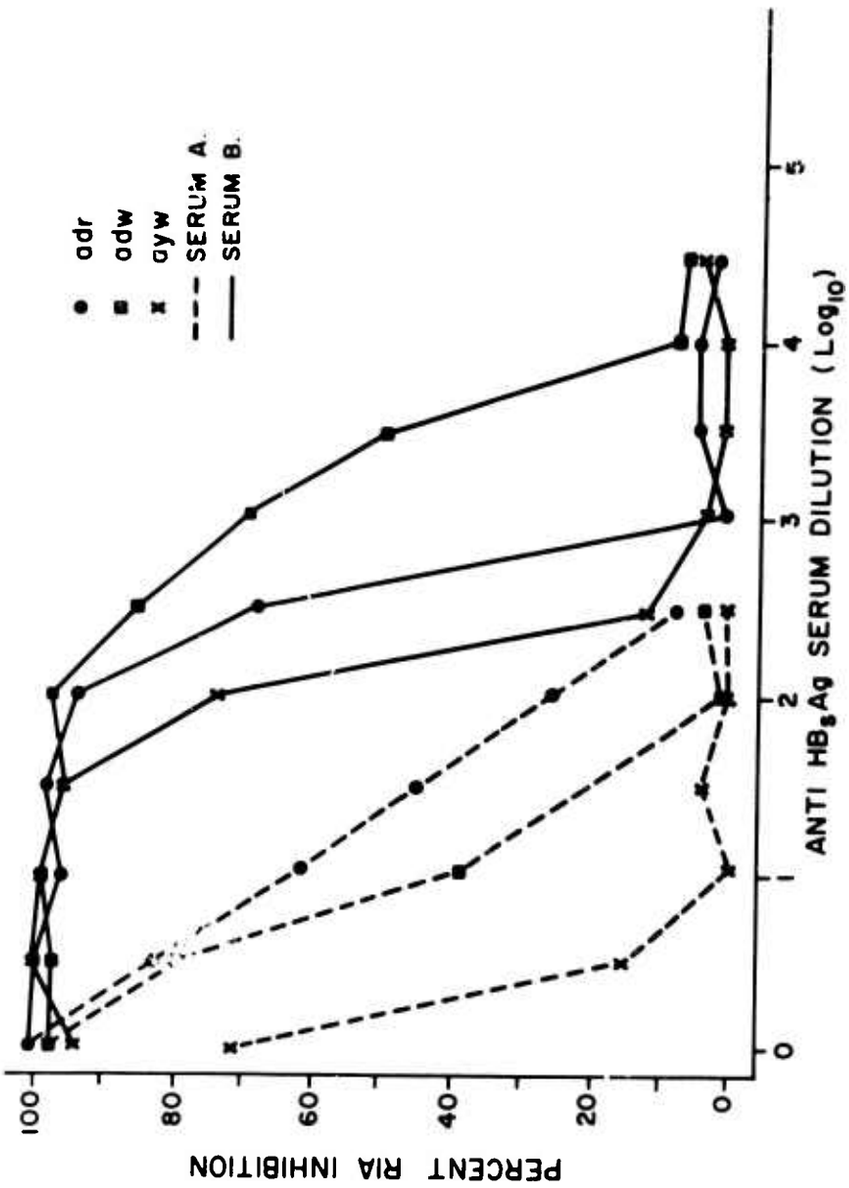


Figure 4. Radioimmune assay inhibition dilution curves on two sera showing differences in titer to three different subtypes of HB$_s$Ag (adr, adw, ayw). Serum A was obtained from a patient approximately 18 months after a documented clinical infection with HB$_s$Ag/adr. Serum B was obtained from a blood recipient following transfusion with at least one unit of HB$_s$Ag/adw positive blood.

Figure 4 shows the RIAI results of two such sera, run simultaneously with each of the three antigens. Serum (A) was obtained from an individual from whom HBsAg/adr was isolated during an episode of hepatitis approximately one year prior to the date the serum was obtained. Serum (B) was obtained from a hospitalized patient who received multiple transfusions. At least one transfusion prior to the time blood was obtained was shown to contain HBsAg/adw. Serum (A) inhibited 50% of the adr antigen activity as indicated by the CPM at a dilution three times that of adw. Serum (B) appeared to contain a higher titer of antibody against adw than to adr, showing a ten-fold difference in titer at the 50% level.

DISCUSSION: In our hands RIAI appears to be sensitive, specific and reproducible in cases where large amounts of anti-HBs are present. In cases of multiply transfused individuals with CF titers or in cases with recent hepatitis B, RIAI is capable of detecting antibody. Furthermore, RIAI is from 40-50 times more sensitive than IEOP.

Unfortunately, RIAI was unable to demonstrate antibody in some sera that titered less than or equal to 1:64 by PHA. The RIAI, however, was often repeatable, suggesting the possibility of false negatives and positives by the PHA method.

RIAI, when run using different antigen positive sera, appears to be capable of identifying the subtype of the antigen with which an individual was infected.

Methods of improving the RIAI sensitivity are being tried. Other procedures for the detection of anti-HBs are being sought.

3. Hepatitis B Antigen in Laboratory Reared Mosquitoes

OBJECTIVE: To determine the duration of carriage of Hepatitis B antigen (HBsAg) by laboratory reared mosquitoes fed on HBsAg carriers.

BACKGROUND: It has been suggested that the high prevalence of HBsAg carriers and anti-HBsAg in tropical areas may be due to the presence of biting insects capable of carrying and transmitting Hepatitis B virus (HBV). Information accumulating over the past several years regarding the ability of insects to act as HBV vectors has been contradictory. The presence of HBsAg has been reported in eight species of wild caught mosquitoes engorged with human blood; however, in unengorged mosquitoes no antigen was detected (16). Conflicting data has been presented by other investigators which suggest that HBsAg does presist in certain mosquito species (Aedes aegypti) for

up to six weeks following an antigen positive blood meal (17). Further, one mosquito species (Culex quinquefasciatus) has been reported by a third investigator to develop antigen in the salivary glands approximately three weeks after feeding on HBsAg positive blood (18). With an insectary capable of raising several genera of mosquitoes, with a number of HBsAg carriers available and with a sensitive radioimmune assay (RIA) to test for antigen, it was felt that the question of persistence and reappearance of HBsAg in laboratory reared mosquitoes could be approached.

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 HbsAg/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-forzen 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 cage at varying times after feeding, quick-frozen and stored at -70°C.

All mosquitoes were tested by RIA ("Ausria" kit, Abbott Laboratories) simultaneously for each mosquito species. Pools of 10 mosquitoes were titurated in 0.5 ml of 0.01M 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. Dilutions of serum of a HBsAg positive volunteer were run simultaneously by RIA to determine the concentration at which antigen could no longer be detected.

PROGRESS: At the time of this writing, Aedes aegypti, Armigeres subalbatus, Anopheles minimus and Culex quinquefasciatus have been tested in the above manner (Figure 1). All unengorged mosquito controls fell within 1 standard deviation of the mean of the negative controls run in the RIA. The first sample of the Aedes and Armigeres species were taken immediately after feeding. The initial sample of the Anopheles and the Culex species were taken 15 hours after feeding. Mosquito pools obtained within 24 hours of feeding show the presence of antigen in all four species. Antigen, as determined by the Ausria test, reached negative levels by 24-48 hours after feeding. In species which could be sampled as long as 15-20 days following feeding, antigen levels did not increase but remained within the limits of unfed mosquitoes. Unfortunately, because of the biting habits of Culex quinquefasciatus, engorged mosquitoes were obtained for only two samples; however, in this species, too, a fall of

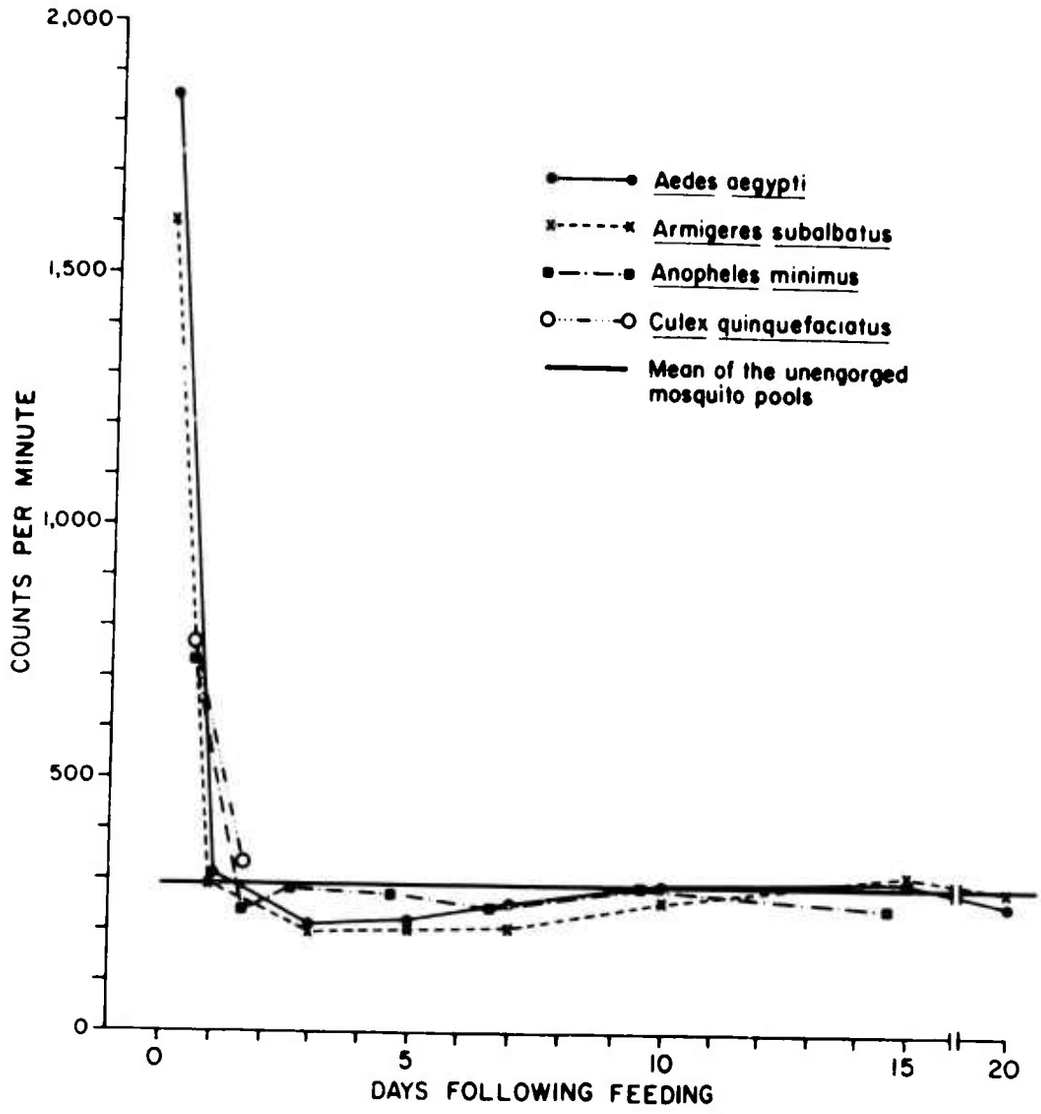


Figure 1. Radioimmune assay for HBsAg on pools of 10 mosquitoes collected following feeding on an HBsAg positive volunteer.

antigen was noted within the first 36 hours. Negative levels were not reached and this species will be re-tested and followed for the full course of the experiment.

The serum dilution curve of the Hepatitis B antigen positive volunteer demonstrated that antigen was no longer detectable at a dilution of 1:30,000. The range of counts per minute seen in mosquito pools would indicate that the pools of 10 mosquitoes contained an amount of antigen equivalent to a 1:1,000 dilution of serum.

DISCUSSION: The Ausria test (Abbott Laboratories) probably is only sensitive for the surface antigen of Hepatitis B virus (HBsAg). There is no evidence that we know of that it has any reactivity with core antigen of HBV. These data indicate that the disappearance of HBsAg from the mosquito pools parallels the digestion and elimination of the blood meal. In the three mosquito species followed for 15-20 days there was no reappearance of HBsAg; however, the presence of antigen in mosquitoes for 24-48 hours may allow them to serve as mechanical vectors if they refeed within this period of time.

It is unfortunate that there were insufficient engorged Culex quinquefasciatus to follow for the full course of 20 days. Studies on this species as well as Aedes albopictus, Anopheles balabacensis, Anopheles maculatus remain to be completed. If, however, the antigen appears to dissipate in all species tested, the question of infection of the mosquito by HBV, with only the production of core antigen, remains a possibility and might provide an explanation of the reappearance of hepatitis B antigen in the salivary glands of Culex quinquefasciatus reported by others. Ways to investigate this question are being determined at present.

4. Anti-Hepatitis B Serum Production in Laboratory Animals

An ability to detect HBsAg subtypes is essential for studies of the transmission and clinical expression of Hepatitis B virus (HBV). The immunodiffusion (ID) method (19) is the most specific method for detecting differences in the antigenic subtypes of HBV. Antigen subtypes are determined by comparing the precipitin lines and spurs of test sera to those of reference antigens after they react with anti-HBs sera. Antisera that react with the subtype specific surface determinants are necessary for this test.

This paper describes the methods we employ to produce antisera to Hepatitis B antigen (HBsAg) to use for the determination of antigen subtypes in immunodiffusion test.

DESCRIPTION: Rabbits of 2.5 to 4.0 kilogram body weight were used to produce antiserum. These rabbits were bled prior to immunization to test for anti-HBs activity by immunoelectroosmophoresis (IEOP) and radioimmune assay (RIA).

Cesium chloride purified selected sucrose fractions of HBsAg/adr, adw and ayw subtypes were used as immunizing antigens. The antigens were prepared by Electronucleonics, Inc. (Bethesda, Maryland) from human plasma previously tested at Walter Reed Army Institute of Research (WRAIR). Each antigen was diluted 1:2 with Freund's complete adjuvant (Difco Laboratory) and thoroughly emulsified with a Vortex super mixer (20).

Five or six rabbits free of anti-HBs were immunized for each subtype of antigen. Each rabbit was inoculated with 0.25 ml of antigen-adjuvant emulsion intradermally into each of 4 sites on the thighs and back. An identical dose of the same antigen was given four weeks later. Test bleeding was done six weeks after the first dose of antigen. Sera were tested for the presence of anti-HBs by IEOP and ID. Rabbits whose sera gave subtype specific precipitin lines were exanguinated. Rabbits with sera that formed weak precipitin lines were boosted with another identical dose of antigen and bled out two weeks later. All of the rabbit antisera were tested again by ID and complement fixation (CF) to determine the anti-HBs titer. Each rabbit serum was also tested for antibody to normal human serum proteins.

PROGRESS: Of five rabbits immunized with HBsAg/adw (EH-016), three produced sera that gave specific reactions with reference antigens (Table 1 and Figure 1). Two other rabbit sera formed definite precipitin lines with reference antigens but weak spurs were observed, suggesting they contained little subtype specific antibody. The anti-HBs CF titers varied from 1:512 to 1:1024.

Of six rabbits immunized with HBsAg/adr (EH-017), only three showed sharp precipitin lines and spurs with reference antigens (Table 1 and Figure 1). In addition, R 07 and R 14 contained anti-normal human serum reactions in low CF titer ($\leq$ 1:2).

Among the anti-ayw rabbit sera, two formed strong precipitin lines and spurs with reference antigens. The anti-HBs CF titers were 1:128 to 1:1024. Unfortunately, all of the anti-ayw rabbit sera contained anti-human serum reactivity.

DISCUSSION: The method of immunization of HBsAg in rabbits proved to be very satisfactory for the production of subtype specific

Table 1. Production of Anti-HBs in Rabbits

Rabbit No.	Immunizing Antigen	Anti-HBs			Anti-normal human serum	
		Immunodiffusion				
		Spur formation	Optimum dilution	CF Titer	ID	CF
R 01	adw EH-016 Fraction 16	Strong	1:2	1:512	Neg**	<1:2
R 02		Strong	1:2	1:512	Neg	<1:2
R 03		Strong	1:2	1:1024	Neg	<1:2
R 04		Weak	Und.*	1:512	Neg	<1:2
R 05		Weak	1:2	1:1024	Neg	<1:2
R 07	adr EH-017 Fraction 16	Strong	Und.	1:128	Neg	<1:2
R 08		Weak	Und.	1:64	Neg	<1:2
R 09		Medium	Und.	1:128	Neg	<1:2
R 13		Weak	Und.	1:128	Neg	<1:2
R 14		Strong	1:2	1:1024	Pos***	<1:2
R 15		Weak	Und.	1:128	Neg	<1:2
R 10	ayw EA-047 Fraction 10 - 14 pooled	None	-	1:1024	Pos	1:2
R 11		Weak	-	1:256	Pos	1:2
R 12		Weak	-	1:128	Pos	1:2
R 16		Medium	1:2	1:128	Pos	<1:2
R 17		Strong	1:2	1:128	Pos	1:4

* Und. = Undiluted

** Neg = Negative

*** Pos = Positive

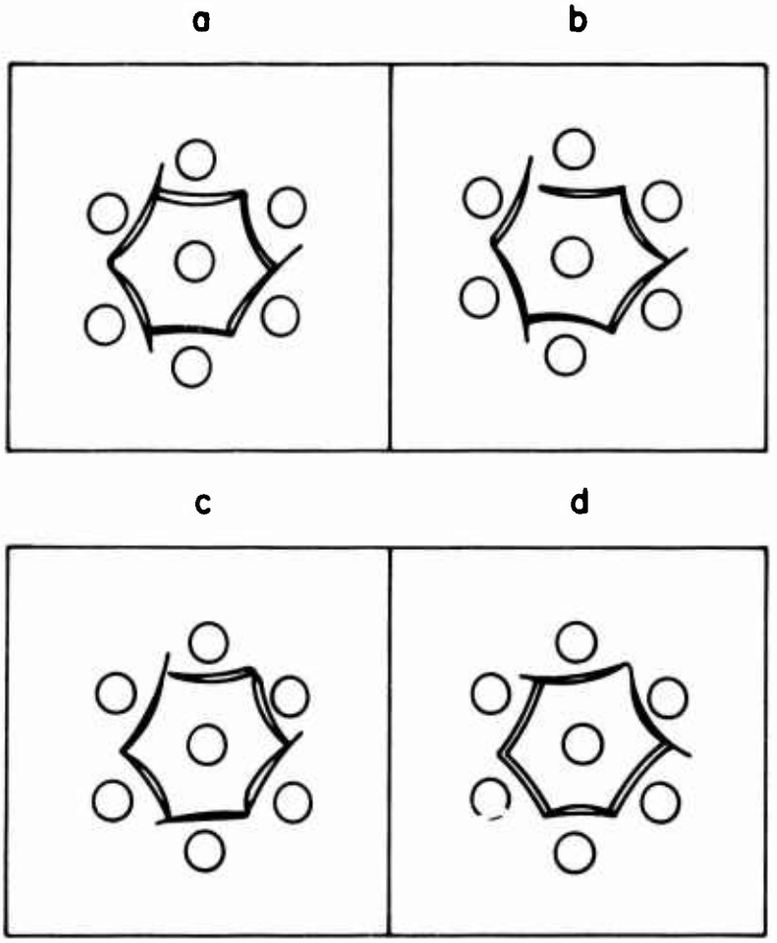


Figure 1. Patterns of immunodiffusion reactions observed with reference HBsAg. In each pattern, reference HBsAg/ayw were placed in the top and right upper wells, HBsAg/adr in right lower and bottom wells and HBsAg/adw in left upper and left lower wells. The central wells contain: a) R 01 anti-adw (1:2); b) R 02 anti-adw (1:2); c) R 09 anti-adr (undiluted); and d) R 17 anti-ayw (diluted 1:2 with normal human serum).

antisera. The reactivity of HBsAg and normal serum protein were different among those rabbits. The anti-adw sera with no anti-human serum response indicated that the antigen used, HBsAg/adw (EH-016), was essentially free of normal serum protein. The adw antiserum produced sharper precipitin lines and spurs with reference antigens than either adr or ayw antisera. The anti-d reactivity of both the adr and adw antisera was stronger than the anti-w and anti-r (Figure 1). Also the production of antibody to normal serum proteins by two rabbits immunized with HBsAg/adr and all rabbits immunized with HBsAg/ayw indicated that those antigens were incompletely purified. Nevertheless, these antisera could be absorbed sufficiently with normal human serum to allow their use for subtyping by ID.

5. Subtypes of Hepatitis B Antigen in Southeast Asia

Hepatitis B surface antigen (HBsAg) has specific antigenic determinants which permit the differentiation of subtypes by immunologic techniques. An attempt was made to determine the subtype of as many antigens as possible detected by this laboratory. New abbreviations for designating antigen subtypes have been adopted as recommended by the U.S. National Academy of Sciences (21). Hepatitis B surface antigen is referred to as HBsAg and antibody as anti-HBs.

This is a continuing study to determine the frequency of each hepatitis B antigen subtype in different study populations.

DESCRIPTION: The method for determining subtypes by immunodiffusion using standard reference antigens and hyperimmune rabbit antisera has been previously described (1).

The following study populations have been sampled and tested:

a. Thai and American patients with acute viral hepatitis based on clinical symptoms, signs and serum biochemical tests of liver function.

b. Thai professional blood donors living in Metropolitan Bangkok.

c. Pregnant Thai women at the time of delivery.

d. Randomly selected residents of an urban low socioeconomic housing area in a Bangkok subdivision (Huay Khwang).

e. Residents of a rural and semi-rural area of Northern Thailand (Chiang Mai province).

f. Young Thai female companions (service girls) of foreign military personnel in South Central Thailand. Most of the girls reported having had intimate contact with Americans.

g. American military personnel entering and leaving the Republic of Vietnam. This group included people with limited or no prior exposure to the intrinsic infections of Southeast Asia.

h. Vietnamese blood donors kindly provided by Mr. William S. Adams, Public Health Advisor, United States Consulate General, Danang.

PROGRESS:

The prevalence of HBsAg in Thai and non-Thai people

Table 1 shows the prevalence of HBsAg in each study population. Among asymptomatic Thai people, the prevalence of HBsAg was higher in professional blood donors than in service girls or pregnant women. Members of the randomly collected urban Thai population had a prevalence of HBsAg almost twice that of the rural population studied. The Vietnamese blood donors showed a higher prevalence of HBsAg than the Thai blood donors; however, the number tested was very small. A very low prevalence of HBsAg was observed in asymptomatic Americans entering and leaving the Republic of Vietnam. 53% of Thai and 39% of American cases of acute hepatitis were associated with HBsAg. The prevalence of HBsAg in cases of acute hepatitis was not significantly different between Thais and Americans ($CHI^2 = 5.0$; $P<0.5$).

The distribution of HBsAg subtypes in people of different nationality

Table 2 shows the results of subtyping of HBsAg found in Thais, Vietnamese and Americans in Southeast Asia. In the Southeast Asian populations, the d determinant was predominant. Among the Thais, the ratio of HBsAg/adr to HBsAg/adw (approximately 8:1) was quite similar in all groups of asymptomatic carriers with one exception. In the rural sample from Chiang Mai, the w determinant was not found. No correlation was observed between socioeconomic status and subtypes of HBsAg. The y determinant was not found in any Thai population sampled, not even in the service girls who had the closest contact with potential y carriers (U.S. military personnel). In the small sample of Vietnamese blood donors studied, only the HBsAg/adr subtype was found. On the other hand, both d and y determinants were found in American servicemen. A different distribution of subtypes was also found in persons of different nationality with acute hepatitis; Thais had only adr while Americans had adr, adw, and ayw.

DISCUSSION: These data suggest that d is the predominant subtype determinant in Thailand. The y determinant was not found on any antigen obtained from Thais and Vietnamese. The third determinant

Table 1. Prevalence of HBsAg in Various Populations Studied in Southeast Asia

Nationality	Study Population	No.	HBsAg Positive		Antigen subtyped			
			No.	(%)	No. tested	%	No. subtyped	%
Thai	Acute Hepatitis	113	60	(53.1)	60	(100)	24	(40)
	Blood Donors	8801	958	(10.9)	101	(10.5)	80	(79.2)
	Pregnant Women	1625	93	(5.7)	51	(54.8)	42	(82.3)
	Service girls	681	51	(7.5)	45	(88.2)	43	(95.5)
	Urban (Bangkok)	695	59	(8.5)	59	(100)	48	(81.3)
	Rural (Chiang Mai)	606	28	(4.6)	23	(82.1)	20	(86.9)
Vietnamese	Blood Donors	35	5	(14.3)	5	(100)	3	(60)
American	Acute Hepatitis	174	68	(39.1)	60	(88.2)	40	(66.6)
	Entering Vietnam	1293	11	(0.85)	11	(100)	10	(90)
	Leaving Vietnam	1072	5	(0.47)	5	(100)	4	(80)

Table 2. Distribution of HBsAg Subtypes in Populations Studied in Southeast Asia

Nationality	Study Population	Antigens Subtyped	Subtype Frequency (%)				
			adr	adw	ad	ayw	other
Thai	Acute Hepatitis	24	92	0	8	0	0
	Blood Donors	80	81	9	10	0	0
	Pregnant Women	42	71	10	19	0	0
	Service girls	43	81	9	9	0	0
	Urban (Bangkok)	48	81	13	6	0	0
	Rural (Chiang Mai)	20	95	0	5	0	0
Vietnamese	Blood Donors	3	67	0	33	0	0
American	Acute Hepatitis	40	20	5	52	23	0
	Entering Vietnam	10	30	30	30	10	0
	Leaving Vietnam	4	25	0	25	25	25*

* Unconfirmed adyw

was r in approximately 80% and w in approximately 10% of antigens, but could not be detected in the remainder.

This study suggests that clinical disease is not related to subtype but is caused by the organism prevailing in the population studied. The absence of the y determinant in all Thais and the w determinant in Northern Thais supports earlier observations that the distribution of subtypes fits into geographic patterns, with ad being the predominant combination in the Far East. For residents of Southeast Asia, determinants w and r are more useful epidemiologic markers than y and d.

6. The Epidemiology of Hepatitis B in a Defined Urban Thai Population: Longitudinal Epidemiological Data

We have followed the changes in hepatitis B antigen (HBsAg) and antibody (anti-HBsAg) in an urban Thai population over a nine month period. For a description of the experimental design and preliminary data of this study see SEATO Medical Research Laboratory Annual Report 1971-1972 and 1972-1973. Samples and information collected from this urban Thai population allowed for determination of the incidence and loss of evidence of hepatitis B virus (HBV) infection between July 1971 and April 1972.

PROGRESS: There were 523 people age one year and older whose paired (July 1971 - April 1972) sera were tested and confirmed by radioimmunoassay (RIA) for HBsAg and by passive hemagglutination (PHA) for anti-HBsAg. The percentage distributions by age and sex of these 523 persons were very similar to the total 683 persons age one year and older in the random sample who were present throughout the nine-month period. Thirty-six (92%) of the 39 people positive for HBsAg in July 1971 remained positive over the nine month period (Table 1). All three who became negative for HBsAg, however, became positive for anti-HBsAg. Four of the 484 people (0.8%) negative for HBsAg in July 1971 (Table 1) had acquired HBsAg by April 1972. Anti-HBsAg titers were negative in both sera of these four individuals.

In terms of antibody (Table 1), 219 (92%) of the 238 persons positive for anti-HBsAg in July 1971 were still positive in April 1972. The 19 anti-HBsAg reverters included 8 males and 11 females. Thirty-one (10.9%) of the 285 persons negative in July 1971 became positive for anti-HBsAg during the interval. Six persons were positive for both HbsAg and anti-HBsAg on one or both collection times.

The paired PHA anti-HBsAg data revealed that, of the 238 people positive for anti-HBsAg in July 1971 (Table 1), 132 (55.5%) had no significant change in titer (less than a four-fold difference) over

Table 1. HBsAg and Anti-HBsAg Results of Sampled Persons in July 1971 and April 1972*

HBsAg		Anti-HBsAg								Total
Jul 71	Apr 72	Jul 71	Apr 72	Jul 71	Apr 72	Jul 71	Apr 72	Jul 71	Apr 72	
		+	+	+	-	-	+	-	-	
+	+	4		2		0		30		36
+	-	0		0		3		0		3
-	+	0		0		0		4		4
-	-	215		17		28		220		480
Total		219		19		31		254		523

* Persons whose paired sera were tested by RIA for HBsAg and PHA for anti-HBsAg.

Table 2. Prevalence of Hepatitis Virus (HBV) Infection in July 1971 and Incidence and Loss of Evidence of HBV Infection between July 1971 and April 1972, by age

Age (years)	No. paired sera tested	HBV prevalence[1] July 1971		HBV incidence[2] July 1971-April 1972		HBV titer loss[3] July 71-April 72	
		No.	%	No.	%	No.	%
1-4	33	7	21.2	6	23.1	2	28.6
5-9	89	28	31.5	6	9.8	2	7.2
10-14	104	44	42.3	6	10.0	4	9.1
15-19	68	41	60.3	5	18.5	1	2.4
20-29	64	40	62.5	3	12.5	2	5.0
30-39	66	44	66.7	3	13.6	1	2.3
40-	99	67	67.7	3	9.4	5	7.5
Total	523	271	51.8	32	12.7	17	6.2

1 Positive for HBsAg, for anti-HBsAg, or for both

2 Acquisition of either HBsAg or anti-HBsAg in persons negative for both in July 1971

3 Reversion to negative of HBsAg (or anti-HBsAg) without acquisition or continued positivity of anti-HBsAg (or HBsAg)

the ensuing nine months; 67 (28.2%) had a significant titer rise and 39 (16.4%) had a significant titer drop, the latter including the 19 people who reverted to negative (Table 1). If a significant titer rise can be taken to mean a re-exposure to HBV, then it is conceivable that about 20% (102 people) in this sample were exposed to HBV during this period. This percentage resulted from adding the 67 persons above with significant PHA titer rises to the 31 persons converting to anti-HBsAg and the 4 persons converting to HBsAg during this period of observation.

Considering the presence of either HBsAg or anti-HBsAg to be evidence of prior exposure to HBV, antigen and antibody results were combined for these 523 people (Table 2). Over 20% of the children age one to four showed serologic evidence of HBV exposure, with HBV prevalence rapidly rising to over 60% by age 15 and remaining fairly constant in the adult years. Only 39% (220 people) were negative for HBsAg and anti-HBsAg on both dates. Acquisition and loss of evidence of infection over the nine months occurred at every age, but is probably seen to the greatest extent under the age of 5 years. The data in Table 2 suggested that it would be appropriate to try to fit a reversible catalytic curve to the age-prevalence data (1). This is displayed in Figure 1. The HBV prevalence results of Table 2 are shown as a histogram. Superimposed on this is the curve fitted to these data using a reversible catalytic model. The fit of this model to the observed data is very good except for somewhat underestimating the prevalence under age 5. (It should be noted that the age scale has been shifted one year to the left in Figure 1 so that prevalence could be estimated from birth). The equation of the fitted curve provides 2 constants: the first, 0.7024, or 70%, is the estimated peak (asymptotic) prevalence attainable under the reversible conditions; the second, 0.099, is a combination of two constants of infection, 0.070 or 7.0% average annual rate of acquisition plus 0.029 or 2.9% average annual rate of loss of evidence of HBV infection. The observed results (Table 2) show a larger gain and loss of evidence of infection over the 9-month observation period than predicted by the model; however, the rates of acquisition and loss estimated by the model express the average of annual rates over the years.

DISCUSSION: The most important result of this study is the evidence that clinically inapparent infection by HBV is a very common occurrence in this population (Table 2). Infections occurred in every age group during the study and between 60 and 70 per cent of subjects age 15 or over showed serological evidence of having been infected at least once in the past. The data suggested that 20% of the population were exposed to HBV during the nine months of observation. Of these, two-thirds showed evidence of re-exposure. Therefore, it is probable that nearly everyone in this community will be infected at least once, if not many times, during his or her lifetime. Further

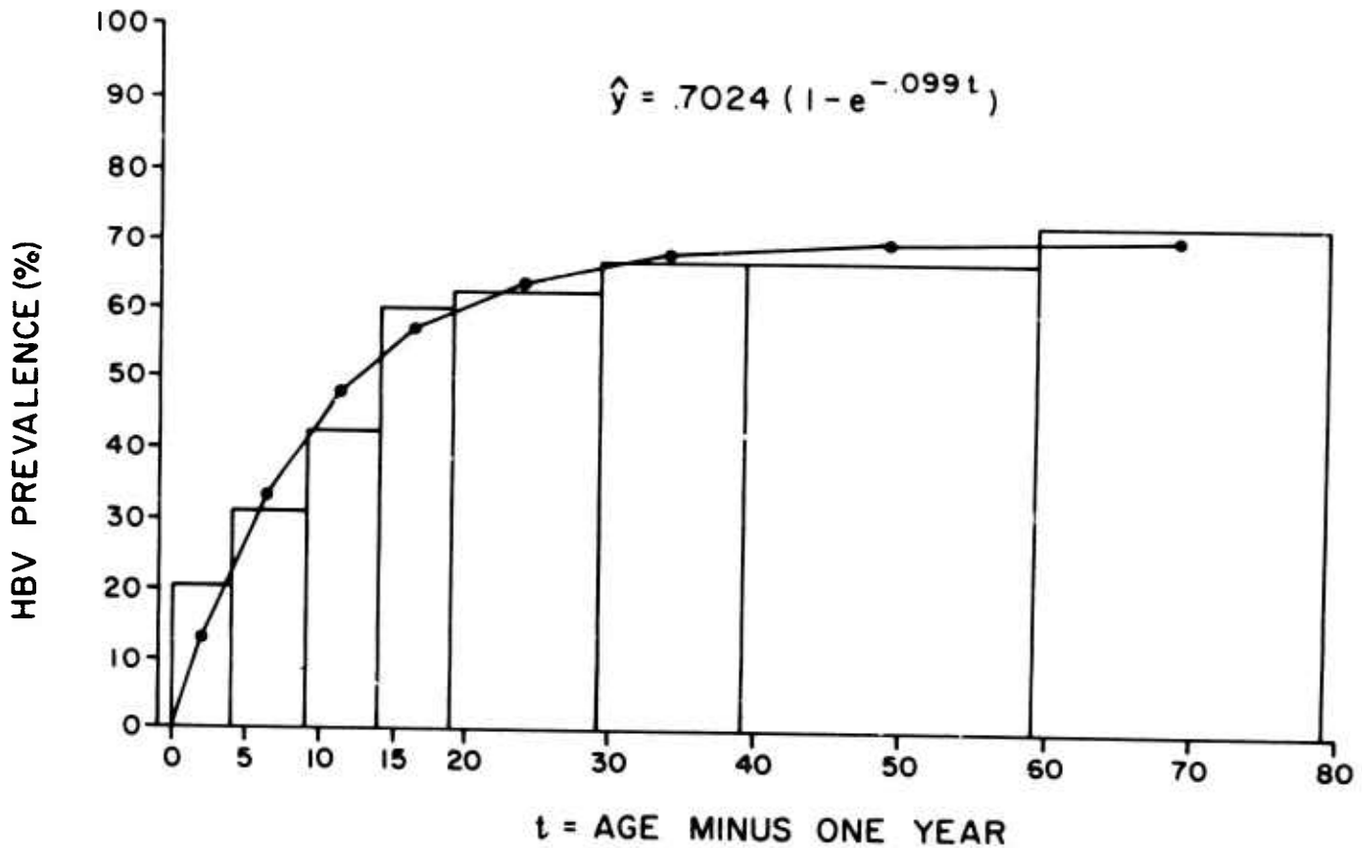


FIGURE 1. HBV PREVALENCE BY AGE (JULY 1971). OBSERVED PREVALENCE PROPORTIONS ARE SHOWN BY THE RELATIVE FREQUENCY HISTOGRAM. THESE DATA WERE FITTED WITH A REVERSIBLE CATALYTIC CURVE (1) WHICH IS SHOWN BY THE SUPERIMPOSED CUMULATIVE RELATIVE FREQUENCY POLYGON, (WITH THE EQUATION ABOVE THE CURVE). NOTE THAT THE TIME SCALE HAS BEEN SHIFTED ONE YEAR TO THE LEFT SO THAT THE EXPERIENCE WITH HBV WOULD BE ESTIMATED FROM BIRTH. (SEE TEXT)

attesting to the widespread exposure and susceptibility to HBV is the finding that nearly every sampled family throughout the study area had at least one member positive for HBsAg or anti-HBsAg. Thus, it may be assumed that the whole population is exposed to HBV from earliest childhood and that susceptibility to infection is universal. Since people of all ages lost serological evidence of HBV infection(s) during the study period, it may also be assumed that reinfections (with or without intervening loss of serologic evidence of infection) are common events. The excellent agreement with the reversible catalytic curve fitted to the July 1971 HBV prevalence data (Figure 1), which requires these same assumptions of universal exposure and reversible susceptibility, further support the conclusion of continuous or at least frequent exposure of all ages to HBV. Because there is also frequent loss of serologic evidence of infection, HBV prevalence by age understandably cannot ever reach one hundred per cent. Indeed, in Huay Khwang, HBV prevalence reaches a plateau in adults close to the 70% maximum predicted by the catalytic model.

7. An Epidemiological Study of Hepatitis B Virus Infection in Bangkok, Thailand

The objective of this study was to determine the persistance, acquisition and loss of hepatitis B antigen and antibody in an urban Thai population. This is a continuation report of the Huay Khwang hepatitis B virus study previously described in the SEATO Medical Research Laboratory Annual Progress Report 1971-1972 pp. 129-139, and 1972-1973 pp. 68-69.

This report concerns the third bleed of the study population, done between September and December 1973. Previously specimens were obtained for testing in July 1971 and April 1972.

PROGRESS: From September through December 1973 all persons previously bled were re-visited in an attempt to do a 2 year follow-up on the study population. Of the original study population of 849 persons in 1971, 7 had died and 276 persons had moved since the 1972 survey and were no longer available for the study. The 566 persons available for this third survey represented 66% of the original study population.

Eighty eight percent (497) of those available submitted specimens; 69 persons refused for various reasons.

Sixty-one persons who had been positive for hepatitis B antigen on one of the previous surveys were again bled on this survey and 60 of these were still positive. Only one person had reverted to negative.

Of the 436 people who were previously negative in 1972, four became positive for antigen. Three of these were asymptomatic. These were children 3, 6, and 8 years of age, two of whom were siblings. The fourth person was a 44 year old, male heroin addict ("sniffer") who was jaundiced and had an SGOT of 665 units/ml, suggestive of acute hepatitis.

SUMMARY: In a two year follow-up survey done on the Huay Khwang urban sample being studied for hepatitis B antigen, 60 of 61 (98%) persons maintained their chronic antigenemia, and 4 of 436 (0.9%) developed antigenemia for the first time.

8. Hepatitis B Antigen in Patients with Liver Disease or Cancer in Bangkok, Thailand

The objective of this study was to determine the prevalence of hepatitis B antigen (HBsAg) in patients with liver disease or cancer.

BACKGROUND: Hepatitis B antigen (HBsAg) provides a marker for epidemiological studies of hepatitis. Since the original discovery of HBsAg, a number of relationships have been described and/or suggested between the presence of the antigen and various disease states other than hepatitis. The SMRL's observations of HBsAg in a blood donor-recipient system have previously been reported in the SEATO Medical Research Laboratory Annual Progress Reports 1971-1972 pp 140-155 and 1972-1973 pp. 70 and are presently being submitted for publication. During the course of the blood bank study it was possible to correlate HBsAg presence with various disease entities.

METHODS: Cases of liver disease, hepatocellular carcinoma, leukemia, and other forms of cancer were tabulated by reviewing recipient card entries under the category referring to diagnosis. The HBsAg status was determined on the basis of the pre-transfusion results, i.e. on the serum sample obtained before the patient received a transfusion. Detection of HBsAg was done by agar-gel immunodiffusion, complement fixation, and immunoelectroosmophoresis.

PROGRESS: A total of 2,602 patients were entered into the study. There were 69 cases of non-malignant liver disease, 22 cases of hepatoma, 211 cases of cancer of other types (excluding leukemia) and 26 cases of leukemia. A review of the cases of liver cancer revealed only 11 hepatomas confirmed by histopathology. The other 9 cases were reclassified into their proper categories (Table 1). Six of those discarded were found to be other diseases of the liver, and three were other forms of cancer.

Table 1 shows the revised totals in each category as well as the number of patients with HBsAg present in their serum.

Table 1. HBsAg Prevalence for Various Disease States

Diagnosis	No. Tested	No. Positive	% Positive
All non-malignant liver disease	75	4	5.6
Hepatoma of the liver	11	1	10.0
Cancer of other types	214	11	5.4
Leukemia	31	3	10.7
Urban population (Huay Khwang)	695	55	8.5
Blood Bank study	2602	175	7.2
Total	3628	249	6.9%

Using a 2XR contingency table the X^2 value is 3.014 with 4 degrees of freedom. This suggests that none of the rates is sufficiently different from the others, and thus implies that none are more likely to be related to HBsAg than the others.

Eighteen patients in the liver disease group had jaundice and/or hepatitis as a diagnosis; three of these (16%) were positive for the antigen.

Two reservations must be considered. First, all of the persons included in the blood bank study were "selected" because they required a blood transfusion. This selection may or may not cause bias in the results. Second, the radioimmune assay (RIA) test was not used for antigen detection, and this, a more sensitive test, might have given somewhat different results.

SUMMARY: Comparing disease states with hepatitis B antigenemia did not show any specific associations in Bangkok, Thailand. This is in contrast to other studies that have related antigenemia to hepatoma, and/or to chronic liver disease.

9. Hepatitis B Antigen and Antibody in "Sexually Active" Thai and American Populations

The objective of this study was to determine the prevalence of hepatitis B antigen (HBsAg) and antibody (anti-HBs) in "sexually active" Thai and American populations.

Hersh et al. (22) have reported transmission of hepatitis B virus (HBV) between hepatitis B antigenemic males and their sexual contacts. Further, HBsAg has been reported to be more prevalent among sexual contacts of antigen carriers than among the general population in

which they reside (23, 24). This suggests that HBV may be sexually transmitted. If this hypothesis is true, then in Thailand, with a high prevalence of HBsAg carriers (6-9%) in the general population, a group of sexually promiscuous women should have more contact with HBV and should therefore have a greater prevalence of HBsAg and/or anti-HBs than a similar group of monogamous women. Further, if the sexually active women consort with a population of susceptible individuals, such as a group of United States servicemen, sexual transmission should occur and a proportion of servicemen should develop either HBsAg, with or without clinical symptomatology, or anti-HBs. This study was designed to determine the point prevalence of HBsAg and anti-HBs in a population of promiscuous Thai women termed "service girls", and compare them with other groups of Thai women of similar age. Also, in association with another study (SEATO Medical Research Laboratory Annual Report, 1973-1974), United States servicemen known to have numerous sexual contacts with these women will be followed longitudinally for signs of the development of hepatitis B infection over a period of twelve months after entering Thailand.

DESCRIPTION: Populations. Members of four populations were studied:

a. Service girls selected from patients attending the Freeland Venereal Disease Clinic, Rayong, Thailand. This clinic was selected because it was in the vicinity of joint Thai-United States military facilities. The women admitted previous sexual activity with both Thais and Americans; however, at this time Americans were their usual consorts. The clinic has a panel of approximately 1,000 women who are seen at weekly intervals.

b. Women visiting a prenatal clinic at the Bangkok Women's Hospital.

c. Women delivering babies at the Bangkok Women's Hospital.

d. United States servicemen entering Thailand. (Serum was collected initially and after periods of 4 and 8 months.)

Serological Studies: Sera were collected from all four groups and submitted for complement fixation (CF), immunoelectroosmophoresis (IEOP) and radioimmunoassay (RIA). Antigens detected by these means were tested for antigenic subtype by agar gel diffusion. Hepatitis B antibody was detected by IEOP. Methods for the above tests appear elsewhere (SEATO Medical Research Laboratory Annual Report 1971-1972, 1972-1973, 1973-1974). Sera were also tested serologically for syphilis by a commercial rapid plasma reagin test (Hynson, Westcott & Dunning, Inc.). All those found to be positive were confirmed by a standard VDRL test.

Table 1. HBsAg and VDRL Positives in Three Thai Populations

Population	Total	HBsAg			Anti-HBs (IEOP)	VDRL
		CF	IEOP	RIA		
Service Girls	681	49 (7.1)*	51 (7.4)	55 (8.1)	68 (10)	79 (11.6)
Prenatal Clinic	1000	60 (7.0)	72 (7.2)	9/100 (9.0)	100 (10)	32 (3.2)
Delivery Room	1625	93 (5.7)	93 (5.7)	7/100 (7.0)	141 (8.6)	ND**

HBsAg (IEOP) CHI^2 (2 df) = 3.3 $0.10 < P < 0.20$

Anti-HBs (IEOP CHI^2 (2 df) = 1.38 $0.40 < P < 0.50$

VDRL CHI^2 (1 df) = 40 $P < 0.001$

* Numbers in parentheses are percentages

** Not done

PROGRESS: Interviews and sera were obtained from 681 service girls at Freeland Clinic; 1,000 consecutive women visiting the prenatal clinic of the Bangkok Women's Hospital; and 1,625 consecutive women presenting for delivery at the Women's Hospital, Bangkok between 0800 and 1200 on weekdays. The Freeland Clinic's population was sampled over a 4 day period. The two groups from Bangkok took approximately a year to accumulate. Serum was obtained from 427 American servicemen on entrance into Thailand and at the time of this writing a second serum has been obtained from 303 of these after 4 months and a third serum from 73 after 8 months in Thailand.

Table 1 presents HBsAg, anti-HBs and VDRL data on the three Thai study groups. There were no significant differences in the prevalence of HBsAg found among any of these populations, and there is little difference between these prevalences and those seen in other Thai populations (SEATO Medical Research Laboratory Annual Report, April 1972 - March 1973 pp 74-76). A comparison of the prevalence of IEOP detectable antibody gives similar findings, with no significant differences between the three groups of women. The VDRL positives, on the other hand, were three times more frequent among the service girls than among the women in the prenatal clinic, giving further evidence of promiscuity in the former group.

The antigen subtype was determined for 21 (41%) of the 51 antigens detected by IEOP in the sera of service girls and in 34 (37%) of the 93 antigen positive mothers in the delivery room (see Table 2).

The distribution of subtypes within each group was almost identical and was not significantly different from that observed in other Thai populations previously studied (SEATO Medical Research Laboratory Annual Report, April 1973 - March 1974). The ay subtype was not found in either group.

At this time only preliminary data may be presented on United States servicemen.

Table 2. HBsAg Subtypes in the Thai Population

Population	Total	HBsAg (IEOP)	Subtype identified	adr	adw
Service Girls	681	51 (7.4)*	21 (41)	18 (85)	3 (15)
Delivery Room	1625	93 (5.7)	34 (37)	30 (88)	4 (12)

* Numbers in parentheses are percentages

Table 3 shows the prevalence of HBsAg, anti-HBs and VDRL positives among the men sampled at 3 different times.

Table 3. HBsAg, Anti-HBs and VDRL Prevalence Among United States Servicemen in Thailand

Time of Sampling	Total	HBsAg	Anti-HBs	VDRL
Arrival	427	1	6	2
4 months	303	1	4	2
8 months	76	0*	2	0**

* The antigenemic individual not yet sampled at 8 months
** The VDRL positive individuals not yet sampled at 8 months

One HBsAg/adw carrier was detected among the 427 men from whom serum was obtained upon arrival in Thailand. This individual has remained positive for the four months he has been followed. In the 303 persons followed longitudinally for 4 to 8 months there does not appear to be a detectable increase in HBV activity despite considerable sexual as well as other contact with the local population as documented by interview data.

DISCUSSION: Among service girls, a sexually promiscuous population, there does not appear to be any higher prevalence of HBsAg or anti-HBs than in control populations of Thai women of approximately the same age group. The prevalence of positive VDRL's among these girls, however, was more than three times that of the control populations, attesting to a higher incidence of venereal disease. Anti-HBs has not yet been tested by a sensitive technique; however, among Thai women, the prevalence of anti-HBs detected by IEOP was higher but not significantly different from any of the two control groups tested.

The lack of any significant difference in the prevalence of HBsAg in these groups of Thai women suggests that venereal transmission of HBV is unlikely. This is also demonstrated by the similiar antibody prevalences among these groups. IEOP only measures high titers of antibody as compared with other methods such as PHA or RIAI (SMRL Annual Report, April 1973 - March 1974) and appears to detect only secondary antibody responses. However if HBV were venereally transmitted a large proportion of service girls would be expected to have had many exposures and therefore should have a higher prevalence

of high titered antibody than a less promiscuous population. This was not found.

The data generated on United States servicemen up to this time cannot be fully interpreted. The incubation period of Hepatitis B is from 6 weeks to 6 months, therefore our follow-up schedule should pick up HBV infections acquired during the first half of a man's sojourn in Thailand. Early findings suggest no marked increase in the incidence of HBV infection as monitored by increasing disease, increasing HBsAg carrier rates or increased conversions of anti-HBs. If transmission of HBV was venereal, with the degree of sexual contact documented in this population, an increase in HBV infection should be evident by this time.

SUMMARY: The prevalence of HBsAg and anti-HBs was determined in three populations of Thai women. No significant differences were detected in the prevalence of HBsAg or anti-HBs despite the known promiscuity of one of these groups. United States servicemen known to have sexual contact with the promiscuous population were followed for up to 8 months after entering Thailand. Early data does not support the hypothesis that HBV can be transmitted to any great extent by venereal contact.

10. Hepatitis B Antigen and Antibody in Umbilical Cord Blood

This is a study of the prevalence of Hepatitis B Antigen (HBsAg) and antibody (anti-HBs) in the sera of women in labor and in umbilical cord blood.

BACKGROUND: Other studies conducted at SMRL have shown that hepatitis B virus (HBV) frequently infects residents of Bangkok at an early age. It is possible that some people are infected during the prenatal period. If so, a prenatal infection might be detrimental to the health of the newborn. In this study the results of hepatitis B serology were compared with demographic information collected by interview, serum biochemical tests, and IgM concentration in the cord blood.

DESCRIPTION: From 17 July 1972 to 15 June 1973, women in labor were interviewed in Women's Hospital, Bangkok. At the time of delivery 10 ml of whole blood was drawn from the mother and from the umbilical cord. Serum was used for all tests.

HBsAg was detected by IEOP, CF and RIA. Anti-HBs was detected by IEOP; SGOT and SGPT determinations were made by the Department of Biochemistry, SMRL. Total IgM concentration of umbilical cord serum was determined by radial immunodiffusion (Hyland Lab).

Table 1. Transaminase Levels in Maternal and Umbilical Cord Sera

Mother	Maternal Serum			Cord Serum		
	No.	SGOT	SGPT	No.	SGOT	SGPT
HBsAg	93	27.3 ± 10.3*	20.2 ± 7.7	90	32.0 ± 11.4	17.4 ± 5.8
Anti-HBs	135	25.4 ± 13.1	18.9 ± 8.4	133	32.1 ± 12.3	16.6 ± 7.0
Negative	138	25.2 ± 13.6	20.0 ± 15.8	136	29.2 ± 9.2	16.3 ± 5.1

* Mean ± one standard deviation

PROGRESS: A total of 1625 pairs of maternal and umbilical cord sera were tested. HBsAg was detected in 93 (5.7%) of the mothers and 10 (0.6%) of the cord sera by RIA. Antigen was never found in the cord serum of a mother who was negative.

Anti-HBs was found in 137 (8.4%) of the mothers and 137 (8.4%) of the cord sera. Although 137 sera were antibody positive by IEOP, none was positive by CF. In every instance when antibody was found in the cord blood, it was also present in the mother; however, the concentration of antibody in the maternal serum was often so low that the serum had to be concentrated about 10 times with polyacrylamide gel (Lyphogel) in order to get a precipitin reaction comparable to that of the cord blood.

For evaluating the interview data, all of the mothers with HBsAg and anti-HBs were compared to 138 negative mothers. Mothers were grouped by their number of pregnancies to see if HBV infection might affect early or late pregnancies differently. The data did not show any relationship between HBsAg or anti-HBs and the age of mother, number of pregnancies, birthweight of last child, number of surviving children, number of people living at home or the reported family income. It was found that, on the basis of small numbers, HBsAg positive multigravidas with a history of abortion were more likely to have antigen in their cord blood than antigen positive mothers without a history of abortion.

There was no difference in the level of SGOT or SGPT between antigen positive and negative sera (Table 1). In 11 pairs of sera where both the maternal and cord blood contained HBsAg, the values of SGOT and SGPT correlated very closely. Similarly, determinations of IgM concentrations in cord sera did not differentiate between antigen positive, antibody positive and negative mothers (Table 2).

A prospective study of HBV infections in infants during the first year of life is underway.

Table 2. Concentration of IgM in Umbilical Cord Sera

Mother	Umbilical Cord IgM			
	No.	Mean (mg%)	Standard deviation	Range
HBsAg	38	6.8	2.8	0-15
Anti-HBs	7	6.5	4.3	0-12.8
Negative	74	6.7	3.3	0-17.1
Normal Thai*	7	6	4	2-10

*Thongcharoen, P. et al: Determination of Human Serum Immunoglobulins in Healthy Thai Subjects. J. Med. Ass. Thailand. 55:347-355, 1972.

F. RABIES

1. Animal Rabies in Thailand

a. RABIES DIAGNOSTIC LABORATORY SERVICE

This laboratory provides rabies diagnostic services to U.S. military personnel in Southeast Asia and in 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 99.8%.

PROGRESS: Of 805 brain specimens examined, 308 (38.3%) were positive (Table 1). The prevalence of rabies in the dog remains high as in previous years (46.4%), and rabies was diagnosed in a substantial percentage of cats as well (18.3%). Rabies was infrequently diagnosed in other pets or in livestock.

b. SURVEY OF WILD RODENTS FOR RABIES INFECTION

DESCRIPTION: Canine rabies is a major public health problem in the Chonburi-Rayong area of Southeastern Thailand. In 1966, wild rodents, particularly Bandicota indicus, which were trapped in this area were reported to be infected with rabies virus (25). In the 1966 survey of 1005 rodents, rabies was reported in Bandicota indicus (7.0 per cent), Rattus rajah (6.3 per cent), Rattus norwegicus (4.7 per cent), Rattus rattus (3.5 per cent), Rattus exulans (2.5 per cent), and Bandicota bengalensis (1.0 per cent).

In 1972 we re-surveyed rodents from this area, capturing animals in many of the same locations and at the same time of year as in the 1966 survey. A total of 704 wild rodents, including 520 Bandicota indicus and 184 Rattus rattus, were captured in four trapping sites between September and December 1972. During 1973 all of the brains, frozen at the time of capture, were examined for rabies using the fluorescent antibody technique and mouse inoculation.

PROGRESS: No evidence of rabies infection was found in any of the 704 rodent brains examined. A virus, which has not as yet been identified, was isolated from the brain of one bandicoot. This virus, which was lethal for mice, was not stainable with fluoresceine isothiocyanate labeled antirabies globulin, and was not neutralized by antirabies immune serum. We conclude that this virus is not serologically related to rabies virus.

Table 1

Summary of Rabies Diagnoses 1 April 1973-31 March 1974

Species	Number of Specimens	Number Positive	Percent Positive
Canine	605	281	46.4
Feline	109	20	18.3
Equine	4	3	75.0
Human	5	2	40.0
Subhuman Primate	18	1[a]	5.6
Bovine	2	1	50.0
Rodents	39	0	0
Rabbits	12	0	0
Bats	7	0	0
Other[b]	4	0	0
Total	805	308	38.3

[a] Pet captive gibbon, Bangkok.

[b] Leopard cat, shrew, civet, deer.

SUMMARY: Rabies was not found in a survey of 704 wild rodents captured in an area of Southeast Thailand where rodent rabies was reported in 1966.

2. The Corneal Test in The Antemortem Diagnosis of Human Rabies

OBJECTIVE: To evaluate the usefulness of the corneal test for the antemortem diagnosis of rabies in man.

BACKGROUND: In a high percentage of animals infected with rabies, the virus can be demonstrated in superficial corneal epithelial cells by immunofluorescent methods (26, 27). In experimentally infected mice, virus has been demonstrated in the cornea even before the onset of clinical signs of disease (26). A number of human cases have been reported in which an antemortem diagnosis of rabies has been confirmed using the corneal test, but no systematic effort has been made to determine the reliability of this diagnostic method in man.

PROGRESS: To date, we have studied four human cases of rabies. One was an American soldier hospitalized in Saigon, Vietnam, and the other three were Thai nationals hospitalized at the Infectious Disease Hospital near Bangkok, Thailand. In each case a tentative clinical diagnosis of rabies had been made based upon clinical symptomatology and history before sampling was initiated.

At the earliest opportunity after a suspected rabies patient was admitted to the hospital, samples were taken for laboratory examination. Corneal impression smears were made by firmly pressing a clean fluoro-slide against the cornea, causing superficial corneal epithelial cells to adhere to the glass. This caused little discomfort to the patient, and usually required no anesthesia or sedation. In the laboratory the corneal impressions were stained with fluorescent antibody in the same manner as is used for brain impressions, except that the time-consuming acetone fixation step is unnecessary and was eliminated (28).

In addition to corneal impressions, samples of serum and saliva were obtained initially, and at frequent intervals during the clinical course of disease. Serum neutralizing antibody titers were determined in mice, and saliva was inoculated into mice intracranially for attempted virus isolation. After death, the diagnosis of rabies was confirmed by applying the standard fluorescent antibody test and mouse inoculation test to the brain. The presence of virus in the saliva, salivary gland, and cornea after death was also determined by mouse inoculation.

Table 1

Laboratory Findings in Four Human Rabies Cases

Patient Description	Day of Disease[e]	Corneal Test	Serum Anti-rabies Titer	Saliva MI[b]	Brain	
					FAT[c]	MI[b]
Case 1. American, male, age 22	8	+	1:5			
	12	+	1:30[d]			
	20	+	1:125[d]			
	21[a]	+			+	+
Case 2. Thai, female age 43	2	+	<1:5			
	3[a]	+			+	+
Case 3. Thai, female age 57	4	+	<1:5	+		
	6	+	<1:5	+		
	8	+	<1:5	+		
	9[a]	+			+	+
Case 4. Thai, female age 57	4	-	<1:5	-	Autopsy not authorized	
	5[a]	-				

[a] Day of death

[b] MI = Mouse inoculation test

[c] FAT = Fluorescent antibody test

[d] Titer determined after administration of hyperimmune serum

[e] The days indicated are the number of days after initial appearance of clinical symptoms as reported by the patient

3. Antemortem and Postmortem Diagnosis of Rabies in Dogs from Fluorescent Antibody Stained Corneal Impression Smears

PURPOSE: To evaluate the corneal test for the early antemortem diagnosis of rabies in dogs.

DESCRIPTION: Recent surveys conducted at the municipal dog pound in Bangkok indicate that 3 to 5 percent of these dogs are incubating rabies as determined after euthanasia by post mortem examination of the brains utilizing the fluorescent antibody test (FAT) and mouse inoculation (MI) (see previous annual reports). This dog population will be utilized to study the ability of the corneal test (26) to identify pre-clinical cases of rabies, and to determine whether there is a reliable correlation between a positive corneal test and infectivity of the saliva. These studies have potentially important implications in the management of animals in quarantine following biting incidents.

PROGRESS: A preliminary study has been conducted utilizing dog heads submitted to the rabies diagnostic laboratory. A total of 35 heads have been examined to date. In addition to the examination of the brain, corneal impressions were prepared by repeatedly pressing a clean fluoro-slide against the cornea. After air drying, the corneal impressions were stained at room temperature with fluoresceine isothionate conjugated anti-rabies globulin of equine origin (Baltimore Biological Laboratory) for 30 minutes, washed with phosphate buffered saline pH 7.6 for 10 minutes, mounted in phosphate buffered glycerine pH 8.5, and examined under the fluorescent microscope. Normal mouse brain (20 per cent suspension) was mixed with the conjugate a short time before staining, and rhodamine counterstain was added. Non-specific fluorescence was evaluated by the examination of impressions stained with conjugate mixed with 20 percent rabid mouse brain. In some cases, impressions were stored at -70°C for up to two weeks before staining with satisfactory results. The time-consuming acetone fixation step, required in processing brain impressions for the FAT, is unnecessary in processing corneal impressions. Corneal impression slides were labelled with coded numbers so the microscopist would be unaware of the results of the brain examination prior to reading the slides. Saliva (buccal washings), parotid salivary gland tissue, and the corneas were also obtained from each head and the homogenated tissues inoculated intracranially into weanling mice for attempted rabies virus isolation.

The results of this study are summarized in Table 1. Of the 35 brains examined, 12 were positive for rabies by FAT and MI. Virus was present in the corneas of 11 (91.7 percent) of the positive dogs as determined by MI. The corneal test was positive in only 5 of the

Table 1

Post Mortem Identification of Rabies Virus in the Brains and Corneas of Rabid Dogs

Case No.	Cornea		Brain		Time of Examination[1]
	FAT[2]	MI[2]	FAT	MI	
65	+	+	+	+	1
79	-	+	+	+	1
92	+	+	+	+	1
104	+	+	+	+	1
111	-	+	+	+	1
114	+	+	+	+	1
132	+	+	+	+	1
68	-	+	+	+	2
80	-	+	+	+	2
108	-	-	+	+	2
109	-	+	+	+	2
115	-	+	+	+	2

1 Number of days between death and the time of laboratory examination

2 FAT = Fluorescent antibody test; MI = Mouse inoculation test

ll corneas known to contain virus. Retrospective analysis of the histories of the dogs in the study indicates that in most cases the corneas which were positive on MI and negative on FAT came from dogs that had died or had been sacrificed more than 24 hours before the laboratory examinations were performed (although adequate refrigeration was provided). Further studies of the possibility that the sensitivity of the corneal test deteriorates with time are being undertaken.

In no case was a corneal test declared positive when the cornea was obtained from any of the 23 dogs in which the brain was negative. This indicates the high degree of specificity of the corneal test in the hands of an experienced FAT diagnostician. The morphology of the fluorescent antibody stained inclusions in the cornea are quite similar in appearance to rabies inclusions in brain, except that the inclusions are quite scanty in many impressions, and the inclusions tend to be smaller in size, and more uniform.

Laboratory analyses of saliva and salivary gland specimens are incomplete at this time. These data will be included in a future report, along with data from additional dogs to be studied.

DISCUSSION: Preliminary post mortem studies indicate that rabies virus may be demonstrated in the corneal epithelium of most dogs which die of rabies or which are sacrificed in the advanced stages of the disease. The fluorescent antibody test ("Corneal Test") was considerably less sensitive than mouse inoculation in demonstrating this virus. It is suggested that post mortem decomposition accounts, at least in part, for this decreased sensitivity. Antemortem studies in dogs are being initiated.

II. BACTERIAL DISEASES OF MAN AND ANIMALS

A. TYPHOID FEVER

1. Chloramphenicol-Resistant *Salmonella typhi*

The objective of this study was to determine the prevalence and degree of chloramphenicol resistance among *Salmonella typhi* isolates from typhoid fever patients at Children's Hospital; and to correlate the *in vitro* pattern of resistance with epidemiological data and clinical response.

Chloramphenicol-resistant *S. typhi* has recently been reported from Mexico (29), India (30), and Vietnam (31). R factor transfer of resistance has been demonstrated in these strains, and is believed to be the manner in which chloramphenicol resistance develops. Typhoid fever is endemic in Thailand. Approximately 100 cases are seen annually at Children's Hospital. Enteropathogens isolated in Thailand are resistant to multiple antibacterial drugs, and this resistance is transferable (32). The occurrence of chloramphenicol-resistant *S. typhi* at Children's Hospital has been suspected for several years but has only recently been documented.

Five of fifty *S. typhi* isolates from hemocultures at Children's Hospital were resistant to chloramphenicol (30 µgm Kirby-Bauer discs) during the period Jan-Jun 1973.

DESCRIPTION: Children with suspected typhoid fever had hemocultures, rectal cultures, and Widal tests performed on the day of admission, day 5, and day 10. Bone marrow cultures were obtained when deemed necessary by the ward physician. *S. typhi* isolates had disc sensitivities performed by the Kirby-Bauer method. Plate dilution minimum inhibitory concentration (MIC) was determined for chloramphenicol, ampicillin, tetracycline and trimethoprim-sulfamethoxazole.

PROGRESS: *S. typhi* has been isolated from 41 patients since the beginning of the project 1 Nov 73. Thirteen of these (32%) have been resistant to chloramphenicol. In each resistant case the same pattern of resistance has been found: resistance to chloramphenicol, tetracycline, streptomycin and sulfadiazine. MIC data for the resistant *S. typhi* isolates are reported in Table 1. Two of the isolates were resistant to ampicillin. All chloramphenicol-resistant isolates were sensitive to trimethoprim-sulfamethoxazole.

Of thirteen resistant cases, eight were from Wang Noi, Ayutthaya, two from Nonthaburi, two from Bangkok and one from Lopburi (see Figure 1). The sensitive cases were from scattered locales, predominantly in the Bangkok area. The home of a family living in Wang Noi, with

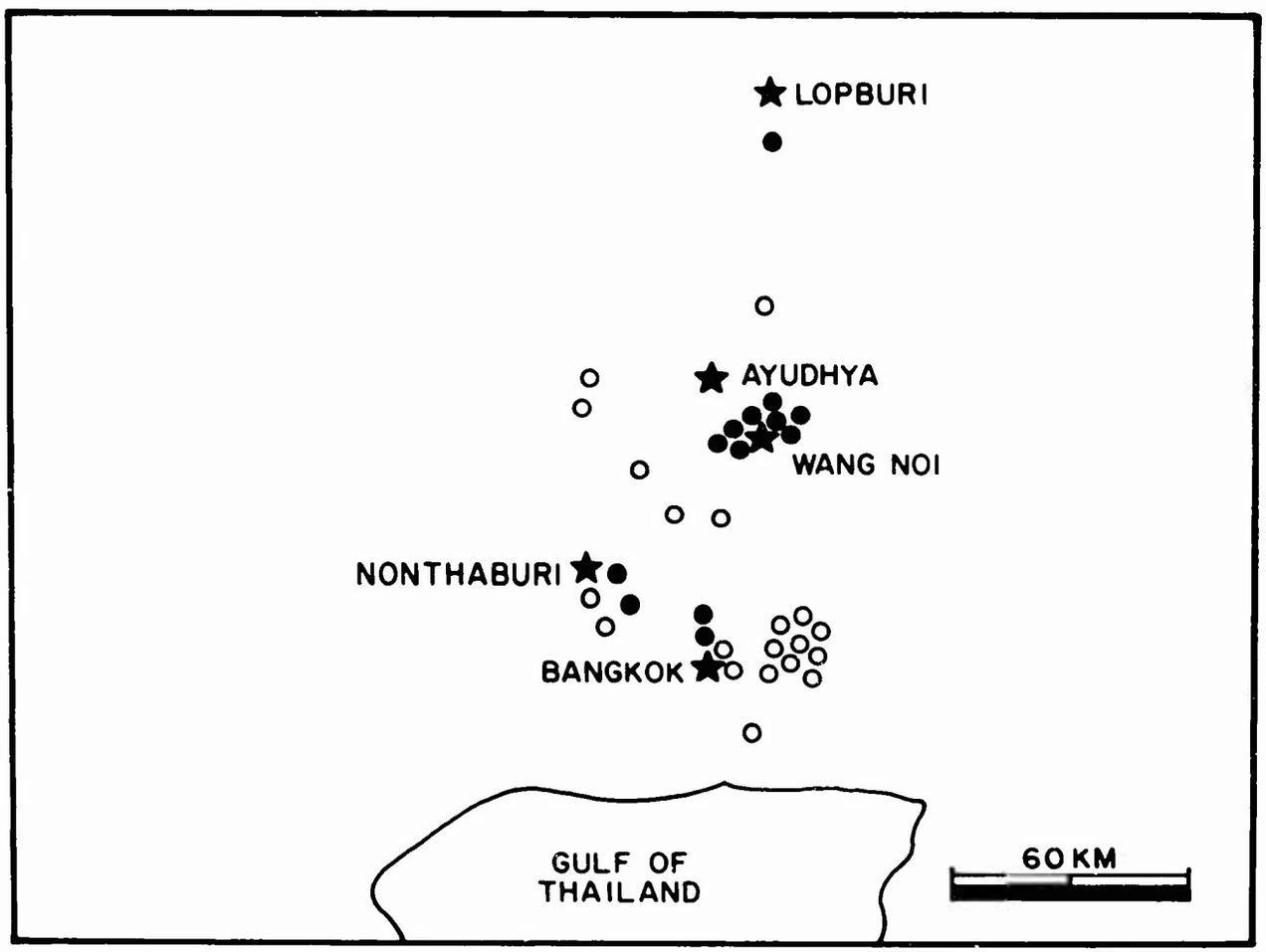


FIGURE 1: DISTRIBUTION OF TYPHOID CASES
RESISTANT (●) AND SENSITIVE (o)

Table 1. MIC Values for Chloramphenicol-Resistant S. typhi

Antibiotic	No. of S. typhi isolates	MIC(μg/ml)
Chloramphenicol	13	256-512
Tetracycline	13	256-512
Ampicillin	11	0.5-1.0
Ampicillin	2	256-512
Trimethoprim (TMP)	13	0.4-1.6
Sulfamethoxazole (SMZ)	13	16384
TMP-SMZ	13	0.1/1.9-0.8/15.2

chloramphenicol-resistant S. typhi isolates from two of their children, was visited and rectal swabs were obtained from the parents and three additional siblings. Canal (klong) water, drinking water and duck feces were also cultured. No Salmonella typhi was found. Conversations with personnel at District Hospital and the District Health Center revealed no recognized problem with typhoid fever, unresponsive to therapy.

One patient with chloramphenicol-resistant S. typhi lived in an orphanage, Baan Rajvithi, in Bangkok. Eighteen contacts, children living in the same cottage as the patient, had rectal swabs taken as did the six food handlers for the orphanage. None were positive for S. typhi.

Four chloramphenicol resistant isolates of S. typhi from 1973 and one resistant isolate from 1965 have been phage typed at the CDC, Atlanta, Georgia. The 1965 isolate was phage type E-1; the four 1973 isolates were untypeable.

Transfer of chloramphenicol resistance to a sensitive strain of E.coli has been demonstrated in the three resistant isolates of S. typhi on which this has been attempted.

Patients from whom chloramphenicol-resistant isolates have been obtained have demonstrated clinical resistance when treated with chloramphenicol. Therapy with ampicillin or trimethoprim-sulfamethoxazole has proved satisfactory in the resistant cases.

DISCUSSION: Chloramphenicol-resistant S. typhi isolates have been obtained from 32% of typhoid fever patients at Children's Hospital. The pattern and degree of resistance resembles closely those isolates

from outbreaks in Mexico, India and Vietnam. Transfer of resistance has been demonstrated. The phage types of the resistant strains in Thailand are untypeable. Chloramphenicol is unsatisfactory in treating patients with chloramphenicol-resistant typhoid, but ampicillin or trimethoprim-sulfamethoxazole are satisfactory alternatives.

The geographical distribution of resistant cases in Wang Noi is interesting. No explanation is available at present. Since Children's Hospital is the only pediatric hospital in Bangkok, pediatric patients with typhoid are often referred there if out-patient treatment has been unsatisfactory. The close proximity of Wang Noi to a major highway leading to Bangkok may facilitate the patient's transport to Bangkok.

2. Prevalence of Glucose-6-Phosphate-Dehydrogenase Deficiency in Typhoid Fever Patients at Children's Hospital

We have performed a study to determine the prevalence of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency among hospitalized typhoid fever patients at Children's Hospital.

BACKGROUND: The clinical staff of Bangkok Children's Hospital, responsible for the management of typhoid fever patients, has noted a high prevalence of G-6-PD deficiency among hospitalized typhoid fever patients. Approximately 35% of 50 typhoid fever patients at Children's Hospital were G-6-PD deficient using the method of Brewer (33). This method may not detect heterozygotes and may be normal if the red cell population is young.

In the present study, the more sensitive methemoglobin elution technique of Gall (34) was used.

Hemolysis as a clinical expression of G-6-PD deficiency following the administration of various drugs, or in association with specific illnesses, is well known. Hemolysis in patients with typhoid fever and G-6-PD deficiency is well recognized.

Thirty-six male African patients hospitalized with typhoid fever had G-6-PD determinations performed. Fourteen were deficient. This incidence (39%) was more than twice the general incidence for G-6-PD deficiency in Accra, Ghana (35).

The relationship between sickle cell anemia and salmonella osteomyelitis is well recognized. Several studies have shown that bactericidal mechanisms which operate to eliminate salmonella are severely impaired in animals with hemolysis.

PROGRESS: Blood was drawn for G-6-PD determination from 34 patients admitted to Children's Hospital with typhoid fever confirmed by culture and Widal test. Fourteen of 34 (41%) were either homozygous

Table 1. G-6-PD Determinations in 34 Typhoid Patients

Sex	Normal	G-6-PD Deficient	
		Homozygotes	Heterozygotes
Male	12	6	0
Female	8	2	6
Subtotal	20	8	6
Total	20	14	

Table 2. G-6-PD Determinations in 15 Non-Typhoid Patients

Sex	Normal	G-6-PD Deficient	
		Homozygotes	Heterozygotes
Male	7	1	0
Female	7	0	0
Subtotal	14	1	0
Total	14	1	

or heterozygous for G-6-PD deficiency (Table 1).

Fifteen patients with suspected typhoid fever but with negative serial cultures for Salmonella typhi and serial Widals of 1:40 or less have had G-6-PD determinations performed. One of 15 was a homozygote (7%)(Table 2).

DISCUSSION: This high prevalence of G-6-PD deficiency among hospitalized typhoid fever patients is remarkable.

The prevalence of G-6-PD deficiency among hospitalized patients with fever, but having no evidence to support the diagnosis of typhoid fever, is approximately the same as the prevalence of G-6-PD deficiency in the general population (10%).

This high prevalence of G-6-PD deficiency among typhoid fever patients suggests that individuals with heterozygous or homozygous deficiency are more susceptible to the Salmonella typhi organism. This might be a direct result of the G-6-PD metabolic defect, or it might be possible that in G-6-PD deficient patients the reticuloendothelial system is already preoccupied with the handling of the products of red cell destruction.

It may be that G-6-PD deficient individuals who acquire typhoid fever are more likely to be hospitalized than G-6-PD normal individuals who acquire typhoid. For this reason a study of typhoid fever out-patients and controls is planned.

B. VIBRIO PARAHEMOLYTICUS GASTROENTERITIS

1. Clinical Observation of Vibrio parahemolyticus Infections in Thailand

Studies have been conducted to determine the prevalence and 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 is a major cause of gastroenteritis in adults in Bangkok (36). At the Bumrasnaradura Infectious Disease Hospital located in Nonthaburi, this organism has been isolated from approximately 25% of the diarrheal patients admitted to the hospital ward. Marine life and the marine environment, including sea water, sea fish, crabs, oysters, etc., have been found heavily contaminated with this halophilic bacillus throughout the year. These findings suggest that sea foods may be the major source of the V. parahemolyticus diarrheal outbreaks in this community. The

The detailed clinical picture of this disease, its mode of transmission in Thailand, and the efficacy of antimicrobial therapy have not been previously described.

DESCRIPTION: All patients admitted to the Infectious Disease Hospital, Nonthaburi, with symptoms of acute gastroenteritis between September 1973 and February 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 as soon as they were identified.

RESULTS:

Clinical Manifestations of V. parahemolyticus Gastroenteritis

Sixty-six patients admitted to the hospital during the study period were found to harbor V. parahemolyticus in their diarrheal stools. Only 42 of these patients were available for the clinical analysis (Table 1). The disease was characterized by acute, profound diarrhea with fever, nausea and vomiting. Consistency of the stool was watery or semisolid without mucus. Only one patient presented with bloody stool. Colicky abdominal pain was a prominent symptom. Fifty-seven (88%) of these patients had a history of sea food ingestion.

Table 1. Clinical Findings of V. parahemolyticus Gastroenteritis

Symptoms	Number of Patients	%
Characteristics of the stools		
Watery	29	69
Semisolid	13	31
Bloody	1	2
Mucus	0	0
Vomiting	32	76
Fever	7	17
Headache	19	45
Abdominal pain	38	90
History of sea food ingestion	37	88
TOTAL	42	

Sensitivity of V. parahemolyticus to Antimicrobial Agents

Sensitivity profiles of the vibrios isolated from the patients are

TABLE 2

Sensitivities* of V. parahemolyticus to 8 Antimicrobial Agents

Antimicrobial Agents	No. of Strains Tested	Sensitive	Intermediate Sensitivity	% Sensitive
Chloramphenicol	66	66	-	100
Tetracycline	66	35	31	100
Ampicillin	66	1	1	3
Colistin	66	6	4	15
Co-trimoxazole	66	66	-	100
Neomycin	66	17	48	98
Erythromycin	66	29	36	98
Streptomycin	66	13	49	94

* Sensitivity is based on Zone-Site Interpretation Chart (2).

presented in Table 2. Using the standardized single disc method of Bauer and Kirby (37), it was found that the majority of the isolates (94% to 100%) were sensitive to chloramphenicol, tetracycline, co-trimoxazole, neomycin, erythromycin and streptomycin. Only 3% and 15% of the vibrios tested were sensitive to ampicillin and colistin respectively.

Antimicrobial therapy trials comparing oral tetracycline, co-trimoxazole and placebos are being continued. Three groups of patients, 14 in each group, were compared in terms of age, sex and severity of the disease at the time of the admission. Data from these trials will be presented at a later time.

DISCUSSION: The patients in this study probably represent only a small proportion of the total illness in the population. Diarrheal patients are hospitalized only if the illness is severe. The clinical syndrome we observed in these patients, therefore, may represent only the severe form of the infection. A complete clinical picture of the mild form of the disease needs to be described.

The fact that patients were suffering from diarrheal symptoms without bacteremia or toxic symptoms suggests that the infection may localize in the lumen of the intestine. Previous experiments on the pathogenicity of V. parahemolyticus by using the infant rabbit model indicated that the organism elaborated toxic substances, presumably the enterotoxins, into the intestinal fluid (36). Enterotoxic substances may play a major role in the pathogenesis of this disease.

SUMMARY: The clinical picture of V. parahemolyticus gastroenteritis and the sensitivities of the organism to eight antimicrobial agents in vitro are described. Patients exhibited a febrile diarrhea with abdominal pain in the majority of the cases. Stools were watery or semisolid without bloody mucus. The majority of V. parahemolyticus strains isolated from the patients were sensitive to chloramphenicol, tetracycline, co-trimoxazole, neomycin, erythromycin and streptomycin, but resistant to ampicillin and colistin.

2. Case Reports of Vibrio parahemolyticus Diarrhea Among U.S. Personnel at the SEATO Medical Research Laboratory

This paper describes Vibrio parahemolyticus diarrhea occurring in U.S. personnel.

BACKGROUND: The bacteriology laboratory at SMRL provides a referral service to the U.S. Army Hospital Bangkok (USAH) for diagnostic bacteriology. The laboratory also processes sporadic requests from professional members of the laboratory itself. Selection factors in the submission of any one specimen are considerable and for that reason the total numbers of various categories of work done are only broad

estimates. In the calendar year January 1972 to December 1972, for instance, there were 54 stool specimens submitted by U.S. personnel for culture and sensitivity examination. These 54 persons were concerned enough about their illness to first see a physician and secondly to provide the requested specimen (if such a specimen was requested). These 54 persons were probably only a few of the vast total of people affected with enteric illness.

METHODS: Bacteriology laboratory records were reviewed for the years 1972 and 1973 and all stool specimens submitted by U.S. personnel were identified and tabulated. Those persons from whom Vibrio parahemolyticus was isolated were identified and interviewed regarding their case histories and symptomatology.

PROGRESS: In 1972 fifteen (28%) of 54 specimens submitted by U.S. personnel had a pathogenic enteric organism grown on culture. Two (3.7%) had Salmonella sp., 2 (3.7%) had Vibrio parahemolyticus and 11 (20%) had Shigella sp.

During the calendar year 1973 there were 68 specimens submitted, 17 (25%) of which were positive for pathogenic organisms. Eleven (16%) had Salmonella sp., 3 (4%) had Vibrio parahemolyticus, and 3 (4%) had Shigella sp. There were also 6 (9%) pathogenic E. coli and 6 (9%) non-agglutinable vibrios isolated.

There were four individuals in the 12 month period, Oct 72 - Oct 73, with culture-proven Vibrio parahemolyticus diarrhea. All four were adult males and all four were assigned to SMRL. Two patients remembered specifically eating a seafood meal within 24 hours of the onset of symptoms. In both of these cases the seafood was obtained and eaten at restaurants at resort areas on the Gulf of Thailand (not in Bangkok city itself).

Case 1: A 24 y.o. male experienced cramping, lower abdominal pain and watery, light-brown stools 20 hours after eating beef curry and noodles with pork at a local noodle shop. The patient continued to have watery stools and intermittent cramping and abdominal pain for the next four days. A stool culture obtained on the fourth day of illness grew V. parahemolyticus. A repeat culture one week after symptoms had abated was negative. During the first two days of illness the patient had eight watery bowel movements.

Case 2: A 34 y.o. male experienced dizziness and nausea with vomiting of green fluid. Later the same evening the patient became dizzy and weak, and during a four hour interval passed four watery, yellow stools. These were accompanied by severe cramping abdominal pain. The patient continued to have cramping pain and watery diarrhea 6-8 times a day for the next four days. The patient was given tetracycline and two days later had formed stools and was essentially recovered. The

patient frequents the same noodle shop as case 1, though no specific food history was obtained. The patient's wife had a similar clinical history for a much shorter duration, but she was not cultured.

Case 3: A 25 y.o. male developed cramping abdominal pain and watery diarrhea 15 hours after eating a seafood meal consisting of steamed crab and breaded shrimp. The patient had three more bowel movements within the next five hours. The cramping abdominal pain was relieved by Lomotil although the diarrhea continued for at least two more days. Both other individuals who had eaten the crab were also ill but were not available for culture.

Case 4: A 36 y.o. male experienced cramping abdominal pain 24 hours after eating a seafood meal which included raw clams (one of which was not eaten because it was obviously spoiled). During the first evening of illness the patient experienced approximately nine bowel movements of brown, watery stool accompanied by cramping abdominal pain. The diarrhea improved over the next two days although the cramping abdominal pain persisted for three to four more days. After one week the patient was essentially well with formed stool and absence of pain.

All four patients present a similar clinical picture: acute onset of cramping abdominal pain with watery diarrhea. One patient had nausea and vomiting; the other three patients denied this symptom. None had bloody diarrhea, and none complained of fever or chills.

SUMMARY: Five (4%) of 122 U.S. personnel submitting a stool specimen for culture and sensitivity had diarrhea due to V. parahemolyticus. This is very similar to the rate described in the Republic of Vietnam among U.S. servicemen. Reports of four cases of V. parahemolyticus diarrhea among U.S personnel stationed at the laboratory are presented.

3. The Occurrence and Transmission of Vibrio parahemolyticus in a Thai Fishing Village Population

OBJECTIVE: To determine the prevalence of Vibrio parahemolyticus in an insular Thai fishing village population.

BACKGROUND: This report is a continuation of the study originally described in the SEATO Medical Research Laboratory Annual Progress Report 1972-1973, pp. 113-114.

PROGRESS: In May 1973 the village of Ban Kho Lan was mapped and a house by house census of the total population was taken.

The village is located on the protected side of the island, on a narrow plain between the sea and nearby foothills. The physical terrain of the location forces the village to assume a long, narrow shape oriented along a North-South axis. There is a concentrated area

of houses in the center on the north side of the market place. This is also the location of the piers. The village area south of the market place extends down a dirt road and is composed of larger houses and yards suggesting greater affluence.

There are a total of 158 families occupying 156 houses. There were an additional 33 houses that were unoccupied at the time the census was taken. Some families have more than one house and change residency depending on the season, apparently to escape or to enjoy the prevailing winds.

There were 991 persons living in the village at the time the survey was made. Age and sex data are shown in Table 1. The sex ratio is essentially unity except for the 16-20 year age group in which the percentage of males is only 34%. This is probably the result of males leaving the insular setting for both military service as well as for the job opportunities and "adventure" of Bangkok or other cities.

Table 1. Age and Sex of the Total Population of Ban Kho Lan, May 1973

Age	Male	Female	Total	% Male
0-5	92	95	187	49
6-10	85	78	163	52
11-15	68	71	139	49
16-20	37	72	109	34
21-25	30	36	66	45
26-30	29	24	53	55
31-35	35	29	64	55
36-40	29	16	45	64
41-45	20	22	42	48
46-50	13	12	25	52
51-55	15	9	24	62
56-60	7	15	22	32
61-65	8	12	20	40
66-70	8	3	11	73
71+	7	8	15	50
Unknown	4	2	6	66
Total	487	504	991	49%

The number of individuals per household ranged from 1 to 13 with an average of just over 6 persons per house. There were only 22 houses that had no persons less than 14 years of age. The largest number of children in any one house was 7 and the median number of children per house was 3. There were a total of 437 persons considered children,

i.e., under 14 years of age.

The village has a surprisingly stable population. The average length of stay on the island is 17.6 years with a range of less than one month (the new teacher and recently appointed midwife) to 82 years. A total of 776 (78%) persons had lived in the village their entire life.

SUMMARY: To date the entire population of Kho Lan village has been censused and the village mapped. A prevalence survey, on a random selection of households, will be done for Vibrio parahemolyticus using cultures of rectal swabs.

C. CHOLERA

1. Brief Investigation of a Cholera Outbreak in Thailand

BACKGROUND: Cholera, a potentially serious problem in Bangkok with its poor sanitation and water supply, had not been reported in the city for the previous four years. In late April 1973 cases began appearing sporadically in Samut Prakarn Province just south of Bangkok, and by early May it was obvious an outbreak was occurring. Interest and concern was expressed by the School of Public Health and a meeting was held at the school to discuss the status of the outbreak and potential avenues of investigation open to the school, with assistance from the SEATO Medical Research Laboratory (SMRL). At that time it was decided that a limited study could be done, investigating the possible role of food handlers in the maintenance and spread of the disease.

DESCRIPTION: Site visits were made to several areas of potential epidemiological importance:

a. The Samut Prakarn Provincial Hospital was visited to substantiate the diagnosis and occurrence of cholera cases.

b. The village of Moo Song in the factory district, where three cases had originated, was visited.

c. One theory for the recurrence of cholera in Thailand was the possible importation of cases from Malaysia by fisherman. Fishing boats coming to an ice plant in the area of Moo Song were thought to be a possible focus of spread. The ice plant was inspected to determine its potential for such a role.

d. An isolated duck farm, where an individual had apparently contracted the disease, was visited and individuals interviewed to determine possible sources of infection.

To investigate the potential role of food handlers in the spread of the disease: "noodle" shops in the area of Moo Song were identified, censused, and mapped; all persons in these places identified as food handlers (usually all members of the family involved with the noodle shop were considered food handlers) submitted a rectal swab, which was placed in Cary Blair transport media and returned to SMRL for bacteriological culture and sensitivity studies; and a short questionnaire was completed on each of these food handlers.

PROGRESS: The above site visits produced the following results:

a. The Samut Prakarn Hospital had seen 104 suspected cases of cholera starting from 18 April 73 (the beginning of the outbreak) until 3 May 1973. Vibrio cholera was confirmed in 33 of these cases (Bumrasnaradura Hospital Laboratory).

b. Moo Song is a narrow village approximately 50 meters by 200 meters located between two large factories, the United Flour Factory and the Scott-Thai paper factory. One end of the village abuts the Chao Phya river and the other faces the road. Each house in the village has its own "klong jars" which are filled from one central water faucet via a plastic hose, the total length of which is made up of many hoses coupled together. Since this hose lies on the ground in waste water, puddles, etc., it is certainly a potential source of drinking water contamination.

c. The ice factory was very clean and is regularly inspected by a Public Health Worker. The water is obtained from a deep well and the ice is only used for refrigeration, not for consumption. Fishing boats are "iced" (filled) in 1.5 hours by a conveyer belt, and the Malayan crew members do not come ashore at any time. Thus the theory of importation was unlikely.

d. On 19 April the second recognized case of cholera ended fatally for a worker on the duck farm. His wife and niece were also bacteriologically positive though apparently asymptomatic. It had been reported that the infected worker had not left the duck farm in the last six months, but 1000 ducks were shipped daily, and numerous persons entered and left the farm every day. It is possible that one of these persons was excreting the organism and spread the disease to the persons on the farm by poor sanitary practices.

Foodhandler Survey: A total of 31 persons were cultured from ten eating places, a food cart, and a food basket carrier. Four males and twenty-seven females were cultured, reflecting the fact that females are more likely to be engaged in this occupation. Three of the persons cultured had a complaint of diarrhea in the previous two weeks, and indeed, two of them had diarrhea at the time the culture was taken. None of the 31 cultures were positive for V. cholera although one

Shigella organism and seven Salmonella organisms were isolated from this group. The Salmonella organisms were all S. derby except for one S. newlands.

SUMMARY: There was no obvious cause found for the recurrence of cholera in Thailand, though there are several possibilities. Cholera could have continued to smolder unrecognized with the occasional clinical case misdiagnosed. Importation to this area is also possible since this factory area absorbs a large number of migrant workers from other areas of Thailand as well as migrants from other nearby countries. Given the characteristics of the organism, the potential for a major outbreak is always present.

In the "chunk" sample of food handlers living and working in a cholera infected area, there were no persons from whom cholera organisms were isolated. There had been no cholera cases in the area during the previous five days and it is possible that asymptomatic carriers could have reverted to negative by the time the survey was made.

A surprisingly large number of persons had Salmonella organisms isolated on rectal swab. The public health significance of food handlers thus infected is very real and emphasizes the need for continued instruction in the use of proper sanitary practices.

D. GONORRHEA

1. Minimum Inhibitory Concentrations (MIC) of Penicillin G and Ampicillin for Neisseria gonorrhoeae

The objective of these studies was to determine the minimum inhibitory concentrations (MIC) of penicillin G and ampicillin for Neisseria gonorrhoeae in Thai males and to determine serum levels of ampicillin and ampicillin combined with probenecid in the single oral dose used to treat uncomplicated gonorrhea.

BACKGROUND: Despite the spread of resistant strains of Neisseria gonorrhoeae throughout the world, penicillin remains the drug of choice for the disease because of its minimal cost and low toxicity. The resistant strains are not absolutely resistant and the majority will respond provided a suitably high serum level of penicillin can be reached and maintained for a long enough perird of time.

A single oral dose of ampicillin appears to be the most convenient and effective treatment for uncomplicated gonorrhea. Oral ampicillin combined with oral probenecid produces a higher serum ampicillin level of longer duration than ampicillin alone. To test the efficacy of this treatment regimen the following study was conducted. The study is divided into two parts:

PART I:

DESCRIPTION: This part of the study was carried out at the V.D. Clinic, Royal Thai Army Hospital, Bangkok, from November 1972 to January 1974. A total of 280 male patients presented with purulent urethral discharge of uncomplicated gonorrhea.

Specimens were obtained with cotton swabs from the urethra and streaked directly on Thayer-Martin agar with hemoglobin, isovitalex and V.C.N. added (TM Media) and on blood agar plates for culture of N. gonorrhoeae and other possible causative organisms. The TM plates were incubated at 35-37°C in candle jars and observed at 48 hrs. Typical oxidase positive colonies of gram negative diplococci were tested for dextrose, maltose and sucrose fermentation. Blood agar plates were incubated anaerobically at 37°C. Organisms were identified after 24 hrs. incubation.

The MIC's of penicillin and ampicillin were determined with initial isolates of N gonorrhoeae by the agar plate dilution technique. The concentrations of ampicillin used were 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 μg/ml. Concentrations of penicillin G 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 IU/ml.

RESULTS: A total of 280 patients were studied. Pure gonococcus isolates were obtained from 33 cases (11.7%). The other 247 cases (88.3%) were mixed infections. The bacteria other than N. gonorrhoeae isolated from mixed infections are listed in Table 1. Penicillin G MIC's and MIC's for ampicillin were determined on 158 isolates. The definition of sensitivity is that proposed by Amies (38) and is based on the average peak blood level after a therapeutic dose of a particular drug has been administered. The term "less sensitive" refers to those organisms which will continue to multiply in the presence of >0.05 IU/ml of penicillin or 0.8 μg/ml of ampicillin. In this study 98% of the isolates were shown to be less sensitive to penicillin and 3.2% less sensitive to ampicillin.

DISCUSSION: This report indicates that the frequency of recovery of strains of N. gonorrhoeae less sensitive to penicillin has increased 10.6% since our report on the year 1971-1972 in which 87.2% of the strains studied in this laboratory were less sensitive to penicillin G (39). Most of the patients in the present study had medicated themselves with antibiotics purchased from drug stores without the prescription of a physician. This antibiotic pressure may be responsible for the increase of less sensitive strains.

The MIC's of ampicillin to gonococcal strains showed fewer isolates with reduced sensitivity (3.2%). Ampicillin given orally is still effective against N. gonorrhoeae.

TABLE 1

Bacteria other than N. gonorrhoeae Isolated from Mixed Infections

Organisms	Number of Isolates	Percentage
Staph. epidermidis	113	46.1
Staph. aureus	9	3.7
Alpha streptococcus	33	13.5
Beta streptococcus		0.4
Gamma streptococcus		4.5
Streptococcus fecalis	6	2.5
Diphtheroids	18	7.3
Micrococcus spp.	52	21.2
Enterobacter cloacae	1	0.4
Pseudomonas spp.	1	0.4
TOTAL	245	100.0

TABLE 2

Minimum Inhibitory Concentration of Penicillin G
for N. gonorrhoeae*

MIC of Penicillin G IU/ml	No. of Strains	Percentage
< 0.05	2	1.1
0.05	2	1.1
0.1	3	1.6
0.2	15	8.2
0.4	18	9.8
0.6	38	20.8
0.8	31	17.0
1.0	31	17.0
1.2	14	7.6
1.4	10	5.5
1.6	6	3.3
1.8	9	4.9
2.0	1	0.5
> 2.0	3	1.6
TOTAL	183	100

* Less sensitive strains = 97.8% (Definition of less sensitive strain = MIC > 0.05 IU/ml).

TABLE 3

Minimum Inhibitory Concentrations of Ampicillin for N. gonorrhoeae*

MIC of Ampicillin µg/ml	No. of Strains	Percentage
0.1	5	3.2
0.2	34	21.5
0.4	55	34.8
0.6	35	22.2
0.8	24	15.2
1.0	4	2.5
1.4	1	0.6
TOTAL	158	100

* Less sensitive strains = 3.2% (Definition of less sensitive strains = MIC > 0.8 µg/ml).

TABLE 4

SERUM CONCENTRATION (μg/ml) AFTER ORAL DOSE OF 2 gm AMPICILLIN WITH AND WITHOUT 1 gm PROBENECID

CASES		HOURS AFTER ADMINISTRATION								
		0	1/2	1	2	4	6	8	10	12
1	WITHOUT	0	0	1 8	13 8	7 9	2 5	0	0	0
	WITH	0	4 6	10 6	ND	12 5	7 9	6 8	3 2	0
2	WITHOUT	0	1 6	1 6	6 1	6 1	2 8	1 6	0	0
	WITH	0	5.3	9.3	4 0	23	5 3	1 7	1.6	0
3	WITHOUT	0	1.6	2.0	7.3	2.3	1.6	0	0	0
	WITH	0	1 6	3.3	6.5	9.2	9.2	4.9	2.4	1.6
4	WITHOUT	0	1 5	8.8	18.5	3.3	0	0	0	0
	WITH	0	12.5	12.3	42	20	7.8	3.5	1.5	1.5
5	WITHOUT	0	0	2.5	9.3	6.1	3.2	1.5	1.5	0
	WITH	0	0	4.8	13.8	26.8	13.8	6.7	4.6	2.1
6	WITHOUT	0	0	5.7	7.5	0	6.7	3.3	1.4	0
	WITH	0	2.7	6.5	11.5	14.5	6.2	2.5	1.5	0
7	WITHOUT	0	1.5	1.5	2.9	1.5	1.5	0	0	0
	WITH	0	0	11.2	24.2	7.2	2.1	1.5	0	0
8	WITHOUT	0	0	0	19.0	5.4	2.1	1.3	0	0
	WITH	0	0	1.5	10.0	6 0	>100	6.5	1.5	0
9	WITHOUT	0	8 8	13.2	3.0	6.0	0	0	0	0
	WITH	0	0	4.0	25.5	29	11.5	3.5	1.4	0
10	WITHOUT	0	0	0	4.3	6.6	2.3	0	0	0
	WITH	0	0	1.6	5.7	38.5	14.5	6.6	2.2	0

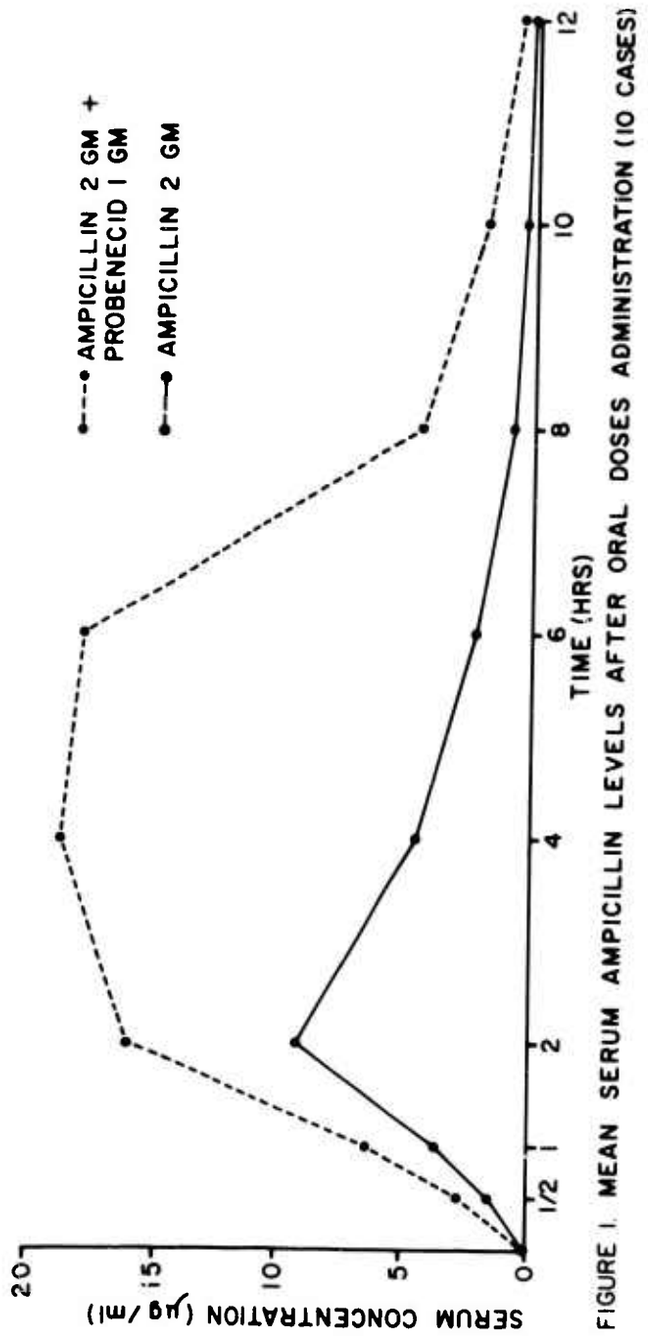


FIGURE 1. MEAN SERUM AMPICILLIN LEVELS AFTER ORAL DOSES ADMINISTRATION (10 CASES)

PART II:

DESCRIPTION: Ten male patients with uncomplicated gonorrhea were studied. All were hospitalized throughout the course of infection at the Royal Thai Army Hospital, Bangkok. Two sets of blood specimens were obtained from each subject. A control specimen of 3 ml of blood was taken from each patient when admitted. Ampicillin (2 gm) was administered orally in a single dose and blood specimens were taken 30 minutes, 1, 2, 4, 6, 8, 10 and 12 hours afterward.

The follcwing day 1 gm of probenecid and 2 gm of ampicillin were given to the same patients and the same sampling schedule was followed.

RESULTS: Ampicillin serum levels after an oral dose of 2 gm with and without 1 gm of probenecid are presented in Table 4. The mean maximum concentration was observed at 2 hours (9.2 µg/ml), and the drug was detectable until 8 hours after administration. When 1 gm of probenecid was administered with the ampicillin, the highest mean serum level observed was 18.7 µg/ml at the 4th hour. Serum levels remained high until 6 hours after administration, then decreased rapidly to 0.05 µg/ml in the samples obtained at 12 hours (Fig. 1).

DISCUSSION: The data presented in Table 4 illustrate that there is a variation in serum ampicillin levels among the ten patients. Many factors such as protein binding of the antibiotic, the status of the gastric mucosa during treatment, presence or absence of food, and the enzyme penicillinase produced by some organisms in the body may play a role in this variation. A single oral dose of ampicillin combined with probenecid produced higher serum levels than ampicillin alone. The drug was detectable in serum at one-half hour and increased to a maximum concentration at 4 hours after ampicillin and probenecid were administered. The serum level remained high until 6 hours and decreased to minimum levels after 12 hours.

2. Studies on the Susceptibility of Gibbons to Gonococcal Infection

These studies were conducted to determine whether the white-handed gibbon (Hylobates lar) would serve as a satisfactory host for experimental infection with Neisseria gonorrhoeae.

BACKGROUND: Attempts to produce experimental gonococcal infection in common laboratory animals have invariably failed in the past (40, 41). Varying degrees of success have been reported in infecting rabbits by inoculating N. gonorrhoeae into the anterior chamber of the eye (42) and by culturing the organism in a golf ball subcutaneously implanted into the rabbit (40). Experimental gonococcal urethritis has been successfully produced by inoculating pus from gonorrhea patients (43) or Type 1 gonococcal suspension grown in vitro into the chimpanzee

intra-urethrally (44). Gonococcal urethritis in chimpanzees resembles urethritis which occurs in humans. The gibbon is often used as an experimental animal in the study of diseases of humans; therefore, it was deemed practical to attempt an experimental infection with N. gonorrhoeae. The study was divided into four parts.

PART I: Cellular Components of Gonococcal Urethral Exudate of Male Patients

INTRODUCTION: Gonococcal urethral exudates of male patients have been widely used as inocula to produce experimental gonorrhea both in human volunteers and in laboratory animals. A study on the phagocytic activity, the cellular composition, and the concentration of viable gonococci in acute gonorrheal exudates was done.

DESCRIPTION: Male patients with urethritis who had intracellular gram negative diplococci in the urethral exudate smear were selected for this study. From each patient, a urethral exudate smear was prepared and a small amount of the exudate was transferred by means of a wire loop into a sterile centrifuge tube containing 1 ml of GCBID medium (GC broth supplemented with 1% Isovitalex and 10% defined supplement of Dr. D. S. Kellogg). The gram stained smears were examined and phagocytosis and extracellular gonococci quantitated.

Phagocytic activity was defined as the per cent of 400 polymorphonuclear leukocytes (PMN) which contained gram negative diplococci, morphologically typical of gonococci and in the focal plane of the PMN. Extracellular gonococci were recorded as the per cent of 500 gonococci distributed randomly in the smear both intracellularly and extracellularly. A differential white blood cell count was performed on 100 WBC per smear. The number of viable gonococci present in the exudate was determined by enumerating colonies on GCBID agar after incubation at 36°C.

RESULTS: Results obtained from the urethral exudates of 12 Thai male patients are given in Table 1. The amount of urethral discharge obtained from each patient was small and variable. The exact quantity of each exudate sample was not measured.

DISCUSSION: The results indicate that almost all gonococci contained in the acute urethral discharge are intracellular. Of the 12 specimens studied, extracellular gonococci ranged from 0 to 15% with an average of 6%. The number of viable organisms varied from 2.4×10^5 to 4.6×10^6 per patient sample. Such variation may have been due in large part to variation in the amount of exudate collected from each patient. Average per cent PMN among the specimens was 97% with a narrow range of distribution from 93 to 98%. Phagocytic activities of these PMN varied from 2 to 51% with an average of 25%. Such variation in the phagocytic activity of PMN is not unexpected and agrees well with in

TABLE I. CELLULAR COMPOSITIONS OF SMEARS PREPARED FROM GONORRHEAL URETHRAL EXUDATES OF MALE PATIENTS.

PATIENT NO.	GONOCOCCI		LEUKOCYTES	
	EXTRACELLULAR PER CENT	VIABLE COUNTS PER SAMPLE $\times 10^5$	PMN PER CENT	PHAGOCYTOSIS PER CENT
1	13	4.4	96	28
2	4	2.4	98	23
3	3	5.6	98	51
4	7	3.5	98	6
5	1	9.8	98	2
6	15	13.0	93	26
7	11	16.0	98	27
8	0	5.1	96	29
9	1	46.0	95	49
10	8	2.8	97	13
11	2	6.4	98	19
12	8	13.0	95	32
AVERAGE	6	14.8	97	25

vitro results reported elsewhere. The study failed to show any correlation between the percentage of extracellular gonococci and the number of viable cells in the exudate.

PART II: Effect of in vitro Incubation at Higher Temperature (39°C) on Type I Gonococci

INTRODUCTION: Inoculation of virulent gonococci, either as a suspension of pure culture or as purulent exudate obtained directly from patients, into sub-human primates (e.g., the gibbon) consistently failed to produce gonococcal infection (45). Gonococci grow optimally at 36°C; therefore, the failure of gonococci to grow and establish infection in the gibbon might be due to the sensitivity of gonococci to higher temperature. Because the normal body temperature of the gibbon is 39°C, we wished to determine the effect of long exposure at this temperature on gonococci.

DESCRIPTION: Both N. gonorrhoeae F62 Type I in pure culture and Type I gonococci contained in urethral exudate obtained from acute gonorrhea patients were used in this study. The gonococcal suspension was prepared and diluted with GCBID medium. Triplicate samples of each dilution were incubated both at 36°C and 39°C. Incubation was carried out for 48 hrs before determining viability.

RESULTS: Figure 1 shows the viability of gonococci at 39°C compared to that at 36°C. The total viable gonococcal colonies counted after 48 hours of incubation at 36°C was taken as 100% viability, and viability of gonococci at 39°C was expressed as per cent of viability at 36°C.

In all 4 specimens, viability of urethral exudative gonococci at 39°C was reduced, e.g. 0.01, 85.3, 63, and 33% of their corresponding viability at 36°C. Viability of N. gonorrhoeae F62 in pure culture was unaffected by incubation at the raised temperature. Colonial typing was performed after 24 hours of incubation. No morphological differences were observed in colonies produced by gonococci in pure cultures or gonococci in exudate after incubation at 36°C or 39°C.

DISCUSSION: Type I gonococci in the urethral exudates demonstrated loss of viability after incubation at 39°C. While there was nearly total loss of viability in one exudate, there was no significant change in two other exudates. Conversely, gonococci in pure culture (F62 Type I) survived incubation at 39°C. It is tempting to associate the sensitivity of exudative gonococci to incubation at high temperature with their exposure to the PMN which were present in abundance in the urethral exudates. In this regard the relatively higher phagocytic rate in the two affected exudative specimens (Table 2) supports this hypothesis; however, direct studies on the phagocytic and bactericidal effect of exudate on gonococci is needed to establish the point.

TABLE 2: Viability of N. gonorrhoeae from Urethral Exudates and in vitro Culture Suspensions of Strain F62.

Exudate No.	Loss of Viability at 39°C (per cent of viability at 36°C	Phagocytic activity* (per cent)
1	99.9	51
2	14.7	6
3	37.0	2
4	67.0	26
N.gonorrhoeae		
F62 (1)	5	-
" (2)	0	-

* Phagocytic activity observed in the urethral exudate from patients with gonococcal urethritis.

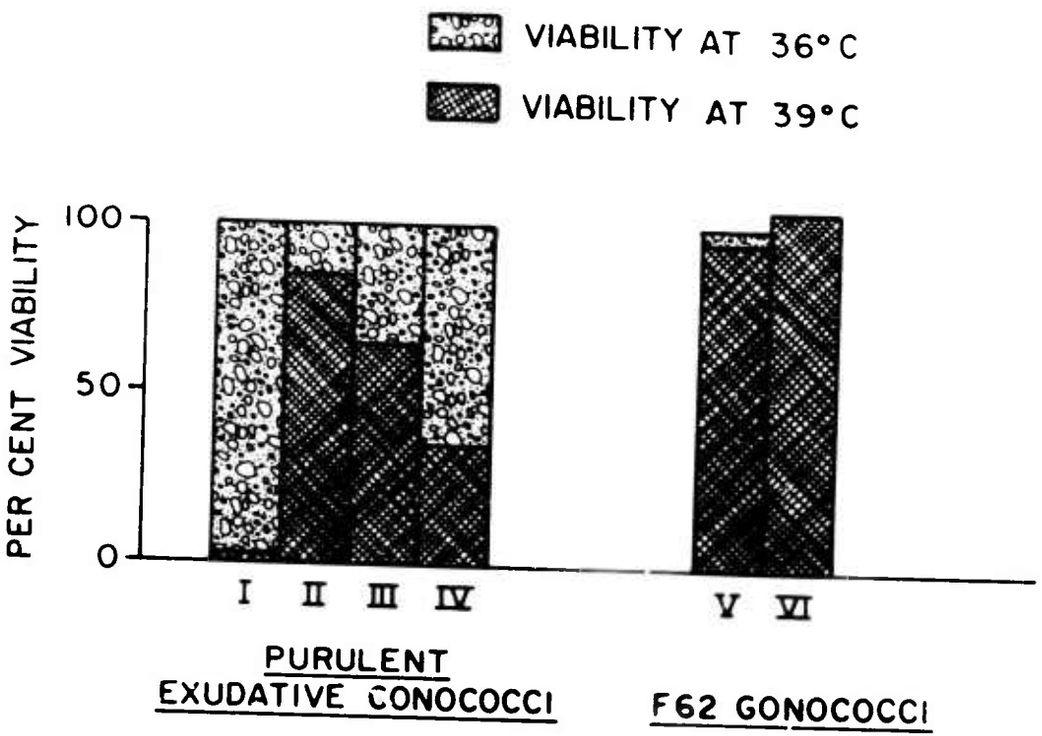


FIGURE 1. VIABILITY OF TYPE I GONOCOCCI AT 36°C ( ) AND AT 39°C ( ) WHICH WAS EXPRESSED AS PER CENT OF VIABILITY AT 36° C. FOUR PURULENT URETHRAL EXUDATES (I – IV) WERE OBTAINED FROM FOUR MALE PATIENTS WITH ACUTE URETHRITIS. N. GONORRHOEAE F62 TYPE I WAS TESTED IN TWO EXPERIMENTS (V – VI). INCUBATION WAS TERMINATED AFTER 48 HRS.

Although the data are limited, the results indicate that in vitro incubation at 39°C for 48 hours does not adversely affect virulent gonococci in pure culture either in viability or in colonial type. Less conclusive is the effect of incubation at 39°C for 48 hours on exudative gonococci from patients. The evidence indicates that gonococci in urethral exudate have reduced viability after incubation at 39°C; phagocytosis may be responsible.

PART III: In vitro Phagocytic and Bactericidal Activity of Gibbon Leukocytes on Virulent Gonococci (F62).

INTRODUCTION: As an alternative to human volunteers, subhuman primates have been used as close-to-man models for gonococcal infections, both successfully in the case of chimpanzees (43) and unsuccessfully in the case of gibbons (45). Since the susceptibility of man to gonococcal infections has been attributed to the relatively low bactericidal activity of his leukocytes compared to that of lower animals, it was necessary to define the interaction of gibbon leukocytes and the virulent gonococci.

DESCRIPTION: Neisseria gonorrhoeae F62 Type I and pooled gibbon leukocytes were used throughout this study. The in vitro phagocytic system of Smith and Wood was employed.

Gonococcal suspension: F62 Type I gonococci were grown on GCBID agar for 15-16 hours at 39°C in candle jars. Bacterial growth was scraped with a glass rod, washed off the plate with warm (39°C) GCBID broth and centrifuged at 1,400 x g at 26°C for 20 minutes. The gonococci were resuspended in warm GCBID broth. The suspension was further agitated in a mixer for 2 minutes and diluted to a final concentration of 5×10^{10} gonococci per ml. The concentration of gonococci was determined by direct count in a Petroff-Hausser Chamber (C. A. Hausser and Son, Pa.). The viability of gonococci was evaluated by enumerating colony forming units on GCBID agar.

Gibbon leukocytes: Heparinized samples of gibbon whole blood were mixed with an equal volume of 6% dextran solution and centrifuged at 1,400 x g, at 4°C for 30 minutes. The buffy coat was harvested, resuspended in Hank's balanced salt solution supplemented with 0.1% glucose (HBG), and recentrifuged as previously described. The buffy coat of the second centrifugation now contained only a small number of red blood cells. Concentration of the leukocytes was determined by counting in a hemocytometer.

Phagocytosis: 1.25×10^9 gonococci, in a volume of 0.025 ml, and 0.1 ml of HBG, were mixed with 2.5×10^8 packed gibbon leukocytes, and 0.06 ml of the mixture was distributed evenly over a 2 x 2 cm area of a glass slide. After incubation in a damp filter paper-lined petri dish chamber at 39°C for 30 minutes, the cells were recovered from

the slide with 5 ml HBG and collected after centrifugation at 180 x g for 3 minutes at 4°C. Smears were prepared and stained with methylene blue. The amount of phagocytosis was expressed as the per cent of 400 polymorphonuclear leukocytes (PMN) that contained one or more gonococci.

For determination of bactericidal activity, incubation of the phagocytic mixture was prolonged. Phagocytic mixtures were recovered after incubation at 39°C for 0, 1, and 2 hours and viability determined by the viable count method. All tests were done in duplicate and the mean was taken as the result.

RESULTS: Table 3 presents the results of two identical experiments. Throughout this study, cultivation of gonococci and incubations of phagocytic mixtures were carried out at 39°C which is the average body temperature of gibbons.

Table 3. In vitro Phagocytic Activity of Gibbon Leukocytes and Viability of F62 Type I Gonococci in the Concentrated Test System after Incubation at 39°C

Experiment No.	Phagocytosis 30 min (%)	Viability after 1 hr (%)	Viability after 2 hrs (%)
1	49	52	50
2	53	42	18
Average	51	47	34

DISCUSSION: High concentrations of gibbon leukocytes phagocytize gonococci efficiently at 39°C, presumably their optimal temperature; they also kill the virulent gonococci intracellularly. Further experiments will be carried out.

PART IV: Inoculation of Gibbons with an Exudative Gonococcal Suspension

DESCRIPTION: Four adult male gibbons were anesthetized and inoculated intra-urethrally via polyethylene catheters with 0.1 ml of gonococcal suspension prepared from pooled urethral discharges of 10 male patients with acute gonococcal urethritis and collected in a centrifuge tube containing 0.5 ml GCBID broth. The number of viable gonococcal colony forming units in each inoculum was determined. The gibbons were observed for any indication of infection, and bacteriological cultures, serum samples, and clinical observations were followed both prior to and after the inoculation.

RESULTS: Each gibbon was inoculated intra-urethrally with 5×10^6 viable gonococcal colony forming units. Bacteriological cultures showed little change in the microflora of the urethra, the conjunctival sac, or the rectum of the inoculated gibbons. Gonococci were absent in all cultures throughout 30 days of observation. During the course of observation, smears prepared from urethral exudates of the gibbons were uniformly negative for gram-negative diplococci, except in the instance where "one extracellular gram-negative diplococcus suspected" was reported in one gibbon on day 4 after inoculation. While bacteriological cultures of urethral exudate of this gibbon did not confirm the presence of gonococci, clinical observation was encouraging, i.e., the penis was reportedly reddened from day 5 to 11 and the normally clear exudate became purulent on day 13 through day 30. Three of the four gibbons developed some degree of inflammatory response as indicated by elevation of WBC and by clear discharges. Results of the serological studies are not yet available.

DISCUSSION: The uniform absence of gonococci in cultures taken at the site of inoculation indicate failure of the dose of 5 million viable gonococcal colony forming units to establish infection in the urethra of the gibbon. Attempts to produce the infection with a larger inoculum of gonococci are underway.

3. Oropharyngeal Gonorrhea During Pregnancy

BACKGROUND: The frequency with which Neisseria gonorrhoeae can be cultured from pregnant patients has been reported to vary from 1-7.3% (45), dependent upon the population studied. These reports have been based primarily upon cultures obtained from the urogenital tract. The finding of N. gonorrhoeae in the oropharynx is not new; however, due to the pandemic of the disease caused by this organism, more attention has been focused on this site. The purpose of this investigation is to determine the frequency of isolation of N. gonorrhoeae from the oropharynx as well as the urogenital tract in a pregnant population.

DESCRIPTION: The study sample consisted of all patients reporting for their first prenatal visit of their current pregnancy to the Obstetrics Outpatient Clinic of the U.S. Army Hospital in Bangkok, Thailand, between 30 August 1973 and 31 December 1973. During this time period 154 patients, all of whom were dependents of U.S. Government personnel stationed or retired in Southeast Asia, came for their first visit. Since complete data on four patients was not available due to Bacillus overgrowth of their cultures, 150 patients constitute the study population.

A throat culture on all patients was obtained at the time of the initial physical examination by swabbing the patient's oropharynx with a sterile cotton-tipped applicator and streaking it directly on freshly prepared Thayer-Martin (VCN) media. The culture specimens from the

cervix and the rectum were obtained in a manner identical to that previously described (46). Culture plates were incubated at 37°C under increased CO_2 atmosphere in candle jars. Cultures were inspected at 24 and 48 hour intervals and those colonies with gross morphology resembling Neisseria species were subjected to gram staining and testing with oxidase reagent. All colonies of gram-negative diplococci that gave positive tests with oxidase reagent were transferred to fresh Thayer-Martin (VCN) media and taken to the SEATO Medical Research Laboratory (SMRL) for confirmation by sugar fermentation. When the presence of N. gonorrhoeae was confirmed, the patients were recalled and those not allergic to penicillin were treated with aqueous procaine penicillin, 4.8 million units intramuscularly, 45 minutes after they had received two grams of probenecid orally. The patients were instructed to have their sexual contacts report for treatment. In the event that a patient was allergic to penicillin, she was treated with erythromycin, 500 mg orally, four times a day for one week. The patients were instructed to return one week after treatment for repeat culture. Serological tests (VDRL) for syphilis were performed on all patients upon their return and at one and three month intervals following the initial isolation.

PROGRESS: Patients that constituted the sample were composed of 61% Thai, 31% Caucasian, and 9% of other ethnic origins. The last group consisted of patients of Negro and Asian descent other than Thai. The patients' ages ranged from 16 to 39 years with an average of 25.5 years. Primigravidas constituted 31% of the population, 32% were secundigravidas and 37% of the patients were gravida three or greater. The average gravidity was 2.4. The patients' gestational age at time of first visit varied from six to thirty-nine weeks with the average patient presenting at 17.2 weeks gestation. A diagnosis of N. gonorrhoeae was made in 24 of the 150 patients studied, giving a frequency of 16% in this population.

The most frequent site for a positive culture was the oropharynx from which 23 of the 24 positive cultures (96%) were obtained. A cervical culture accounted for the one remaining positive culture, and none of the rectal cultures were positive. There were no patients that had positive cultures from more than one site. Average age of those patients with positive cultures was 24.2 years (range 19-30 years). Ethnic origins were 54% Thai, 33% Caucasian and 12% other races. The average gravidity was 2.1 with 29% primigravidas, 42% secundigravidas and 29% gravida 3 or greater. The percentage of secundigravidas in the infected group was significantly higher than in the study sample, but the average ages of these patients (25.1 years for the infected secundigravidas and 24.8 years for all secundigravidas) was found to be not significantly different. Patients who had positive cultures presented for their first clinic visit between 6 and 30 weeks gestation with the average being 14.9 weeks. This was not significantly earlier

than for the study population as a whole. Follow-up evaluation of the patients with N. gonorrhoeae revealed that none of these patients developed a positive serological reaction for syphilis. There was no case in which the patient, after antibiotic therapy, was found to have a subsequent positive culture for N. gonorrhoeae. The gestational and postpartum courses of all patients with positive cultures were completely uneventful and their resultant infants manifested no evidence of disease.

DISCUSSION: The finding that 16% of the patients studied were harboring N. gonorrhoeae was greater than what was expected, whereas 1 positive cervical culture out of 150 patients agrees with previous studies of military dependent populations (46). The major difference between this study and previous studies was the incorporation of the oropharynx as a culture site and this site accounted for the majority (96%) of the positive cultures. The high frequency of positive throat cultures may be partially explained by the fact that the gonococcus is an organism that has an apparent predilection for glandular tissue as demonstrated by the presence of the organism in Skene's and Bartholin's glands and the glands of the endocervic canal. The means by which the oropharynx becomes infected is not presently known; however, direct inoculation through fellatio is a distinct possibility. The question of the practice of fellatio was confirmed by some, but not all, of the patients with positive oropharyngeal cultures. A large percentage of our patients who had positive cultures from the oropharynx were of Thai origin which is predominantly of the Buddhist faith and, although the practice of fellatio is not condemned in Buddhist teachings, it is generally not condoned. Oral to genital transmission of N. gonorrhoeae has been shown to occur (47). Oral to oral transmission of this organism has not been demonstrated at the time of this report.

The possibility that the organism gains access to the glandular oropharyngeal tissues by lymphatic spread is questionable, but possible. This would entail the organism traveling via the lymphatic channels to these areas and, although one might propose that the organism would become trapped and phagocytized in the numerous lymph nodes between the urogenital tract and the oropharynx, systemic manifestations of gonococcal infections such as arthritis and pericarditis have been reported in patients with venereally acquired gonococcal infections. The greater number of infected patients being secundigravidas compared to the entire group is an interesting finding for which we have no explanation. One might propose that these patients would fall into an age range where venereal disease was most common; however, the ages of the infected secundigravidas did not differ significantly from the non-infected secundigravidas nor from the study population as a whole.

We conclude that:

a. The oropharynx may be a major asymptomatic reservoir for the gonococcus and significantly contribute to the pandemic of the disease caused by this organism.

b. The oropharynx should be routinely cultured for N. gonorrhoeae in sexually active persons to more effectively determine its prevalence.

SUMMARY: A prenatal population of military dependents was surveyed to determine the incidence of asymptomatic gonorrhea. A total of 150 patients were sampled over a 4 month period and 24 patients had cultures positive for N. gonorrhoeae. Of the 24 positives, 23 were obtained from the oropharynx and 1 from the cervix. No positive cultures were obtained from the rectum and no patients had more than 1 positive site.

E. MELIOIDOSIS

1. Two Pediatric Cases of Melioidosis

BACKGROUND: SEATO Medical Research Laboratory has carried out extensive research on the geographic distribution of Pseudomonas pseudomallei in soil and water throughout Thailand. Large scale surveys have been conducted in search of clinical cases and serologic evidence of infection. From 1964-1970 only two clinical cases of melioidosis were detected (see Annual Reports, 1965-1970). Two brothers, ages 26 and 16, had Pseudomonas pseudomallei isolated from their sputa by guinea pig inoculation techniques. Both patients had respiratory symptoms and chest x-rays consistent with a diagnosis of pulmonary melioidosis.

PROGRESS: During January and February 1974 two cases of melioidosis were seen at Bangkok Children's Hospital.

Case 1

History: A 10 year old Thai female presented with fever, weight loss and generalized weakness of 5 months duration.

Physical Examination: T 38.8°C, pulse 116/min, respiratory rate 28/min, BP 100/60 mmHg, Wt 14 kg. The patient was pale, lethargic, and had edema of both upper palpebrae. A soft tissue (2 cm) mass was noted at the glabella and two (3 cm) soft occipital masses were present. The patient was extremely emaciated. No other significant physical findings were noted.

Laboratory Findings: Hgb 4 gm%, WBC 17,800, Diff. 83%n 17%L. Chest x-ray revealed right lower lobe pneumonia with 2 cm abscess cavity in the right middle lobe.

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Clinical Course: The patient died 24 hours after admission to the hospital while receiving high doses of penicillin parenterally. Post-mortem examination revealed abscesses in spleen, pancreas, liver and lung. The gross and microscopic findings were consistent with systemic melioidosis. Pseudomonas pseudomallei was isolated from blood, glabella, soft tissue abscess and bone marrow.

Case 2

History: A 15 month old Thai female presented with fever and cough of 3 weeks duration. Hemoptysis was observed on the day of admission.

Physical examination: T 38.8°C, pulse 110/min, respiratory rate 64/min, Wt 6.5 kg. There was marked dyspnea and coarse rales were heard in both lungs.

Laboratory Findings: Hgb 7.5 gm%, WBC 58,400, 83%N 11%L, 6%M. Chest x-ray revealed bilateral pulmonary infiltration predominately involving the upper lobes.

Clinical Course: Pseudomonas pseudomallei was cultured from a percutaneous lung aspirate. The child died 48 hours after admission despite parenteral administration of chloramphenicol and penicillin. No autopsy was performed.

The identification of both isolates of Pseudomonas pseudomallei was confirmed at the Walter Reed Army Institute of Research.

DISCUSSION: These two cases of melioidosis in the pediatric age group are the first ones recognized at Children's Hospital. Reports of clinical melioidosis in Thailand are rare. Clinical awareness of the disease and supporting bacteriological laboratory facilities are necessary to make the diagnosis. The finding of more cases of melioidosis at Children's Hospital may elucidate the epidemiology, clinical presentation and natural course of melioidosis in Thailand.

F. BACTERIAL MENINGITIS

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 gram stain and culture results from the infected body fluid.

BACKGROUND: Bacterial capsular antigens of pneumococcus, Hemophilus influenza type b, and meningococcus have been detected in body fluids of patients with disease due to these bacteria. Specific bacterial antigens have been detected in cerebrospinal fluid (CSF), subdural

fluid, synovial fluid, serum and urine using CIE and specific antisera. The CIE technique may detect infection even when gram stains and cultures are negative. It has been estimated that CIE gives false negative results in about 10% of CSF specimens (CIE negative but culture positive).

Purulent meningitis is common at Children's Hospital, Bangkok. Cases seen annually number 80-120. Gram stains and bacterial cultures of the CSF have been negative in over 50% of the cases seen in the past six months. Prior treatment with antibiotics is one reason for the failure to recover organisms in culture.

Empyema cases are also common at Children's Hospital. Cases seen annually number 80-100. Gram stains and cultures are negative in approximately 50% of the cases.

Hemophilus influenza type b and pneumococcus are the most frequent bacterial isolates from CSF; pneumococcus and staphylococcus are the most frequent isolates from empyema specimens.

DESCRIPTION: Gram stains and cultures were performed on CSF specimens from cases of suspected purulent meningitis and on pleural fluid specimens from cases of empyema using routine bacteriological methods. CIE using antisera against pneumococcus and antisera against Hemophilus influenza type b was carried out by a previously described method (48). Follow-up CSF and empyema specimens had cultures and CIE performed. Subdural and pericardial fluids were also studied when available.

PROGRESS: Hemophilus influenza type b antigen has been detected in 13 CSF specimens. Positive culture confirmed 11 of these specimens (Table 1). Gram stains were positive on only three occasions. There has been only 1 false negative (CIE negative but culture positive). Hemophilus influenza type b antigen has persisted in the CSF for 2-5 days. Subdural effusions from 2 of these 14 patients have had CIE detectable H. influenza type b antigen. One patient with a purulent pericardial effusion has had detectable H. influenza type b antigen in pericardial fluid.

Pneumococcal antigen were detected in 4 CSF specimens (Table 2); 3 were confirmed by gram stain and 2 have been confirmed by culture. There has been 1 false negative (CIE negative but culture positive). Pneumococcal antigen persisted in the CSF for 11 days (1 patient) and 13 days (1 patient). Cases of tuberculous meningitis and salmonella meningitis have not cross-reacted with the pneumococcal or Hemophilus influenza type b antisera.

Pneumococcal antigen has been detected by CIE in 15 cases of empyema (Table 3). Of these 15, 6 have had both positive gram stains and positive cultures for pneumococcus; 2 have had positive gram stains but

Table 1. Presence of Hemophilus influenza type b Antigen in CSF, Subdural Fluid and Pericardial Fluid

Case	Source	Gram Stain	Culture	CIE	Duration
1	CSF	+	+	+	4 days
2	"	+	+	+	3 days
3	"	+	+	+	5 days
4	"	-	+	+	4 days
5	"	-	+	+	
6	"	-	+	+	
7	"	ND	+	+	
8	"	ND	+	+	
9	"	ND	+	+	
10	"	ND	+	+	
11	"	ND	+	+	
12	"	ND	-	+	3 days
13	"	ND	ND	+	2 days
14	"	-	+	-	
15	Subdural	-	-	+	9 days
16	"	-	-	+	
17	Pericardial	ND	ND	+	4 days

ND = no determination

Table 2. Presence of Pneumococcal Antigen in CSF

Case	Gram Stain	Culture	CIE	Duration
1	+	+	+	13 days
2	+	-	+	11 days
3	+	-	+	
4	-	+	+	
5	-	+	-	

Table 3. Presence of Pneumococcal Antigen in Empyema Fluid

Case	Gram Stain	Culture	CIE	Duration
1	+	+	+	
2	+	+	+	
3	+	+	+	
4	+	+	+	
5	+	+	+	
6	+	+	+	
7	+	-	+	
8	+	-	+	10 days
9	-	+	+	11 days
10	-	+	+	
11	-	-	+	13 days
12	-	-	+	
13	-	-	+	
14	-	-	+	
15	-	-	+	

negative cultures; and 2 have had negative gram stains and positive cultures; 5 have had negative gram stains and negative cultures. CIE detectable antigen has persisted from 10-13 days in 3 patients studied. There have been no cross reactions in empyema cases with isolates of E. coli or Staphylococcus aureus. There have been no false negatives (CIE negative but culture or gram stain positive).

DISCUSSION: CIE is a useful clinical laboratory procedure for detecting capsular bacterial antigens of pneumococcus and Hemophilus influenza type b in infected body fluids. This technique can detect bacterial antigen when gram stains and cultures are negative. Results using CIE are more rapidly available than culture. In the experience at Children's Hospital CIE results are more reliable than gram stains, cultures or the combination of gram stain and culture. This technique is particularly useful in cases of purulent meningitis or empyema that have received prior antibiotic therapy, in which gram stains and cultures are often negative.

III. PARASITIC DISEASES OF MAN AND ANIMALS

A. FILARIASIS

1. Survey for Bancroftian Filariasis in Kanchanaburi Province

The objective of these studies was to determine the incidence of microfilaremia among villagers within Sangkhlaburi district, Kanchanaburi Province, and to assess the potential of this area for more detailed studies of bancroftian filariasis.

BACKGROUND: Harinasuta, et. al., (49) examined blood films from 1,549 people from 10 villages along the river Kwae Noi in Kanchanaburi Province. Approximately 13% of these were positive for microfilariae. In addition, they found various larval stages of Wuchereria bancrofti in 2 species of Aedes and 3 species of Anopheles mosquitoes; however, third stage larvae were found only in mosquitoes of the Aedes (F.) niveus group. This group of mosquitoes is in a state of taxonomic confusion, and it is suspected that as many as 5 species may presently be identified as Aedes niveus. Thus, the identity of the vector of bancroftian filariasis in this area is in doubt.

DESCRIPTION: Three site visits were made to the Sangkhlaburi district during this reporting period. On the first visit in November 1973 the principal objective was to collect mosquitoes of the Aedes (F.) niveus group for taxonomic study. On the second and third visits, in December 1973 and March 1974 respectively, blood films were taken to determine the prevalence of microfilaremia in inhabitants of several villages located along the Kwae Noi river.

PROGRESS: From 9-15 November 1973 adult mosquitoes were collected

Table 1. Mosquitoes Collected in Sangkhlaburi District, Kanchanaburi Province - November 1973

Species	No. Collected
Biting man inside houses	
Aedes (S.) albopictus	2
Mansonia (Mn.) dives	3
Resting inside houses	
Aedes (S.) aegypti	2
Armigeres (Armi.) subalbatus	2
Armigeres (Lices) magnus	1
Culex (C.) pipiens quinquefasciatus	3
Culex (C.) vishnui complex	1
Culex (Culicio) pallidothorax	1
Heiz sp.	8
Biting man outside houses	
Anopheles (An.) barbirostris	3
Anopheles (C.) maculatus	1
Anopheles (C.) philippinensis	1
Aedes (S) albopictus	28
Aedes (S) annandalei	2
Aedes (S) desmotes	2
Aedes (S) gardnerii imitator	6
Aedes (F) niveus group	1
Armigeres (Armi) subalbatus	1
Culex (C) vishnui complex	4
Culex (C) whitmorei	5
Mansonia (Mn) dives	60
	137

Table 2. Results of Blood Film Examinations for Microfilariae, (MF) in 7 Villages of Sangkhlaburi District, Kanchanaburi Province - December 1973 and March 1974

Village	Males			Females			Both Sexes		
	No. Exam.	No. MF-Pos	Per cent Pos	No. Exam.	No. MF-Pos	Per cent Pos	No. Exam.	No. MF-Pos	Per cent Pos
Ku Phra[illegible]	30	9	30	39	4	10	69	13	19
Lawa	21	1	5	22	1	5	43	2	5
Ni-tha[illegible]	93	0	0	131	1	0.8	224	1	0.4
Nong Pa Dong	18	3	17	10	1	10	28	4	14
Kui Yatho	24	6	25	30	5	17	54	11	20
Via Ka Dee	[illegible]	3	23	12	3	25	25	6	24
Koeng Chada	[illegible]	2	13	11	0	0	26	2	8
Totals	214	24	11	255	15	6	469	39	8

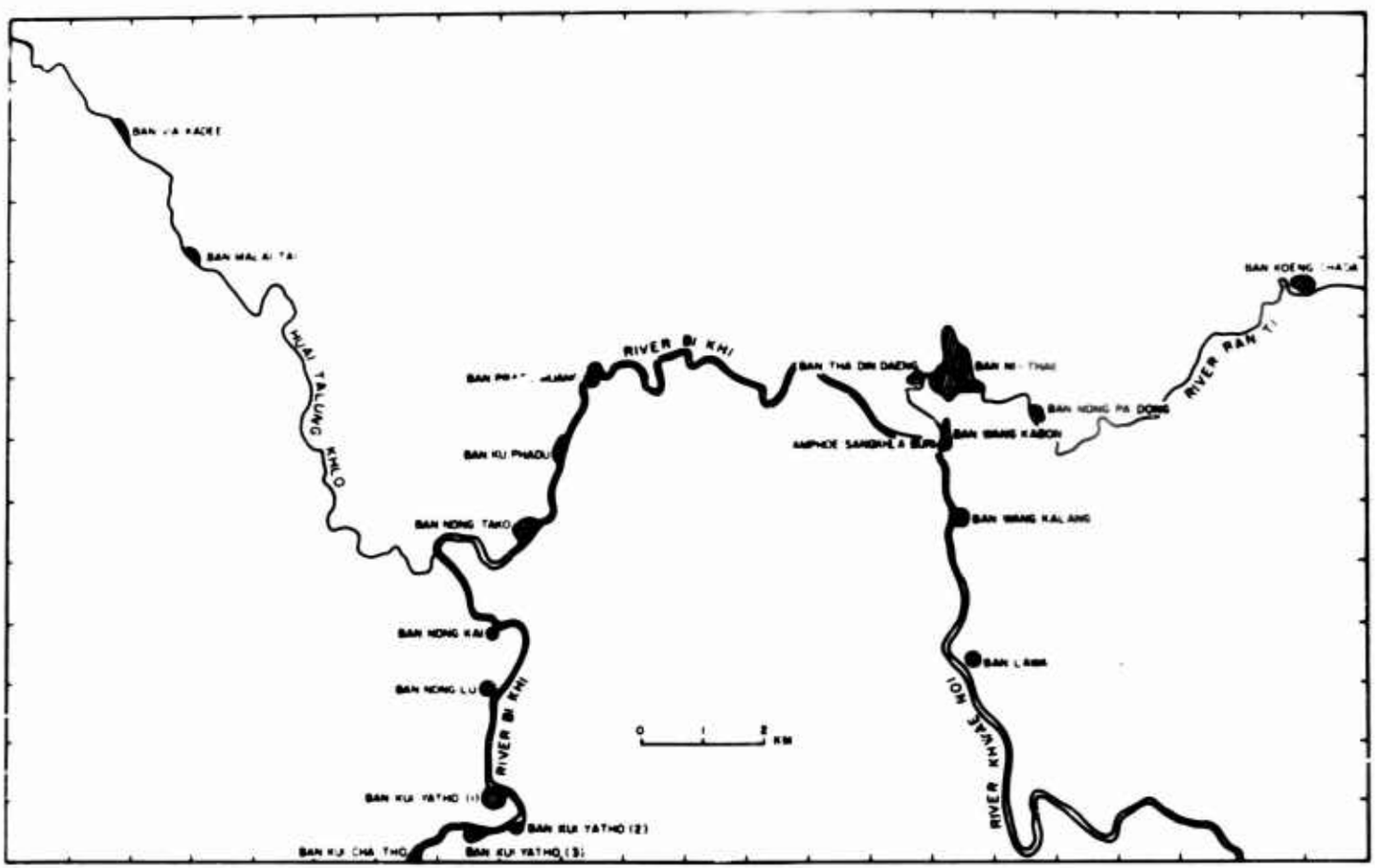


FIGURE 1. MAP SHOWING THE DISTRIBUTION OF VILLAGES ALONG THE RIVER KHWAE NOI AND SOME OF ITS TRIBUTARIES, SANGKHLABURI DISTRICT, KANCHANABURI PROVINCE.

while biting man, both inside and outside houses, as well as resting inside houses (Table 1). Only 1 adult specimen belonging to the Aedes (F.) niveus group was collected. These collections were made in the dry season when the populations of these mosquitoes were probably at their lowest levels. Harinasuta and his co-workers collected only 5 specimens of Mansonia, all of which were M. uniformis, in Sangkhlaburi district over a 13 month period. No M. uniformis were collected in November 1973; however, M. dives was the most common species collected biting man, with 63 of the 137 adults collected belonging to this species. Various members of the genus Mansonia are considered important vectors of bancroftian filariasis in Southeast Asia and other parts of the world, and it is possible that M. dives is involved in transmission of filariasis in Sangkhlaburi district as well. All of the adult specimens listed in Table 1 were dissected for filarial parasites but were found negative.

Results of blood-film examinations for microfilariae taken from seven villages within the Sangkhlaburi district are shown in Table 2. Of the 469 slides examined, 39 or 8% of the total number examined were positive for Wuchereria bancrofti. The incidence of microfilaremia ranged from 0.4% for Ni-thae village to 24% for Via Ka Dee (Fig. 1). The youngest person found to have circulating microfilariae was an 18 year old female from Ni-thae; however, in Ni-thae over 50% of the sample population were less than 15 years of age, whereas all persons sampled in the other villages were older than 15 years. Only 6% of the slides from females had microfilariae, 11% of the blood films from males were positive.

B. GNATHOSTOMIASIS

1. Chemotherapy of Gnathostomiasis

OBJECTIVE: To find drugs with chemotherapeutic activity against Gnathostoma spinigerum.

BACKGROUND: These studies are a continuation of work reported in previous years. Drugs with activity against other parasitic diseases are being evaluated for possible chemotherapeutic activity against Gnathostoma spinigerum in experimentally infected mice.

METHODS: ICR mice were infected by oral administration of 5 advanced third-stage larvae of G. spinigerum. After infection the test drug or combination of drugs, dissolved in distilled water, was administered orally 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 counted in the liver and/or body muscles, and results recorded.

The drugs tested were: Metrifonate (O, O-dimethylhydroxy-2, 2, 2-trichlorethylphosphonate); Flagyl or Metronidazole (1-β-hydroxyethyl-2-methyl-5-nitromidazole); Astiban (sodium antimony dimercapto-succinate); Ambilhar or Niridazole (1-(5-nitro-2-thiazolyl)-2-imida-zolidinone); and a combination of Astiban and Ambilhar.

RESULTS:

Metrifonate: This drug was administered in five oral doses given at two week intervals. The results are shown in Table 1. Doses of 20, 40, 80, and 120 mg/kg were ineffective in reducing the number of larvae found in treated mice.

Flagyl: We previously administered five oral doses of 200-1600 mg/kg of Flagyl to gnathostome infected mice without effect. This year we administered a five day oral course of 200 mg/kg/day, but split the daily dose into three equal portions similar to the way in which this drug has been used with success in man for the treatment of dracuncu-liasis and amebic liver abscess. The results are shown in Table 2. No reduction in the number of gnathostome larvae in treated mice was observed.

Astiban: Gnathostome infected mice were treated with five daily oral doses of 640 mg/kg or with a single oral dose of 1920 mg/kg. The results are shown in Table 3. The drug is judged ineffective in these regimens.

Ambilhar: Daily oral doses of 25-100 mg/kg for 5 days have previously been judged ineffective in gnathostome infected mice. This year mice were treated with five daily oral doses of 640 mg/kg or with a single oral dose of 1920 mg/kg. Results are shown in Table 4. At 1920 mg/kg, 8 of 15 mice developed diarrhea with streaks of blood in the feces as a result of drug toxicity, and 1 died. No reduction in the number of larvae was observed in the treated mice at either dose.

Astiban and Ambilhar in Combination: Infected mice were given a single oral dose of 1920 mg/kg of astiban beginning on the follow-ing day, ambilhar was given orally at 640 mg/kg every other day for four doses. Results are shown in Table 5. A lower percentage (44%) of advanced third-stage larvae was found in treated mice than in controls (58%), although no mice were cleared of larvae entirely. The possibility that this represents a chemotherapeutic effect is being investigated further.

SUMMARY: Oral administration of metrifonate, flagyl, astiban or ambilhar was ineffective in the chemotherapy of Gnathostoma spinigerum in experimentally infected mice. Combined therapy with astiban and ambilhar had a modest chemotherapeutic effect which is being investi-gated further.

TABLE 1

Treatment of Gnathostoma spinigerum Infected Mice with Metrifonate*

Drug dose (mg/kg/day)	Number of mice treated	Mice positive with larvae Number (%)	Third-stage larvae found Number (%)	Time of Necropsy** (days)
20	16	16 (100)	43 (54)	12-20
Control	10	10 (100)	26 (52)	21
40	15	15 (100)	48 (64)	5-20
Control	8	8 (100)	27 (68)	7-20
80	16	16 (100)	48 (60)	6-20
Control	10	10 (100)	33 (66)	4-20
120	19	19 (100)	51 (54)	4-20
Control	10	10 (100)	28 (56)	20

* Five doses at two week intervals.

** Days after last dose of drug.

TABLE 2

Treatment of Gnathostoma spinigerum Infected Mice with Flagyl

Drug dose (mg/kg/day)	Number of Mice treated	Mice positive with larvae Number %	Third-stage Larvae found Number %
200*	17	16 (94)	49 (58)
Control	7	7 (100)	18 (51)

* Three doses per day for 5 days

TABLE 3: Treatment of Gnathostoma spinigerum Infected Mice with Astiban

Drug dose (mg/kg/day)	Number of Mice Treated	Mice positive with larvae Number (%)	Third-stage Larvae found Number (%)	Time of Necropsy (Days)***
640*	15	15 (100)	38 (51)	18
Control	10	10 (100)	29 (58)	19
1920**	15	15 (100)	40 (53)	7-16
Control	10	10 (100)	28 (56)	7-15

TABLE 4: Treatment of Gnathostoma spinigerum Infected Mice with Ambilhar

Drug dose (mg/kg/day)	Number of Mice Treated	Mice positive with larvae Number (%)	Third-stage Larvae found Number (%)	Time of Necropsy (Days)***
640*	13	13 (100)	32 (49)	1-18
Control	10	10 (100)	28 (56)	19
1920**	15	14 (93)	43 (57)	1-15****
Control	10	10 (100)	30 (60)	7-15

* For 5 days
** For 1 day
*** Days after administration of last drug dose.
**** 8 mice developed bloody diarrhea after drug was administered indicating toxicity.

TABLE 5

Treatment of Gnathostoma spinigerum Infected Mice with an Astiban-Ambilhar Combination*

Group	Number of Mice Treated	Mice positive with Larvae Number	(%)	Third-stage Larvae found Number	(%)	Time of Necropsy** (Days)
Treated	15	15	(100)	33	(100)	13
Control	10	10	(100)	29	(58)	14

* One dose Astiban (1920 mg/kg body weight) followed on the next day by 4 doses of Ambilhar (640 mg/kg body weight) administered every other day.

** Days after administration of last drug dose.

2. Experimental Detection of Gnathostomiasis Antibody by Gel-Diffusion

OBJECTIVE: To develop a gel-diffusion test to detect gnathostomal antibody in laboratory animals and humans and to document the immune response of these hosts to active infections with this nematode.

BACKGROUND: Methods of transmission and reservoir hosts for Gnathostoma spinigerum have been well documented (50). Although some work has been done with this nematode using precipitin ring tests and the Sarlis phenomenon, methods of detecting gnathostomiasis have not been completely worked out (51, 52). In the present study, detection of an anti-gnathostome antibody in cats and rats artificially infected with G. spinigerum is reported.

DESCRIPTION: Two studies were conducted to determine whether a suitable G. spinigerum antigen could be successfully extracted from advanced third-stage larvae, and to see if this antigen could be used satisfactorily in detecting antibody in artificially infected rats and cats.

Parasite Antigen Source: Material used to prepare the antigen was obtained from experimentally infected mice harboring G. spinigerum third-stage larvae. The mice were exsanguinated or allowed to die and the larvae dissected immediately from the tissues and placed in phosphate buffered saline (PBS), pH 7.5. This material was freeze-dried and stored until needed for antigen preparation.

Preparation of the Antigen: Freeze-dried G. spinigerum third-stage larvae were weighed out in 500 mg portions, placed in a glass homogenizer and de-lipidized with absolute alcohol and anhydrous ether. Following de-lipidization the homogenate was extracted by constant stirring at 2-5°C in PBS (pH 7.5) overnight. The extracted material was then centrifuged at 15,000 rpm for 15 minutes and the resulting supernate used as the antigen.

Double-Diffusion Test: The double-diffusion test was conducted by the method of Ouchterlony (53). Hyperimmune serum for use as a positive control was obtained from rabbits (54) immunized with the antigen extracted from advanced third-stage G. spinigerum larvae. Equal portions of antigen and rat or cat serum were then added to the reaction chambers and allowed to react for 2-5 days before recording results.

EXPERIMENT NUMBER ONE:

PROGRESS: Five hundred rats were each infected orally with ten G. spinigerum third-stage larvae obtained from exsanguinated donor mice. Experimental rats were killed in pairs every two days and the serum tested for anti-gnathostome antibody using immuno-diffusion with the advanced third-stage larval extract as the antigen.

TABLE I

The immune response of experimentally infected rats harboring G spinigerum third stage larvae

DAYS AFTER INFECTION WITH G. SPINIGERUM																														
DAYS	0		11		13		15		17		19		21		23		25		27		29		31		33					
RAT #	-	-	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34				
RESULTS	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	NS	NS	-	+	+	-	+	+				
DAYS	35		37		39		41		43		45		47		49		51		53		55		57		59					
RAT #	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60				
RESULTS	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	NS	NS				
DAYS	61		63		65		67		69		71		73		75		77		79		81		83		85					
RAT #	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86				
RESULTS	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-				
DAYS	87		89		91		93		95		97		99		101		103		105		107		109		111					
RAT #	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112				
RESULTS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
DAYS	113		115		117		119		121		123		125		127		129		135		141		145		147					
RAT #	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	135	136	141	142	145	146	147	148				
RESULTS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+				
DAYS	149		151																											
RAT #	149	150	151	152																										
RESULTS	+	-	+	+																										

N.S. = NO SAMPLE

Anti-gnathostome antibody was detected as early as 13 days (Table 1) after infection and could still be detected as long as 151 days after infection. In rodents G. spinigerum rarely develops past the third-stage larval phase. Since the third-stage larvae were used as the antigen in this experiment, the positive reactions observed in the gel-diffusion test were presumably due to antibodies developed against G. spinigerum larval forms.

Comparative studies using sera from rabbits immunized with crude antigen and sera from actively infected rats indicated that antibodies were present against the third-stage larval antigen in both, although specific serological identity has yet to be proven (Fig. 1, 3). In one rabbit (Number 5) a minimum of three distinct precipitin bands could be detected while in the rat model only two have been detected thus far. In the rat, this second band first appeared 71 days after infection and may be due to antibody against antigen released during the worms' maturation.

Up to now it has not been conclusively demonstrated that the antibody detectable in the rat system is identical to that seen in the control rabbit but present evidence suggests they are the same.

EXPERIMENT NUMBER TWO:

PROGRESS: Three adult cats (120, 132 and 135) were selected for the study. Cats 120 and 132 had been infected previously for different experiments in April and May 1972 and in February and August 1973 and both had previously shown G. spinigerum ova in their stools. Cat 135 had never been used before and served as a positive control. Cat 135 was infected on 17 July 1973 for the first time with 205 third-stage larvae, 155 larvae by active skin penetration and 50 by oral administration. Weekly bleedings on all the cats except 135 were commenced on 28 September 1973 and have been continued to the present (Table 2). Cat 120 which had already been infected for almost 1 year and 10 months demonstrated positive double-diffusion tests on the first day of bleeding. Similarly, cats 132 and 135 which had been more recently infected showed positive results (Table 2). Positive results were obtained in cat 132 and 120 until November 1973. Cat 135 first became negative on 2 November 1973 although one more positive immuno-diffusion test was obtained on 16 November 1973. This cat then became negative and remained so until his death on 15 February 1974. At necropsy of cat 135 we found only immature adult stages of G. spinigerum (Table 3). G. spinigerum ova were never recovered from the stools of cat 135, ruling out the possibility of adult nematodes in the stomach.

Cats 120 and 132, which were previously infected, produced results similar to cat 135 except that they were positive in the gel-diffusion test for a longer period of time (Table 2). Cat 120 was sacrificed to determine if it also harbored the immature adult or the adult stages of

TABLE 2

IMMUNO-DIFFUSION RESULTS ON G. SPINIGERUM INFECTED CATS

DATE	CAT # 120 (inf. Apr & May 72)	CAT # 132 (inf. Feb & Aug 73)	CAT # 135 (inf. Jul 73)
28 Sep 73	Positive	Positive	No specimen
5 Oct 73	Positive	Positive	Positive
12 Oct 73	Positive	Positive	Positive
19 Oct 73	Positive	Positive	Positive
26 Oct 73	Positive	Positive	Positive
2 Nov 73	Positive	Positive	Negative
9 Nov 73	Positive	Positive	Negative
16 Nov 73	Positive	Positive	Positive
22 Nov 73	Positive	Positive	Negative
30 Nov 73	Negative	Positive	Negative
14 Dec 73	Negative	No specimen	Negative
21 Dec 73	Negative	Negative	Negative
28 Dec 73	Negative	Negative	Negative
4 Jan 74	Negative	Negative	Negative
11 Jan 74	Negative	Negative	Negative
18 Jan 74	Negative	Negative	Negative
25 Jan 74	Negative	Negative	Negative
4 Feb 74	Negative	Negative	Negative
8 Feb 74	Negative	Negative	Negative
15 Feb 74	Negative	Negative	Died
22 Feb 74	Negative	Negative	
1 Mar 74	Negative	Negative	
7 Mar 74	Killed		

TABLE 3

NECROPSY RESULTS ON G. SPINIGERUM INFECTED CATS

CAT #	Total Larvae found	Right Front leg		Left Front leg		Right hind leg		Left hind leg		Abdomen		Back		Stomach	Diaphragm
		Fat	Muscle	Fat	Muscle	Fat	Muscle	Fat	Muscle	Fat	Muscle	Fat	Muscle	Tumor	Muscle
120	30	–	4	–	3	1	5	2	7	5	1	1	1	–	–
135	45	–	–	–	–	–	–	–	–	–	7*	–	–	32*	6*

*ALL IMMATURE ADULTS

PRECIPITIN REACTION USING RABBIT, RAT, AND CAT ANTISERA AGAINST G. SPINIGERUM THIRD-STAGE LARVAL ANTIGEN

Fig. 1

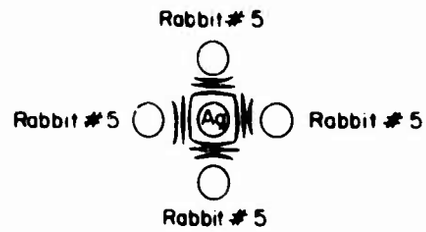


Fig. 2

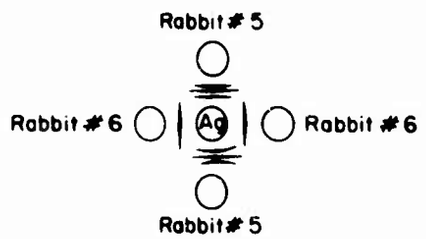


Fig. 3

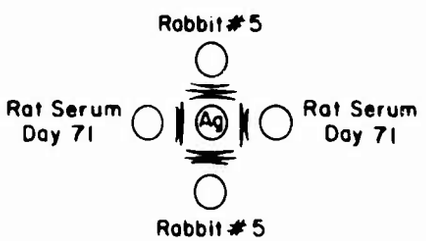
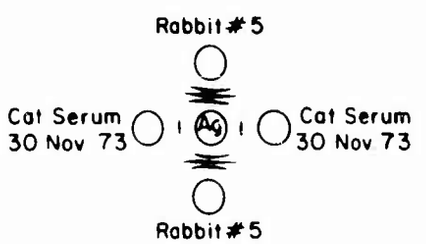


Fig. 4



this parasite because maturational stage differences in this case may have been responsible for the negative gel-diffusion tests observed. At necropsy all the worms found were encysted advanced third-stage larval forms, even though 1 year and 10 months had elapsed since last exposure to infection (Table 3). This development is noteworthy, since, once the definitive host becomes infected, the advanced third-stage larvae usually develop into immature adults within a few months. All the larvae found in cat 120 had become encysted and were not actively migrating. To date, cat 132 remains negative in gel-diffusion tests, although he has been positive for gnathostome eggs in the stool since Feb 1974.

DISCUSSION: The double-diffusion test appears to be helpful in detecting third-stage larval infections in the rat host. The nature of the specific antibody being detected is presently unknown. The ability to detect antibody against the third-stage larvae of this parasite for as long as 151 days in the rat is interesting. The technique may be applicable in human infections where the third-stage larval parasite is present. The negative double-diffusion test results in cat 135 after November 1973 may have been due to differences in the antigenic stimulus being presented after the advanced third-stage larvae molted into immature adult forms. A definitive answer as to whether there are qualitative antigenic differences between the different stages of this parasite could be obtained by further study. Negative gel-diffusion results in cat 120 after November 1973 may have been because the encysted larvae were no longer providing sufficient stimulus for the level of precipitating antibody to be detectable by immuno-diffusion. This theory is supported by the fact that cats 121, 122, and 123, infected in July 1973 for purposes other than our experiment, showed positive gel-diffusion tests even after cats 135, 132, and 120 had become negative. Cat 122 at necropsy showed actively migrating larvae. In order to detect active third-stage larval infections of G. spinigerum by gel-diffusion, actively migrating larvae and stage specific antigens may have to be present. Studies are now being conducted in this area. Comparative studies with immune cat serum and immune positive control rabbit serum have shown results similar to the rats; however, serological identity of the anti-gnathostome antibody in both systems to a common antigen has not yet been demonstrated.

SUMMARY: An antigen extracted from advanced third-stage G. spinigerum larvae was used to experimentally detect anti-gnathostome antibody in artificially infected rats and cats. In rats, precipitating antibody could be detected as early as 13 days after infection and as long as 151 days after infection. Studies with the gel-diffusion test using third-stage larval antigen could detect the larval migratory phases of this nematode in the definitive host; but, when this parasite molted into an adult or encysted as a third-stage larva, detection by gel-diffusion was no longer possible. The immuno-diffusion test using third-stage larval antigen could be useful in detecting human infections

where the third-stage larval parasite is present. Larval migratory periods of up to 10 years or more have been reported in humans (54). Studies on the cross-reactivity of this antigen with other helminthic infections are also being conducted and studies of immunological detection of gnathostome infection in primates are being planned.

IV. DRUG ABUSE STUDIES

1. A Survey of Thai Student Drug Use

OBJECTIVE: To determine the prevalence of illicit drug use as reported by a sample of Thai students.

BACKGROUND: There currently exist no empirical data on the prevalence or incidence of drug abuse in Thailand, although some informal estimates are available. In 1972, 300,000 individuals were estimated to be addicted to drugs (55). The Director of one of Bangkok's two drug rehabilitation programs indicates that 200 students seek assistance every day for drug abuse (56), although this includes users of some "soft" drugs and individuals who have appeared more than one time. In 1973, it was estimated (57) that 300,000 students were addicted to drugs. It was also estimated that more than half of the country's 43,000 prisoners are addicted to narcotic drugs (58). There have been no formal surveys of drug use by a Thai student population.

DESCRIPTION: We surveyed 1613 students. These students attended schools in the Chiang Mai and Nakorn Rajsima provinces and one school in Bangkok. Classrooms from schools in Chiang Mai and Nakorn Rajsima provinces were selected by a stratified random sampling procedure to give proportional representation of students by grade, kind of school and type of specialization. The grade levels surveyed were MS3, 4, 5 and technical college, years 1-4. Students were randomly drawn from selected classrooms to give proportional representation by sex. An additional 304 students (50% of total enrolment selected at random) from the Demonstration School under the Chulalongkorn Teacher's Training Faculty in Bangkok were also surveyed. These were students in grades MS2-5. The average age of all students was 16.9, range 13-25.

A series of 15 pairs of questions included in the questionnaire were designed so that students could respond in a mutually exclusive (i.e., inconsistent) manner. These question pairs were used to evaluate consistency of students' responses. Any student who responded inconsistently on four or more pairs of these questions was eliminated from the study; 106 students were eliminated for this reason along with 1 additional student who failed to complete his questionnaire. Data reported here are based on responses from 1506 students from Chiang Mai, Nakorn Rajsima and Bangkok who provided complete and consistent questionnaires.

RESULTS: To facilitate discussion results will usually be presented by comparing two kinds of subjects. The first kind is labelled "drug user." These are students who reported ever having used an illicit drug. The second kind is labelled "non-user." These are students who reported never having used any illicit drug. Alcohol and tobacco are not considered illicit drugs in Thailand. All entries in the tables are percentages.

Twenty-three percent (23%) of all students indicated "ever use" of an illicit drug. Six percent (6%) of all students indicated that they are currently using an illicit drug. The average ages were 17.9 (range 13-23) and 16.6 (range 13-25) years for the drug user and non-user, respectively.

Tables 1, 2 and 3 indicate the following: Males are more likely to report using drugs than females and drug users are relatively less likely to be living with parents. The latter finding is due to the higher age of drug users, who are at greater risk of living away from home. Parents of drug users are more likely to smoke tobacco or drink alcoholic beverages. Table 4 presents frequency of reported drug use by drug type. Students who report "ever use" of an illicit drug also report more frequent use of tobacco and alcohol. Marijuana is the illicit drug most frequently reported used. Table 5 indicates that drug users are more likely to report involvement with police and Table 6 indicates they are more likely to have had sexual intercourse. Differences reported in Tables 5 and 6 cannot be attributed to the higher age of drug users, as they were maintained when users and non-users were compared within each age level. Table 7 indicates that drug users report receiving somewhat less love and supervision from parents than peers received. Table 8 indicates that most drug users report getting their drugs from a friend or acquaintance rather than by self-purchase.

Table 9 indicates that about one-fourth of drug users were "self-initiates" into drug use, and about half indicate that a friend or acquaintance first introduced them to drug use. Table 10 indicates that drug users are more likely to have close friends using drugs, and Table 11 indicates that drug users are also more likely to report that their acquaintances are using drugs. About 65% of users and half of non-users indicate that at least some of their acquaintances are using illicit drugs. Table 12 provides estimates of other students' drug use. Users estimate slightly greater numbers of students are using drugs than non-users estimate. About 75% of all students report that at least some students are using illicit drugs. Table 13 indicates that that drug users are more likely to turn to friends and non-users to parents when they seek help for personal problems. Few individuals selected teachers as a first choice for this kind of help. Table 14 indicates that about one-fourth of all students do not wish to learn more about drugs, but about half of all students do. At the same time

TABLE 1

Sex and Drug Use

Subject	Sex	
	Male	Female
User (n = 359)	94%	6%
Non-User (n = 1147)	70%	30%
Combined (n = 1506)	76%	24%

TABLE 2

Place of Living and Drug Use

Subject	Place		
	With both parents	With relative	Other
User (n = 359)	61%	21%	18%
Non-User (n = 1147)	67%	18%	15%

TABLE 3

Parental Use of Tobacco and Alcohol

Subject	Tobacco		Alcohol	
	How many parents use:		How many parents use:	
	At least one	Neither	At least one	Neither
User (n = 359)	78%	22%	65%	35%
Non-User (n = 1147)	64%	36%	48%	52%

TABLE 4

Frequency of Reported Drug Use[1]

Drug Type	Frequency of Use						
	never	used but stopped	<1 time/ month	<1 time/ week	1-3 days /week	4-6 days /week	daily
alcohol[2]							
user (n=359)	15	32	30	17	5	1	1
non-user (n=1147)	70	19	8	3	0	0	0
tobacco[2]							
user (n=359)	9	26	5	6	10	5	38
non-user (n=1147)	65	21	4	3	2	1	3
amphetamines (n=1506)	98	1	0	1	0	0	0
barbiturates combined (n=1506)	96	3	1	0	0	0	0
hallucinogens (n=1506)	99	1	0	0	0	0	0
marijuana (n=1506)	79	16	2	1	1	0	1
opiates (n=1506)	99	1	0	0	0	0	0
others (n=1506)	99	1	0	0	0	0	0

1. All values are per cents; due to rounding errors, row totals may not equal 100%.

2. These per cent values are of the total student population (n=1506).

TABLE 5

Involvement with Police after Age 13

Subject	Number of Incidents		
	Never	One time	More than once
User (n = 359)	81%	12%	7%
Non-User (n = 1147)	94%	4%	2%

TABLE 6

Experience with Sexual Intercourse

Subject	Experience	
	None	Some
User (n = 359)	41%	59%
Non-User (n = 1147)	87%	13%

TABLE 7

View of Parental Love and Supervision after Age 13

Subject	Love Received from Parents			Supervision Received from Parents		
	more than peers	same as peers	less than peers	more than peers	same as peers	less than peers
User (n = 359)	25%	63%	10%	28%	50%	21%
Non-User (n = 1147)	33%	59%	4%	41%	49%	6%

TABLE 8

Method of Drug Acquisition

Source	Per Cent
buying[1]	21
friends give	50
acquaintance give	9
other	5
no answer	15

[1] Only two per cent of these report illegal activity to provide money for purchase.

TABLE 9

Source Suggesting First Use of Illicit Drugs

Source	Per Cent
self	25
neighborhood friend	12
school friend	25
acquaintance	14
other	6
no answer	18

TABLE 10

Drug Use by Close Friends at Home and School

Subject	Friends from Neighborhood			Friends from School		
	no close friends or none use	at least one uses	no answer	no close friends or none use	at least one uses	no answer
user (n=359)	45%	25%	30%	54%	27%	19%
non-user (n=1147	63%	9%	28%	80%	7%	13%

TABLE 11

Estimate of Acquaintances' Drug Use

Subject	Percent of Acquaintances Estimated to be Using Illicit Drugs					
	1-20%	21-50%	51-70%	> 70%	some but can't say	none or don't know
user (n=359)	13	2	2	1	46	35
non-user (n=1147)	9	1	0	0	38	51

TABLE 12

Estimate of other Students' Drug Use

Subject	Percent of other Students Estimated to be Using Illicit Drugs					
	<5%	5-10%	11-20%	21-50%	>50%	some but can't say
user (n=359)	21	12	7	5	4	49
non-user (n=1147)	25	7	4	2	1	60

TABLE 13

Source of Help for Personal Problems

Subject	Source from which Students Seek Help				
	parents	friends	teacher	other	no answer
User (n=359)	32%	57%	3%	6%	2%
Non-User (n=1147)	62%	24%	3%	5%	1%

TABLE 14

Desire to Learn more about Drugs of Abuse

Subject	Expressed Desire			
	know enough	don't wish to learn more	do wish to learn more	no answer
User (n=359)	19%	21%	42%	18%
Non-User (n=1147)	8%	23%	53%	17%

TABLE 15

Source of Information Concerning Drugs

Subject	Source Selected as One of First Two Choices							
	none	home	school	friend	TV radio	news-paper	drug store or doctor	other
User (n=359)	2%	10%	50%	50%	12%	23%	14%	8%
Non-User (n=1147)	13%	11%	69%	19%	18%	30%	14%	3%

users are more likely to report that they know enough about drugs.

To the statements in Table 15 individuals were allowed to select up to two "sources of information about drugs." Users receive information from school, friends, and to some extent the mass media (TV, radio, newspapers); non-users are more likely to receive information from school or the mass media. Table 16 is based on responses from non-users only. Individuals were allowed to select up to two of the reasons for not using drugs listed. Fifty-one percent (51%) of individuals selected "not interested" as one of their first two reasons for non-use and 61% selected "risk of physical damage" as one of their first two reasons for not using illicit drugs. Few individuals selected other reasons.

Table 16. Why Non-Users Choose Not to Use Drugs

Reason	Percent Selecting as First or Second Choice
not interested	53
don't know where to buy	3
parents'influence	8
friends' influence	1
risks of legal problems	5
religious	4
risk of physical or mental damage	61
other	5

DISCUSSION: We present results based on those questions felt to be most appropriate for the purposes of this paper, thus responses to all 49 questions are not presented. Reporting of drug use is sex related in Thailand with 94% of the users being male while 76% of the total student body surveyed is male. This may be typical of results found when drug use is just beginning to become widespread and has not yet been readily adopted by the female student.

Drug users are less likely to be living with their parents or a relative. This is a reflection of the fact that heaviest drug use occurred among technical school (vocational) students living in dormitories not under the direct influence of their parents. It is not primarily the result of broken homes.

There is a weak relationship between reported drug use and use of alcohol and tobacco by parents. Users are more likely to come from a family where one or both parents utilized these licit drugs. Blum (59) has attributed this to a learned orientation toward drug use. There is, however, virtually no illicit drug use, according to student report, by parents of either the drug users or non-users.

It is clear from Table 4 that users smoke a great deal more tobacco products, and in addition consume more alcoholic beverages, than non-users. This is a typical finding in surveys of drug use and points up one difficulty of controlling illicit drug use; the drug user not only uses illicit drugs, but has established a pattern of heavy use of legal psychoactive drugs. This orientation to several kinds of drugs which alter the individuals' consciousness makes the behavior especially difficult to extinguish. Perhaps intervention at a very young age, even before alcohol and tobacco are first used (by age 13 in this survey), is called for. Twenty percent (20%) of students indicated "ever use" of marijuana with 5% of students indicating current use. Some experimentation with other illicit drugs is reported but little current use. Thus, it may well be that the student population studied here has not yet been infected with the problem of widespread illicit drug use.

As is typically found, drug users are more likely to have been involved with illegal activities brought to the attention of the police. It is important to note, however, that only a minority of drug users (19% vs 6% for non-users) actually had such involvement. Thus, it would be an error to characterize the user as a "criminal." Also, drug use is not a "lower-class" phenomenon. Table 6 indicates that drug users are more likely to have had sexual intercourse.

Drug users report receiving less love and supervision from parents than non-using peers receive from parents. The implications of this may be that in at least some cases disturbed home life has been a factor contributing to drug usage. Whether these are real differences or merely those that are perceived by the drug user is not clear. It may reflect the importance of providing affection and supervision to children as a means to help establish maximum internalization of parental and societal norms. At the least, parents must be involved with any drug prevention program, perhaps by pointing out the parents' role in the genesis of many behavioral problems (including drug use).

Involvement with drugs is a social phenomenon (59, 60) often spread through relationships established within specific social networks. Thus, it is not surprising that Tables 8 and 9 indicate that "friends" are the primary agent reported as suggesting first use of illicit drugs. The drug use reported in this paper could be described as social or "experimental," a kind of drug use for which social rewards (peer acceptance, status, etc.) could well provide the primary impetus for

continuation. Prevention programs would probably gain most if they emphasized the role of "friends" in the spread of drug use and relied heavily on students as transmitters of education materials. Almost no illegal activity was reported to gain money for financing of respondents' drug use, as only 2% of those reporting using illicit drugs indicated illegal activity was involved to get money to buy those drugs. As little use of addicting drugs is reported, this result is not expected.

As expected drug users are more likely (although the differences are not large) to have friends who use drugs. Friends who use drugs are equally likely to be reported (by both non-users and users) as coming from a home neighborhood as from a school. This fact may indicate that any drug prevention program which is administered solely through school systems would not have maximum impact. Thus, if these "friends" from the neighborhood are not students then other methods must be utilized to reach them. These other methods presumably would be through the mass media, preferably newspapers.

Surprisingly, users' and non-users' estimates of other students' drug use and of their friends' drug use did not show large differences. Drug users typically over-estimate the extent of drug usage and non-users under-estimate the extent of drug use. We found 6% of students reporting current use of some illicit drug and an additional 17% reporting ever use of some drugs. Table 11 indicates that both users and non-users have over-estimated the amount of student drug usage (assuming our figures are accurate). That most students are aware that some drugs are being used is indicated by the "some but can't say" column in this table. At least half of all students did indicate that "some" students are using drugs. This is an entirely accurate statement. That the user and non-user did not indicate large differences in amount of friends' drug use may indicate that drug use, or at least frequent use, by a large part of the study population is not occurring. This agrees with the figures presented earlier.

A clear difference was observed between users and non-users as to the source of help for personal problems. Friends are more likely to be sought by the drug users and parents sought by the non-users. The implications of this are: a) drug education prevention programs might utilize "friends" to help educate other students; and b) because teachers (and other school sources) were selected as a first choice by very few students, the credibility of school authorities as sources of counselling may be very low. This may be an area to improve through teacher education and training.

Table 14 indicates that about half of all students expressed a desire to learn more about drugs. This points to a need for more thorough education programs. These might be directed through schools and at the same time presented through mass media (radio, TV, newspapers). As we

see in Table 15 "schools" have been the primary source of information about drugs for most individuals, especially non-users of drugs. Users, however, have also relied greatly on friends and newspapers for information. Drug stores, doctors, parents and other sources apparently do not play a significant role in this kind of education.

Table 16 was based on responses from non-users only. Important reasons for not using drugs are "risk of physical or mental damage" and "no interest"; no other reason was largely selected for not using drugs. Surprisingly, friends' and parents' influence did not play a major role. The physical and psychological effects of drugs should be emphasized in any education/prevention program; an accurate picture of these effects might serve to deter a significant proportion of individuals from using drugs.

SUMMARY: In a survey of drug use by 1506 Thai students about one-quarter reported using illicit drugs, and six percent (6%) reported current use. This extensive drug experimentation, in addition to alcohol use, indicates a need for active preventive efforts to abort future epidemic drug use in this drug-saturated environment. We indicate that a desire for drug education is expressed by students, that this should be administered through schools and mass media for maximum impact, and should stress physiological and psychological effects of drug use.

2. Prediction of Illicit Drug Use by United States Servicemen

For a description of the objectives of this study see the SEATO Annual Progress Report, March 1972.

PROGRESS: A 59 item questionnaire designed to predict involvement with illicit drugs has been administered to 450 Army and Air Force troops upon their arrival in Thailand. Interviews are being scheduled for each of these individuals at 4-month intervals during their first year in Thailand. As of 1 May 1974 all individuals had been interviewed one time and about one-third received a second or third interview.

It is anticipated that data collection will be completed by 15 November 1974. At that time a classification of each individual as a non-user or drug abuser will be made. These clinical classifications will be based on the clinical interviews. Each individual will also be statistically classified as a drug abuser or non-user by use of discriminant coefficients derived from a discriminant analysis of the 59 item questionnaire (61). Clinical classifications will be compared with statistical classifications to provide evaluation of the efficacy of the questionnaire instrument for predicting drug use.

SUMMARY: A questionnaire designed to predict involvement with drugs has been administered to 450 individuals. These individuals are being

interviewed at four month intervals during their first year in Thailand, and all have been interviewed at least one time. Drug use classification based on the clinical interview and the questionnaire will be compared to evaluate the predictive validity of the questionnaire.

3. Perceived Distance from Father and Its Relation to Drug Use in a Soldier Population

For a description of the objectives of this study see the SEATO Annual Progress Report, March 1973.

PROGRESS: Each of 75 military personnel was administered the father distance scale questionnaire. Individuals were divided into three groups for analysis: Group 1 consisted of individuals who did not use drugs; Group 2 consisted of individuals who were confirmed drug abusers; and Group 3 consisted of individuals who had psychiatric problems without involvement with drugs. We predicted that individuals in Group 2 would be more father distant than individuals in Group 1 but would have father distance scores similar to individuals in Group 3.

A preliminary analysis indicates that drug users (Group 2) did have significantly higher father distance scores than the non-users (Group 1). Contrary to our expectation, Group 2 subjects were also significantly more father distant than individuals in Group 3 (individuals with psychiatric problems).

Knowledge of an individuals' relations with his father or authority can be used by the therapist for the treatment of drug use related to emotional problems, and this knowledge can be obtained from an individuals' score on the father distance scale. A detailed report of findings and recommendations for therapy will be included in next year's Annual Report.

SUMMARY: Three groups of subjects (made up of non-drug users, drug abusers, and individuals with psychiatric problems) were administered the father distance scale questionnaire. Drug users were found to be more father distant than both non-users and psychiatric patients. Recommendations for therapy will be included as part of the final report of this study.

4. A Study to Define the Prevalence of Methaqualone Drug Abuse among American Soldiers in Thailand

BACKGROUND: Methaqualone (Mandrax, Parest, Qualude, Sopar, Tuazole) abuse is a serious military health problem in Europe. This drug is not used by military medical facilities and its identification in urine therefore implies abuse in most instances. A study on the abuse of methaqualone by American teenagers in Bangkok has shown the problem did exist in Thailand (62). The present study was undertaken to determine

the prevalence of methaqualone use among American servicemen in Thailand.

METHOD: We assumed that urine specimens submitted under Golden Flow were representative of all American servicemen in Thailand, as specimens are randomly sampled from all areas of the country where troops are stationed. During the 50 day period of data collection two groups of urine samples were examined. Group 1 consisted of all samples suspected of containing morphine. These were positive by the Free Radical Assay Technique (FRAT). Approximately 2000 of the samples submitted were positive by the FRAT and, therefore, examined for the presence of methaqualone.

Group 2 consisted of 2000 specimens which were assigned a laboratory accession number ending in 9; this was 10% of the total number of specimens submitted. These were examined for the presence of methaqualone (63).

RESULTS AND DISCUSSION: Twelve of the specimens from Group 1 contained methaqualone. None of these had an accession number ending in 9. All samples from Group 2 were negative for methaqualone.

It was concluded that illicit use of methaqualone is not a major problem among American servicemen in Thailand. Use of this drug appears to be limited to those individuals who are taking some other drug, usually an opiate, with or without medical advice.

V. MISCELLANEOUS STUDIES

1. A Serological Survey for Infectious Agents Causing Clinical and Subclinical Infections in United States Military Personnel in Thailand

OBJECTIVE: To determine the incidence of infection, with serologically identifiable agents common to a tropical environment, in a group of susceptible American soldiers stationed in Thailand.

BACKGROUND: Studies on fevers of unknown origin among American personnel in Vietnam indicate arboviruses, scrub typhus, and leptospirosis to be major causes. Little is known, however, of the incidence among susceptible American troops of subclinical disease caused by a variety of infectious agents commonly found in tropical environments. Such knowledge requires specific serologic tests. Many diseases in the subclinical form lead to decreased efficiency, loss of productivity, and have considerable impact upon a unit's performance. The incidence of some of these diseases in a group of men during their tours in the tropics may be ascertained by serial serological studies for diseases such as amebiasis, selected arbovirus diseases, gonorrhea, hepatitis B, leptospirosis, lymphogranuloma venereum, malaria, scrub typhus, and syphilis.

By coupling this serological survey to periodic interviews we hope, in addition, to assess the effect of attitudes and behavior on the development of these infections. Disease acquisition might be associated with length of time in country, exposure to indigenous populations, residence in civilian areas, utilization of drugs, and personal attitudes towards disease and disease prevention.

Systematically collected information of this nature is of importance to both command and medical personnel involved with American troops. This information might suggest types of precautions necessary for military personnel entering tropical environments.

DESCRIPTION: This study interfaces with a study being undertaken by the Department of Neuropsychiatry (SEATO Medical Research Laboratory Annual Report 1971, pp 264-265) designed to validate a psychometric test to predict drug abuse among American troops.

The population being studied was drawn from servicemen, ages 18-26, in grades E1-E5 in either the United States Army Support 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 the previous duty station, previous tropical experiences, immunization history, home of record, place of upbringing, etc. Answers were also sought to certain attitudinal questions relating to diseases.

During the ensuing year each serviceman will be interviewed three times at approximately 4 month intervals. A serum sample will be collected at the time of each interview. During the interviews questions will be asked relevant both to drug utilization and to medical problems, including questions on the degree of interaction with indigenous populations, and inquiries into illness of any sort during the interview period will be made.

General information as to the health of these men is also being obtained through coordination with command medical personnel and by review of medical records. Sera will be submitted for serological tests for amebiasis, selected arbovirus diseases, gonorrhea, hepatitis B, leptospirosis, lymphogranuloma venereum, malaria, scrub typhus, and syphilis. Serological information will be related to information acquired from each man through interviews and medical feedback.

PROGRESS: Subjects were enrolled in this study between April and December 1973. Questionnaires were completed by 434 individuals and serum was obtained from 424. The first of three interviews with each subject was completed in April 1974. All second interviews will be completed by August 1974 and the third by November 1974. After that time the data will be collated and analyzed.

2. Study of Vertebrate Reservoirs of Disease

BACKGROUND: Vertebrate animals potentially involved in the epidemiology of human disease are identified for investigators engaged in field studies. Information is supplied on their life history and ecology especially as they relate to arthropod-borne diseases. This information is based upon our own studies because of unreliability of the available literature on mammals owing to confusion on scientific names.

DESCRIPTION: Although Thai mammals have been extensively collected in the past and are well represented in museums, studies have been hampered by the poor preparation of their skulls and teeth. SEATO Laboratory has therefore built up a new and extensive collection, preserving only those specimens with perfect skulls that have been cleaned by our colony of dermestid beetles. This collection is now divided among SEATO Laboratory, Applied Scientific Research Corporation of Thailand (Center for Thai National Reference Collections, which was started by our contribution), British Museum (Natural History), and Smithsonian Institution. In those museums can be found the specimens which fully document the taxonomy of rodents which follows.

Thai rats and mice are notable for their great numbers of individuals and of species, and for the close association of many of them with man. There are 36 species, most of which support ectoparasites capable of transmitting disease. Eleven of them live in warehouses, homes or ricefields and thus constitute an economic as well as a medical problem. All that we have learned about the nomenclature, ecology, distribution, karyology, and ectoparasites of these rodents is now in press as a chapter on the Family Muridae (Rats and Mice) contributed by Marshall to the book "Mammals of Thailand" by Lekagul and McNeely (Association for the Conservation of Nature of Thailand). It consists of a fully illustrated key; diagrams of chromosomes, palatal ridges, footpads, mammae, tail color patterns; average measurements; and species accounts. It is illustrated with photographs of the skulls and of the live animals.

The following checklist is extracted from that chapter. The subgenus, a category that may be unfamiliar to the reader, is a group of species that possess in common a certain skull shape, similar karyotype, and the same species of lice in the genera Hoplopleura and Polyplax. Recent discoveries are Dr. Bahmanyar's taking of Bandicota bengalensis and B. savilei together at Myingyun, Burma, and our capture of B. savilei and B. indica in the same fields at Korat, Thailand, thus proving that there are three species of bandicoots; Mr. Kitti Thonglongya's capture of true Rattus niviventer at the summit of Doi Angka which knocks over the names of related species like a row of dominoes; and William A. Neill's trapping a new species of rat at the Saraburi bat cave, to be named Rattus hinpoon, the limestone rat.

CHECKLIST OF RATS AND MICE OF THAILAND

FAMILY MURIDAE Rats and Mice

SUBFAMILY I. MICROTINAE Meadow-mice

Genus 1. Eothenomys

1. Eothenomys melanogaster Père David's Vole. Summit of Doi Inthanon.

SUBFAMILY II. MURINAE Old World Rats and Mice

A. Arboreal Rats and Mice

Genus 2. Vandeleuria

2. Vandeleuria oleracea Long-tailed Cane Mouse. Throughout Thailand except for the peninsula; in cane.

Genus 3. Chiromyscus

3. Chiromyscus chiropus Fea's Tree Rat. Three specimens from Chiangmai and Loei.

Genus 4. Hapalomys

4. Hapalomys longicaudatus Marmoset Rat. Lives in bamboo internodes; two specimens from western Thailand.

Genus 5. Chiropodomys

5. Chiropodomys gliroides Pencil-tailed Tree Mouse. Lives in bamboo internodes; common throughout Thailand.

B. Terrestrial Rats and Mice

Genus 6. Bandicota

6. Bandicota indica Great Bandicoot. Ricefields throughout Thailand except the peninsula.

7. Bandicota savilei Lesser Bandicoot. Foothill localities except the north and peninsula. Lives in teak forest and is a despoiler of corn fields and vegetable gardens.

Genus 7. Mus

Subgenus 1. Leggadilla

8. Mus shortridgei Shortridge's Spiny Mouse. Dry dipterocarp forest across northern and central Thailand.

Subgenus 2. Coelomys

9. Mus pahari Gairdner's Shrew-mouse. Tak and Chiangmai, in the evergreen forest.

Subgenus 3. Mus

10. Mus caroli Ryukyu Mouse. Ricefields throughout Thailand as far south as Huahin.

11. Mus cervicolor Fawn-colored Mouse. Ricefields and grass beneath deciduous dipterocarp forest throughout Thailand except the peninsula.

12. Mus cookii Cook's Mouse. Grass within forests of the north; also in hill rice plantations.

13. Mus musculus castaneus Asian House Mouse. Warehouses of Thonburi and Trang.

Genus 8. Rattus

Subgenus 1. Berylmys White-toothed Rats

14. Rattus berdmorei Lesser White-toothed Rat. Marshy grass in forests throughout Thailand except the central plains and peninsula south of Huahin.

15. Rattus mackenziei Kenneth's White-toothed Rat. Two specimens, from forests of Tak and Chiangmai.

16. Rattus bowersi Bower's Rat. Mountain evergreen forests throughout Thailand.

Subgenus 2. Rajah Rats

17. Rattus whiteheadi Whitehead's Rat. Lowland evergreen forest of the peninsula.

18. Rattus rajah Brown Rajah Rat. Two specimens from Trang.

19. Rattus surifer Yellow Rajah Rat. Abundant in forests throughout the Kingdom.

Subgenus 3. Niviventer Group

20. Rattus langbianis Langbian Rat. Collected by Kitti Thonglongya in deciduous forest at Chongmek, eastern Thailand.

21. Rattus cremoriventer Pencil-tailed Rat. Forests of peninsular Thailand.

22. Rattus niviventer White-bellied Rat. Moss forest at the summit of Doi Angka and Doi Pahompok, Chiangmai Province.

23. Rattus rapit orbus Long-tailed Rat. Evergreen forests of Khao Luang, Nakornsritammarat Province, and Doi Inthanon, Chiangmai Province.

24. Rattus fulvescens Chestnut Rat, Bonhote's Rat (including Rattus fulvescens bukit which was formerly called "Rattus niviventer bukit"). Forests throughout Thailand: in the north becoming reddish through intergrading with Rattus fulvescens huang; Rattus fulvescens fulvescens in the mountains of the west; a dull colored long-tailed population at Khao Yai National Park; the rest of the country occupied by the common Rattus fulvescens bukit.

25. Rattus hinpoon nov species Limestone Rat. Limestone cliffs of Saraburi Province, central Thailand. The only endemic terrestrial species of mammal in Thailand.

Subgenus 4. Rattus House Rats

26. Rattus norvegicus Norway Rat. Buildings and warehouses of cities in central and southern Thailand. Absent from Chiangmai.

27. Rattus nitidus Himalayan Rat. In houses of mountain villages in the north.

28. Rattus losea Lesser Ricefield Rat. Ricefields and vegetable gardens throughout Thailand.

29. Rattus argentiventer Greater Ricefield Rat. Ricefields of the central plains and peninsula.

30. Rattus remotus Island Rat. Secondary forest of Samui Island and adjacent islands, Suratthani Province.

31. Rattus koratensis Claden's Rat. Evergreen forests of mountains exclusive of peninsular Thailand.

32. Rattus exulans Polynesian Rat. Houses throughout Thailand.

33. Rattus rattus Roof Rat. The native, non-European forms with 42 chromosomes, with subspecific names such as jalorensis, diardii, robinsoni, and Rattus rattus thai, are found in wild, domestic, island,

and agricultural habitats throughout Thailand. Especially destructive to rice and coconuts.

Subgenus 5. Stenomys Aquatic Giant Rats

34. Rattus mülleri Müller's Giant Rat. Wet portions of lowland evergreen forest of the peninsula.

Subgenus 6. Leopoldamys Long-tailed Giant Rats

35. Rattus edwardsi Edwards' Rat. Evergreen forest of Phu Kradung National Park, Loei Province.

36. Rattus sabanus Noisy Rat. Evergreen forests throughout the Kingdom.

3. Mosquito Fauna of Thailand

OBJECTIVE: To collect, identify, catalogue and redescribe the mosquito species of Thailand. Information is also 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 318 mosquito collections were made in six provinces of Thailand. The majority of these collections were made in Chonburi, Trat, Chantaburi, Kanchanaburi, Nakhon Ratchasima and Pratoomthani provinces. These collections resulted in 2,414 pinned adults, 3,557 slide mounts of larvae, larval and pupal skins, and 49 slide mounts of terminalia. Progeny rearings of the Culex vishnui subgroup were obtained from 194 individual gravid females, from which a total of 1,894 pinned adults, 2,735 slide mounts of larvae, and larval and pupal skins were obtained. An additional 185 collections of adult and larval mosquitoes from Chiangmai were made, and a total of 705 Aedes larvae and pupae were received from SEAMP for mounting.

Anopheles: During this period "An Illustrated Key to the Anopheles Larvae of Thailand" was published. This key includes 42 plates of illustrations and is designed to provide entomologists, malariologists and public health workers with a reliable means of identifying

Anopheles larvae. A total of 57 species and subspecies are considered based on records from Thailand. Anopheles stricklandi and whartoni, previously known only from Malaysia, represent new records for Thailand.

Anopheles balabacensis, minimus and barbirostris, important vectors of malaria and filariasis, were collected from many areas at Sangkhlaburi, Kanchanaburi province during this period.

Culex: Studies of the subgenus Culex were continued. Species of the Culex vishnui subgroup were collected from animal bait collections. Progeny rearings from these mosquitoes were carried out in an effort to find more reliable adult characters for separating the various species of this important complex. Collections of Culex alienus, annulus, perplexus, pseudovishnui and tritaeniorhynchus were obtained during this period.

Aedes: Work is continuing on the subgenera Stegomyia and Finlaya. A new subgenus of Bothaella, first recorded in Thailand, is currently known from the following provinces: Chiangmai, Kanchanaburi, Lampang and Nan. The first record of the collection of an adult of Aedes eldridgei, a newly described species, was obtained when females of this species were collected biting man in bamboo groves and deciduous forest at Amphur Tha Muang, Kanchanaburi province. Also collected in the same area were Aedes (Stegomyia) albopictus, annandalei, desmotes, gardnerii imitator and scutellaris malayensis, Aedes (Finlaya) albolateralis, Armigeres (Armigeres) malayi, Heizmannia chengi and mattinglyi.

4. Laboratory Animal Disease in Thailand: Its Occurrence and Importance to Comparative Medicine

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 maintained in Thailand, and in developing animal 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: There was little evidence of disease among laboratory rodents during this report period. The annual production of rats,

mice, and guinea pigs has been maintained at levels comparable to previous years, as have indicators of production efficiency such as conception rate and yield per breeding female. Production was significantly lower during the hot season as in previous years. Ceiling fans have been installed in all breeding rooms in an effort to increase air circulation. The number of rodents necropsied and the distribution of gross pathologic lesions according to organ system is shown in Table 1. The most frequently observed gross lesion was lobar pneumonia.

Table 1. Summary of Rodent Breeding Colony Pathologic Findings for 1973

Species	Number Examined	Pulmonary Pathology	Gastro-Intestinal Pathology	Genito-urinary Pathology
Guinea Pig	50	5	2	4
Mouse	100	9	-	-
Hamster	75	15	5	-

Bacteria isolated from the lungs or feces of mice, hamsters, and guinea pigs as part of the disease screening program are shown in Table 2. The coliform group was most frequently isolated, followed in frequency by Proteus spp.

A number of spontaneous deaths occurred among primates in the laboratory during the year. The cause and frequency of deaths in rhesus monkeys during the initial 45 day quarantine period after arrival in the colony are summarized in Table 3. Rhesus monkeys arrive directly from the wild in India, and over 90% of them have intestinal parasites upon arrival. These animals are stressed by capture, transportation, and adjustment to a different environment, and are quite susceptible to enteritis which is often complicated by secondary bacterial infection. Bacterial enteritis accounted for most of the losses due to intestinal symptoms. Most of the losses due to pulmonary disease are attributed to primary measles virus infection with secondary bacterial complications. This was determined from clinical signs and confirmed by observation of pathologic lesions of interstitial and giant cell pneumonia. Virus isolation has not been attempted. Two cases of tuberculosis were diagnosed by tuberculin testing and confirmed by postmortem examination. Four deaths occurred among gibbons during this report period. These are categorized in Table 4.

Table 2

Bacterial Isolates Identified in Laboratory Rodents 1973

BACTERIAL ISOLATE	Mouse (100)		Hamster (75)		Guinea Pig (50)	
	Lung	Stool	Lung	Stool	Lung	Stool
Staphylococcus aureus	4(4%)	0	0	0	0	0
Staphylococcus epidermidis	4(4%)	0	6(8%)	0	8(16%)	0
Staphylococcus sp.coagulase+	1(1%)	0	0	0	0	0
Micrococcus sp.	5(5%)	0	3(4%)	0	0	0
Micrococcus tetragenes	0	0	1(1%)	0	0	0
Enterobacter aerogenes	0	4(4%)	0	7(9%)	0	4(8%)
Escherischia coli	0	52(52%)	0	13(17%)	0	14(28%)
Escherischia coli mutabile	0	18(18%)	0	1(1%)	0	1(2%)
Paracolobactrum aeroginoides	0	0	0	0	0	1(2%)
Paracolobactrum intermedium	0	0	0	0	0	1(2%)
Streptococcus fecalis	3(3%)	0	1(1%)	0	3(6%)	0
Alpha-hemolytic streptococci	1(1%)	0	1(1%)	0	0	0
Pseudomonas aeruginosa	0	7(7%)	0	0	0	3(6%)
Pseudomonas aeruginosa	0	7(7%)	0	0	0	3(6%)
Pseudomonas spp.	0	1(1%)	0	1(1%)	0	0
Proteus mirabilis	0	30(30%)	0	1(1%)	0	0
Proteus morganii	0	1(1%)	0	0	0	1(2%)
Proteus spp.	3(3%)	1(1%)	0	0	0	0
Bacillus spp.	0	0	1(1%)	0	0	0
Hafnia group	0	1(1%)	0	1(1%)	0	11(22%)
Herellea spp.	0	1(1%)	0	0	0	0
Citrobacter spp.	0	0	0	0	0	2(4%)
Providencia group	0	1(1%)	0	0	0	3(6%)
Salmonella paratyphi B	0	0	0	0	0	2(4%)
No pathogens isolated	85(85%)	17(17%)	62(82%)	33(44%)	31(62%)	10(20%)

Table 3

Rhesus Monkey Losses During the Initial 45-Day Quarantine Period

Month	Animals Received	Number of Deaths	Intestinal Disease	Pulmonary Disease	Encephalitis
January 1973	85	7 (8.2%)	7	1	0
March 1973	85	4 (4.7%)	2	0	0
June 1973	85	3 (3.5%)	3	0	0
October 1973	85	9 (10.6%)	6	3	0
January 1974	85	5 (5.9%)	4	3	0
March 1974	85	10 (10.6%)	3	5	2
Total	510	38 (7.4%)	25	14	2

Table 4. Summary of Gibbon Necropsy Findings

Principle Pathologic Finding	Number of Animals
Enteritis (Shigella flexneri)	1
Enteritis	1
Traumatic Injury (cervical fracture)	1
Granulocytic Leukemia	1

5. Intestinal Parasitism in the Rhesus Monkey

OBJECTIVE: To determine the incidence of intestinal parasites in rhesus monkeys arriving from India, and to evaluate the efficacy of the treatment used to control them.

BACKGROUND: Over 500 rhesus monkeys per year are brought into the animal colony at the SEATO Medical Research Laboratory. These animals are received in groups of 85 every 60 days, come directly from the wild in India, and weigh 2-3 kg upon arrival. They are usually heavily parasitized, are highly susceptible to viral and bacterial infection, and have been stressed by transportation and captivity when they arrive. In February 1973, a one year study was initiated to monitor the incidence of internal parasites in rhesus monkeys from India. Monkeys are quarantined for 45-60 days in our colony before issue to investigators.

PROGRESS: A fecal specimen was taken from each animal on the day of arrival and examined by the flotation method for ova and parasites. Each monkey was then treated by oral administration of thiabendazole (100 mg/kg body weight) on the next day. Fecal specimens were collected ten days after treatment and again examined for ova and parasites. All positive animals were re-treated with 100 mg/kg thiabendazole. Fecal specimens were again obtained at the end of the quarantine period. The results of these examinations are shown in Table 1.

DISCUSSION: The data presented in Table 1 were collected from 510 monkeys received over a 12 month period (Feb 1973 - Feb 1974). Over 95% of the animals received were infected with parasites; over 90% with helminths, and over 75% with protozoans. Strongyloides sp. and Trichostrongylus sp. were the most commonly identified helminths. The incidence of helminths dropped to less than 10% after one treatment with thiabendazole, and to 5% after a second treatment. All helminths except Trichuris sp. were virtually eliminated after two treatments. The incidence of Trichuris sp. remained at the same relatively low

Table 1

Intestinal Parasites Found in Rhesus Monkeys

PARASITE FOUND	Before treatment		After first treatment		Upon release from quarantine	
	No. Pos.	% Pos.	No. Pos.	% Pos.	No. Pos.	% Pos.
Helminths						
Strongyloides sp.	341	66.9	17	3.3	3	0.7
Trichostrongylus sp.	148	29.0	2	0.4	0	-
Ancylostoma sp.	43	8.4	8	1.6	2	0.5
Trichuris sp.	26	5.1	19	3.7	17	4.0
Nematode (Unspecified)	17	3.3	13	2.5	1	0.2
Ascaris sp.	1	0.2	1	0.2	0	-
Gastrodiscoides sp.	1	0.2	0	-	1	0.2
Streptopharagus sp.	0	-	0	-	1	0.2
Capillaria sp.	0	-	2	0.4	0	-
Protozoans						
Entamoeba coli	233	45.7	159	31.2	142	33.4
Entamoeba histolitica	156	30.6	148	29.0	172	40.5
Balantidium coli	127	24.9	107	21.0	82	19.3
Iodamoeba buetschlii	103	20.2	46	9.0	44	10.4
Endolimax nana	46	9.0	55	10.8	40	9.4
Chilomastix mesnili	9	1.8	17	3.3	17	4.0
Giardia lamblia	3	0.6	35	6.9	28	6.6
Unidentified	6	1.2	12	2.4	2	0.5

level as before treatment.

The incidence of protozoans remained at approximately 75% throughout the quarantine period. The protozoans identified are commonly found in wild primates, appear to cause no serious clinical problems, and were not specifically treated. There was no significant change in parasite incidence or distribution with the season of the year.

CONCLUSION: The incidence of intestinal parasites in wild rhesus monkeys from India was over 95%, with no significant seasonal variation. Thiabendazole given orally in a single dose of 100 mg/kg was shown to be highly effective against helminths, with a second dose ten days later virtually eliminating all helminths except Trichuris sp. This regimen of treatment was just as effective as the longer course of treatment (daily oral administration of 50 mg/kg for 10 days), required much less time and effort in catching and medicating animals, and placed less stress upon the animals being medicated.

6. Gibbon Menstrual Cycle and Breeding Study

OBJECTIVE: To gather information about normal reproduction and breeding habits of gibbons in captivity.

BACKGROUND: A colony of 45 gibbons (Hylobates lar) is being maintained at the SEATO Medical Research Laboratory for use in the medical research projects of the laboratory. An active breeding program has been conducted for the past several years, and 22 gibbons have been born in the colony. Insofar as it is consistent with the other research objectives of this laboratory, observations of the reproductive and breeding habits of these animals have been made on a regular basis.

PROGRESS: Vaginal swabs were taken daily from ten adult female gibbons and the time of menstruation was recorded. In the 110 menstrual periods observed, the duration of menses was 1-4 days, and the interval between menses was 15-107 days (Table 1). The most common time interval between menstruations was 15-25 days (67.4% of the cases). Eight pairs of gibbons were allowed to mate naturally during the year. In an effort to gain information about breeding habits, 21 matings were observed during normal duty hours (7:30 am - 4:30 pm). The duration of actual copulation was recorded in 18 of the matings and is shown in Table 2. In most matings copulation time was less than 3 minutes. In all 21 matings, both female and male were facing the same direction. The position of each animal is shown in Table 3. The most common position was the male vertical and the female horizontal. Eleven matings were observed on the flat elevated seat, two on the floor of the cage, five hanging from the cage wall, and one on the exercise bar.

Monthly rectal palpations were performed on all breeding animals during the year, and the size of the uterus and developing fetus recorded. These observations are presented in Table 4. Pregnancy can be confirmed

Table 1

The Duration of the Sexual Cycle in Female Gibbons

Gibbon Identification Number	D_1	I_1	D_2	I_2	D_3	I_3	D_4	I_4	D_5	I_5	D_6	I_6	D_7	I_7	D_8	I_8	D_9	I_9	D_{10}	I_{10}	D_{11}	I_{11}	D_{12}	I_{12}	D_{13}	I_{13}	D_{14}
S-2	1	23	1	39	2	19	2	17	2	20	1	17	1	18	1	19	2	17	2	32	1	3	1	28	1	[illegible]	3
S-81	2	52	2	15	3	107	1																				
S-42	1	25	1	24	2	34	1	23	2	29	1	54	1	21	1	26	3										
S-43	1	103	2	24	1	94	2	45	2	20	3	9	1														
B-14S	1	20	2	24	1	17	1	23	1	70	1	25	2	30	3	25	1										
B-99S	2	20	3	17	1	23	3	34	1	24	2	18	2	17	1	23	1	21	1	36	2	21	1				
B-85	2	18	1	38	1	20	1	20	1	18	1	19	1	18	1	40	3	37	2	21	3	22	2	18	2	[illegible]	4
B-88	1	21	2	22	1	20	1	43	1	21	2	63	1	40	1	43	2										
P-2	2	21	3	17	3	19	2	30	3	19	1	15	1	35	1	42	2	55	1								
P-3	1	20	1	49	2	23	1	20	1	22	1	20	2	22	2	20	2	20	1	25	1	46	3	21	1		

D_1 & D_2 = Duration of menses in days

I_1 & I_2 = Interval between menses in days

TABLE 2

GIBBON COPULATION TIME

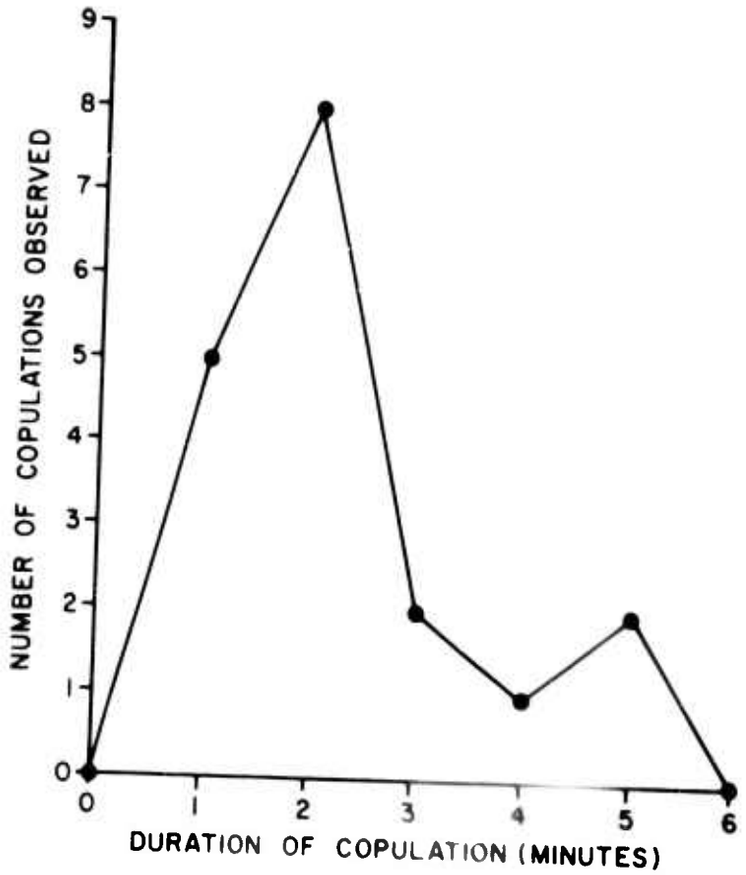


TABLE 3

COPULATION POSITIONS

[illegible]ON DURING COPULATION	NUMBER OF MATINGS OBSERVED
MALE VERTICAL AND FEMALE HORIZONTAL	13
MALE VERTICAL AND FEMALE VERTICAL	6
MALE HORIZONTAL AND FEMALE HORIZONTAL	1
MALE HORIZONTAL AND FEMALE VERTICAL	1

Table 4

Fetal Development

Gibbon Identification Number	Approximate Uterine Size*												
	Mar 73	Apr 73	May 73	Jun 73	Jul 73	Aug 73	Sep 73	Oct 73	Nov 73	Dec 73	Jan 74	Feb 74	Mar 74
B-4	A	A	A	A	A	B	P	P	P	P	D	N	N
B-6	B	B	B	P	P	P	P	D	N	N	N	N	N
B-7	A	A	B	A	A	A	A	A	B	B	P	P	P
B-11	B	B	C	P	P	P	D	N	N	N	N	W	
B-59	A	A	A	B	A	A	A	B	P	P	P	P	D
B-89							A	A	A	A	A	A	A
PC-1							A	A	A	A	A	A	A
S-61	C	P	D	N	N	N	N	W					
V-175	A	A	A	A	A	B	C	P	P	D	N	N	N

*

A less than 2.5 cm in diameter
B 2.5 to 4 cm in diameter
C 4 to 5 cm in diameter
D month of parturition
N Lactating mother; not palpated
P confirmed pregnant; not palpated
W offspring weaned

by rectal palpation as early as the third month of gestation. Table 5 presents data concerning seven gibbons born during the past year.

DISCUSSION: Eight female gibbons were maintained in outdoor breeding cages, and access to males permitted during the reporting period. Seven young were born to six mothers. Two new breeding pairs were established and have not yet produced young. The ten females being observed for normal menstrual cycles serve as a reservoir of new breeding stock, and enables an evaluation of the menstrual pattern to be made before the animals are mated.

Table 5. Newborn Gibbons, 1973 - 1974

Delivery Date	Female Number	Male Number	Baby Number
1 Jan 73	B-59	B-8	Pc-16
19 May 73	S-61	P-16	Pc-18
26 Sep 73	B-11	S-58	Pc-19
23 Oct 73	B-6	B-12	Pc-20
26 Dec 73	V-175	S-18	Pc-21
2 Jan 74	B-4	B-8	Pc-22
10 Mar 74	B-59	B-12	Pc-23

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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 0B 6491	74 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
73 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. CONTRIBUTING	CARDS 114F			

11. TITLE (Provide 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 PRECEDING 74	7	171
FISCAL YEAR CURRENT 75	8	264

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide Each with Security Classification Code) (U) Mass Spectrometry

(U) Drugs of Abuse; (U) Toxicology; (U) ESR; (U) Chromatography; (U) Immunoassay

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

23. (U) To develop and assess analytical techniques for the detection, identification and quantitation of drugs of abuse in biological fluids for use in the military population..

24. (U) Efforts will be concentrated on the development and optimization of simple, rapid and accurate systems of drug detection for operational laboratory use. Some of the techniques utilized will be thin layer chromatography (TLC), gas liquid chromatography (GLC), high pressure liquid chromatography (HPLC), free radical assay technique (FRAT), mass spectrometry (MS), radioimmunoassay (RIA), hemagglutination inhibition (HI), and enzyme multiplied immunoassay technique (EMIT). Use of appropriate quality control measures will be stressed.

25. (U) 73 07 - 74 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 TLC method for detecting methaqualone in urine was developed and implemented. A new technique of using alpha-hydroxymethaqualone to methaqualone ratio has been shown to be effective in improving detection. A screening method for cocaine and metabolites in urine using FRAT and EMIT has been evaluated and found effective. A GLC method for urinalysis of cocaine metabolites has been developed. Work continues on the development of analytical methods for 11-hydroxytetrahydrocannabinol. Procedures reported in the literature have not proved as effective as claimed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* 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 3A762 A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Assay methodology for drugs of abuse

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The technical objectives of this work unit are to develop and evaluate analytical methods for the detection, identification and quantitation 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. Methadone Analysis by GLC and 3 immunoassays.

2. Cocaine Analysis

3. Detection and Identification of Methaqualone Metabolites by Thin Layer Chromatography.

4. Evaluation of the Use of Solid Extractants for Drugs of Abuse in Urine.

5. Immunoassays for Drugs of Abuse.

6. Synthesis of Drug Metabolites.

7. Effects of Drugs of Abuse on Lysogenic Bacteria

8. Quality Control.

9. Instrumental methodology development and evaluation of a portable fluorometer for morphine analysis

10. Effects of 5,5-Diphenylhydantoin (Dilantin) on amino acid pools in rat brain

11. Valium (Diazepam) levels in plasma of anephric and normal individuals

12. Morphine glucuronide analysis

13. Gas chromatographic analysis of ecgonine as esters

14. Pharmacokinetics of procainamide in renal failure

1. Methadone analysis: A comparision of gas-liquid chromatography and immunoassay techniques.

Methods for the detection and quantitation of free methadone in urine are thin-layer chromatography (TLC), gas-liquid chromatography (GLC), spectrophotometry and immunoassays. Each of the first three methods require considerable expenditure of time and effort, for pH adjustment, extraction and concentration while the immunoassays are performed directly on urine. These techniques were compared to determine the limitations for their use for methadone analysis both qualitative and quantitative.

Urine from 113 patients on methadone maintenance was analyzed by TLC, GLC and three immunoassay techniques - Free Radical Assay Technique (FRAT), Enzyme Multiplied Immunoassay Technique (EMIT) and a Modified EMIT (EMIT-M). In the latter case a urine blank is included with no enzyme from the kit to correct for endogenous lysozyme activity in the urine. The results of the comparison are shown in the table below.

	METHOD				
	TLC	GLC	FRAT	EMIT	EMIT-M
Number positive	109	110	113	112	110
Mean conc. ± SEM		6.0 ± .5[a]	8.4 ± 1.1[b]	8.2 ± 1.2[b]	6.2 ± .9[b]
GLC vs Immunoassay significance			p<.02[c]	p<.10	ns

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[a] µg/ml methadone

[b] Expressed as µg equivalent of methadone per ml

[c] Using paired "t" test

Of the 113 specimens analyzed by GLC, 92 (81%) contained methadone metabolite I, and only 3 (2.6%) contained metabolite II.

The GLC results were lower than the FRAT ($p<.02$) results, but did not differ significantly from the EMIT results. The mean concentrations by GLC and EMIT-M were numerically similar. The EMIT results were significantly higher ($p<.01$) than the EMIT-M results.

Each technique has advantages and disadvantages. GLC is the most sensitive (0.2µg/ml) but is technically the most difficult and is the most time consuming. TLC is the least sensitive, does not produce reliable quantitative results and is affected by the presence of other drugs, but is simpler to implement and is reasonably good for screening work. The immunoassays, since they are performed directly on urine, provide the most rapid and are technically the simplest method of analysis. The man-hours required for GLC, FRAT and EMIT are in the ratio 10:1:1. The equipment costs are in the ratio of 2:7:1. The GLC and EMIT-M results were comparable and further study of the modifications to the immunoassay methods is indicated. EMIT-M and EMIT are essentially equal in equipment and costs. EMIT-M requires about 40 seconds longer time per analysis than EMIT and one additional aliquot of reagents.

2. Cocaine analysis

A sensitive, accurate and reliable method for the quantitation of cocaine and its metabolites - ecgonine and benzoyl ecgonine in urine by GLC was developed. Urine from six rhesus monkeys given 1 mg/kg doses of cocaine was extracted and the extracts hydrolized to convert cocaine and benzoyl ecgonine to ecgonine which then served as a measure of the total amount of cocaine and its metabolites excreted. The mean concentration of cocaine and its metabolites in the pooled urinary output at 0-24, 24-48, and 48-72 hours post injection was 2.205 ± 0.304 mg/ml, 1.21 ± 0.364 mg/ml and 0.585 ± 0.251 mg/ml respectively. The mean total ecgonine recovered for all animals was 3.365 ± 0.512 mg, or 51% of the administered dose. In spiked human urine, mean ecgonine recovery was $41 \pm 3.9\%$. In spiked monkey urine mean ecgonine recovery was $54 \pm 2.4\%$. A concentration versus GC response curve for ecgonine concentrations between 0.0133 mg/ml and 3.0 mg/ml is linear with a correlation coefficient of 0.99937.

3. Detection and identification of methaqualone metabolites by thin layer chromatography

Methaqualone is an abused sedative - hypnotic drug which is absorbed rapidly by the gastro-intestinal tract and excreted in the urine primarily as monohydroxy metabolites. An improved method was developed for the detection of methaqualone metabolites in urine by thin-layer chromatography.

The method requires 5 ml of urine and involves the hydrolysis by 10 percent sodium periodate, adjustment of pH to 9.5, chloroform extraction and development on silica gel TLC plates by ethylacetate-methanol-ammonia (85:10:5) or by chloroform-acetone-ammonia (50:50:2). The metabolites were identified as a pattern of four spots viewed under long UV light and visualized by iodoplatinate spray. A level of 3 micrograms of each metabolite is required for detection by UV viewing; 10 micrograms of each metabolite is required for detection by iodoplatinate spray only. Over 300 positive and 150 negative urine specimens have been analyzed by this method. Agreement with other testing methods demonstrate high reliability of the thin-layer method.

4. Evaluation of the use of solid extractants for the detection of abused drugs in urine

The analysis of urine for drugs of abuse requires a preliminary extraction to separate the drugs from urinary constituents. Previous studies in this laboratory described the characteristics and control of variables for efficient drug extraction with XAD-2 resin. These studies were continued and expanded to include the most common drugs of abuse.

Commerical XAD-2 resin columns (2 gm resin/column) were activated by washing with methanol and water and stored overnight in a refrigerator under water. Radioactive drugs (^{14}C labeled) and cold compounds (not radio-labeled) were added to normal urine to give 10,000 to 20,000 CPM and drug concentrations to 0.5 µg/ml morphine, 1 µg/ml methadone, and 5 µg/ml amphetamine. Urine (20 ml) was added to each and permitted to flow through at a controlled rate of 2 ml/min. The column was washed with water and the drugs were eluted with chloroform -isopropanol solvent. All fractions of the urine, the resin, and a radio labeled solution not extracted and used as a reference were counted in a liquid scintillation spectrometer to determine recovery. The results are shown below:

Recovery of Drugs of Abuse
At Different Stages of XAD-2
Resin Extraction (Percent)

Drug	Adsorption	Urine Effluent	Water Wash	Organic Eluate	Resin
Morphine	98-99	1-2	<1	89-94	1
Methadone	98-99	<1	<1	95-98	1-2
Secobarbital	96-98	2-3	1	95-97	<1
Amobarbital	90-96	4-9	1-2	86-93	<1

The XAD-2 resin method was compared to a chloroform-ispropanol extraction procedure performed simultaneously.

Recovery of Drugs of Abuse
in Organic and Aqueous Phases
of Liquid-Liquid Extraction (Percent)

Drug	PHASE	
	Organic	Aqueous
Morphine	92-95	2-5
Methadone	97-98	1-2
Secobarbital	83-94	2-4
Amobarbital	87-91	1-2

The adsorption of the studied drugs on activated XAD-2 resin columns was 90 to 99 percent. The drugs were eluted from the resin using chloroform - isopropanol (75:25 volume ratio).

The yields with liquid - liquid extraction were lower overall than the resin columns, but still in an acceptable range for TLC identification. If the eluant or liquid extractant were cholorform alone, the overall yield dropped to about 82% by resin columns and near 80% with liquid-liquid procedures. If the dried organic extracts were used for TLC or GLC analysis, tl. less efficient chloroform extraction was preferred because it contained less interfering substances which complicated interpretation of results.

In another study, XAD-2 was used to determine the practicality of a single extraction of morphine, amphetamine and phenobarbital from urine with the resin. A number of drug screening laboratories have reported variable and low extraction efficiencies using the resin. The study showed that more than 90% of the ^{14}C amphetamine and more than 80% of the ^{14}C phenobarbital placed on the resin column can be extracted. To achieve these results, controlled eluant flow rates, adjusted urinary pH (7.5 - 9.0) and selection of elution solvent were required. These efficiences were equal to or better than those obtained for four standard liquid/liquid extraction techniques, and the resin extraction technique required 66% less organic solvent. The conditions for the resin extraction involved 20 ml of buffered urine passed over 2 gm of XAD-2 resin in a 0.9 x 5 cm column and the elution of adsorbed drugs from the column with 20 ml of ethylacetate: dichloroethane: isopropanol (5:3:2 volume ratios) at a flow rate of 2.1 ml/minute. This procedure is flexible and seems to have wide applicability in drug screening laboratories.

5. Immunoassays for drugs of abuse

The Free Radical Assay Technique (FRAT), Enzyme Multiplied Immunoassay Technique (EMIT), and Radioimmunoassay (RIA) methods have been used to detect opiates and cocaine metabolites in urine specimens and have been evaluated as analytical tools for a variety of research and clinical applications.

a. The possibility of correlation between hepatitis and opiate use was investigated. Urine samples (100), from hepatitis patients (31) and normal controls (69) some of which were obtained from patients on an orthopedic ward, were analyzed by FRAT for morphine. The specimens from the hepatitis patients were negative for morphine. The control specimens from the orthopedic ward patients, who had been given morphine as an analgesic, were positive for morphine.

b. Urine specimens (1000) collected at Ft. George G. Meade, Maryland, in a collaborative screening study with the Division of Neuropsychiatry, WRAIR, were analyzed by FRAT for cocaine. All specimens were negative for cocaine metabolites. Urine specimens from patients who had been administered cocaine were used as positive controls. The control specimens were positive for cocaine metabolites.

c. Evaluation of commercial RIA kits for detection of drugs of abuse in urine.

Two commerical drug screening kits were evaluated - the ^{125}I - LSD detection kit from Collaborative Research, Inc. and the ^{125}I - Barbiturate Abuscreen Kit, manufactured by Roche Diagnostics.

(1) The screening kit for lysergic acid diethylamide (LSD) in urine was found to be a tedious, complex and imprecise technique. The very involved and cumbersome procedures make the assay impractical for use as mass screening method. The lack of chemical specificity and the lack of a satisfactory confirmatory procedure make the use of the RIA for LSD impractical.

(2) The Roche Abuscreen ^{125}I - Barbiturate kit was found to be a valid and useful test for qualitative drug screening for large numbers of specimens. Extensive testing of the kit demonstrated that with careful pipetting techniques the assay gives excellent results for detection of pentobarbital, aprobarbital, amobarbital, secobarbital, amobarbital and phenobarbital in urine.

d. Evaluation of the EMIT immunoassay

The EMIT system was evaluated as a urine screening method for drugs of abuse. The objective of the evaluation was to determine the efficacy of the technique for qualitative urine screening. This objective included an estimate of the probability of false positives in a screening effort and the effect of various test parameters such as sample pH, salt concentration, specific gravity and subject fasting on the outcome of the tests. The evaluation produced the following conclusions:

(1) The detection of morphine, amphetamine, barbiturates and benzoyl ecgonine by the EMIT method was dependent on the pH and the salt concentration of the urine sample, and these parameters should be controlled in drug assays.

(2) Of the 200 urine samples assayed, no false positives were found for morphine and methadone. False positives were detected with amphetamine reagent (6%), barbiturate reagent (secobarbital, 19%) and benzoyl ecgonine reagent (7.5%). The EMIT system must be used in conjunction with confirmatory methods for the detection of these drugs.

(3) Urine samples adjusted to 0.4 or 0.8 M NaCl or KCl suppressed the reactivity of morphine, methadone, amphetamine, barbiturate and benzoyl ecgonine reagents.

(4) Urea concentrations of up to 0.8 M in the urine samples had no effect on the detection of morphine, methadone, amphetamine, barbiturates or benzoyl ecgonine.

(5) Fasting or normal diet had no effect on the detection of morphine in urine.

(6) The EMIT system is unreliable for the quantitation of methadone, amphetamine, barbiturates or benzoyl ecgonine in urine.

6. Synthesis of metabolic compounds

a. Metabolites

The analysis of metabolites requires the availability of reliable standards for accurate instrument calibration. The control of the purity of physiological standards is difficult. A considerable effort has been made to synthesize those compounds needed for the analytical procedures. The principal metabolites that have been synthesized are:

(1) 3'-hydroxy methaqualone

(2) 4'-hydroxy methaqualone

(3) 6-hydroxy methaqualone

(4) alpha-hydroxy methaqualone

(5) N-acetyl-p-amino-hippuric acid

(6) N-acetyl procaine amide

b. Haptenes

Opiate coupled albumin moities have been synthesized for use as radioimmunoassay antigens.

(1) Morphine-3-succinyl-bovine serum albumin

(2) Morphine-6-succinyl-bovine serum albumin

(3) 3 - O-carboxy methylmorphine-human serum albumin

(4) Morphine monohemisuccinate-bovine serum albumin

7. The effects of drugs of abuse on lysogenic bacteria

An attempt was made to develop a method of detecting abused drugs in body fluids by observing the inhibition of bacterial growth in the

presence of these drugs. The method involved inducing cells of Escherichia coli K_{12} (R) with ultraviolet light or mitomycin C and culturing them in various concentrations of drug. Turbidity measurements were used as indicators of drug effect.

The following drugs were tested by this method:

Opiates - Morphine sulphate, 3,6 - diacetyl morphine, codeine and cocaine

Barbiturates - Phenobarbital, amobarbital, secobarbital and pentobarbital

Amphetamines - Methamphetamine and dextroamphetamine

None of these drugs were found to induce phage in E. coli K_{12} (R) at concentrations of 10^{-2}M to 10^{-4}M. Only phenobarbital and pentobarbital exhibited any drug toxicity and that was at 10^{-2}M. The conclusion was drawn that this bioassay method could not practically compete with the microgram and even nanogram sensitivities of the instrumental and immunoassay methods and offers no advantages over them.

8. Quality control

The support of a quality control program for U.S. Army Medical Department contract drug screening laboratories was terminated. The support involved the preparation, analysis, shipment and analytical follow-up of urine specimens used to determine the competence of screening and confirmation analytical procedures in urine testing laboratories. Approximately 1000 specimens were prepared, analyzed and distributed during this fiscal year. Additionally this laboratory participated in the quality control program sponsored by the Center for Disease Control. Participation was both as a practicing laboratory and as a reference laboratory.

9. Evaluation of a Portable Fluorometer for Screening Morphine in Urine

A portable fluorometer manufactured by the Space Sciences Division of the Whitaker Corporation was extensively evaluated for use as a screening instrument for morphine in urine. Spiked and metabolite-containing urine specimens were used in the test. A total of 102 analyses were made using physiological urine with total morphine concentrations of from 0.5 µg/ml to 1.0 µg/ml. The Whitaker Instrument did not detect the morphine. FRAT and RIA methods easily detected the morphine in these samples. The instrument detected morphine in a group of unhydrolized, "spiked", urine samples containing morphine in concentrations from 0.25 µg/ml to 5.0 µg/ml. When the same samples were hydrolized, morphine could not be detected fluorometrically because of the increased non-specific fluorescence.

It is concluded that the instrument is minimally sensitive for detection of morphine but there must first be developed simple, effective means of freeing conjugated morphine in physiological samples without the production of interfering background material. There is not now a practical method available (with the exception of an enzymatic procedure requiring an extended period of incubation).

10. Effects of 5,5-Diphenylhydantoin (DPH) on Amino Acid Pools in Rat Brain

A study was made to determine free amino acid patterns in various tissues from rats after the administration of DPH and carbon labeled (^{14}C) compounds. Glucose, pyruvate, leucine, butyrate or lactic acid each labeled with ^{14}C were injected I.V. in rats. The free amino acids of brain, liver and serum from control and DPH injected rats were analyzed. The colorimetric amino acid profile of each specimen estimated the total amount of each free amino acid in tissue. The results showed no differences between the control and experimental animals. The amino acid pool remained constant.

In duplicate experiments 3 ml aliquots containing ^{14}C - labeled amino acids were collected. Glucose and pyruvate were similarly incorporated into aspartic acid, glutamine, glutamic acid and gamma amino butyric acid. The difference in incorporation seen in two of labeled glucose and pyruvate into the amino acids groups of rats (control and experimental) was more observable in brain tissue. The amino acid concentrations tended to be variable and to be varying within a larger amino acid pool that did not vary as markedly as did the aspartic acid, glutamine, glutamic acid and gamma amino butyric acid. The three other compounds, lactic acid, butyrate and leucine showed differences in glucose and pyruvate incorporation in amino acids between control and experimental rats. Further studies are planned to attempt to elucidate this observation.

11. Valium (Diazepam) levels in plasma of anephric and normal individuals

Approximately 150 plasma specimens from normal individuals and patients in various stages of renal insufficiency who had received 10 mg of valium (Diazepam) were analyzed. This study was conducted to investigate possible variations of drug metabolism as a function of renal insufficiency. Preliminary evaluation of results indicate no significant differences between normal patients and those with renal insufficiency.

12. Morphine glucuronide analysis

Work was continued on the direct analysis of glucuronides. Opiates are generally excreted in urine as glucuronides.

These glucuronides are cleaved either by enzymatic means or by acid hydrolysis prior to analysis - techniques which destroy all the information about the nature of the compounds and their metabolic history. Isolation techniques and analytical procedures for the intact glucuronides are required in order to study the pharmacokinetics of opiates.

Several approaches have been attempted - derivatization (trimethyl silylethers, mixed esters - trimethyl silylethers and permethylation) and silylation primarily. One method that produced results involved the use of a mixture of pyridine - acetonitrile (1:1) as the solvent and a silylation mixture, N,O-bis (trimethylsilyl trifluoro acetamide), trimethyl silyimidazol and trimethylchlorosilane (3:2:1). For a 50 µg sample, 100 µl of solvent and 100 µl of the silylation agent mixture were used. Heating of the reaction mixture for 60 minutes was required. This heating time was critical. A small gas chromatograph peak was observed at about 10 minutes (retention time). With the following gas chromatograph conditions:

Column: 6 feet by 2 mm (I.D.) glass, Apollo type, packed with 3% OV-1 on gas chrom Q 100/120 mesh.

Temperature: Oven - 275°C, injection port - 300°C, detector - 300°C.

Detector: Flame ionization

Carrier: 40 ml/min. helium

Further refinement of the procedure and mass spectrometric confirmation of the peak are planned.

High performance liquid chromatography has been extensively investigated as a tool for glucuronide analysis. A number of attempts at optimizing the various instrumental parameters for glucuronide analysis have been made with discouraging results.

13. Analysis of ecgonine as esters

A preliminary study has been made to determine the practicality of analyzing ecgonine as its ester. The ester was formed by reaction with dimethylformamide acetal and analyzed by gas chromatography. The gas chromatograph conditions were as follows:

Column: 6 feet by 2mm (I.D.) glass, Apollo type, packed with 3% OV-1 on gas chrom Q 100/120 mesh.

Temperature: Oven 188°C, injection port - 280°C
detector - 280°C

Carrier: Helium, 40 ml/min.

Detector: Flame ionization

Five esters are formed in this manner, and they are listed below along with their gas chromatographic retention times.

Ester	Retention Time (Min.)
Methyl	5.93
Ethyl	7.24
n Propyl	9.94
t butyl	8.31
n butyl	13.63

The n butyl ester was studied in greater detail. The linearity was excellent over the range of 0.2 to 1.0 µg (on column), and the retention time variation in 4 injections was 13.18 to 13.26 minutes. Recovery of ecgonine from spiked water samples was about 40 percent by chloroform - isopropanol extraction.

14. Procainamide in renal failure

To investigate the effect of end stage renal insufficiency and hemodialysis on the serum half life of procainamide, 500 mg of procainamide was given orally to normal volunteers and to dialysis patients. Procainamide was assayed by spectrophotometry and spectrophotofluorometry. Mean half life in normal subjects was 3.2 hours by spectrophotometry and 3.5 hours by spectrophotofluorometry. Mean half life in patients was 11.3 hours by spectrophotometry and 16.0 hours by spectrophotofluorometry. The difference in measured half life between patients and normal volunteers was significant with $p<0.001$ (student t test). Half life of procainamide during dialysis was 4.3 hours compared to 9.6 hours off dialysis. This difference was significant with $p<0.001$. Both methods of assay gave higher levels of procainamide when the metabolite, N-acetylprocainamide, was present in serum but spectrophotometry was affected less. Thus, end stage renal insufficiency greatly prolongs the half life of procainamide. Secondly, procainamide is readily dialyzable. Thirdly, N-acetylprocainamide is hydrolyzed in 1 N HCl to procainamide during routine serum determinations.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Assay methodology for drugs of abuse

Literature Cited.

Publications:

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3. Gorodetsky, Charles W., Angel, Charles R., Beach, Douglas J., Catlin, Don H., and Yeh, Shu-yuan, Validity of screening methods for drugs of abuse in biological fluids. 1. Heroin in urine. Clin. Pharmacol. Therap. 15: No. 5, 461-472, 1974.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6492	74 07 01	DD DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISTR INSTR	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	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	3A762758A833	00	102
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114 (f)			

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 74	4	158
	CURRENT 75	4	194

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) 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) The specific objective of studies managed within the work unit is to specify the probable impact of drug abuse on military performance using laboratory models and performance measures logically equivalent to [illegible] of military performance. In addition, data are obtained from studies managed [illegible] 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) 73 07 - 74 06 A series of studies of the effects of delta-9-THC, the presumed principal active component of marihuana, indicate clearly that behavioral tolerance to the drug reflects learning rather than any of the phenonena usually described as tolerance. Studies of heroin self-administration in baboons indicate a profound and debilitating reduction of food intake. Initial unit dose is a powerful determinant of ultimate intake level, however, considerable exposure to the drug may be necessary before sustained stable intake is attained. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon requestor's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE [illegible] FORMS 1498A 1 NOV 68 AND 1498-1 1 MAR 68 FOR ADMIN 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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Associate: CPT Steven R. Hursh, MSC; CPT Frederick J. Manning, MSC; CPT George R. Sessions, MSC; Mary Carol P. Boren, Ph.D.; Timothy F. Elsmore, Ph.D.

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 unit 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, 111 and 122. Specific objectives of studies managed within the work unit is 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

Tolerance to the effects of tetrahydrocannabinol (THC) on spaced responding by monkeys

Previous work in this lab, and in others, has established that many of the effects of THC, in a variety of species, disappear rather quickly with repeated dosing. This is a striking contrast to reports by human marihuana users that they need smaller, rather than larger doses to achieve the effects they desire. We have suggested that this discrepancy stems from contingencies of reinforcement: tolerance will be seen when the drug effect is clearly detrimental to the subject, but not when the effect is neutral or beneficial. As in most experiments using non-human subjects, THC typically reduces the rate of reinforcement of monkeys rewarded only for spacing lever presses at least 60" apart. Although this is the most sensitive assay for THC of all the behaviors we have examined, the effect typically dissipates after 10-20

exposures to the drug. The present experiment attempted to hold rate of reinforcement constant during a series of THC injections by changing the spacing requirement from 60 sec to 52 sec beginning on the 1st drug treatment day. The typical shift toward shorter interresponse time was apparent in the first THC session, and was undiminished after 20 sessions. Half the monkeys were then returned to a DRL 60 schedule, and spacing quickly approximated pre-drug performance. The remaining monkeys showed no changes in spacing during these sessions. This experiment supports the notion that a substantial portion of the tolerance to behavioral effects of THC is due to the general tendency of organisms to maximize reinforcement density and not specifically related to the changes in the organisms, such as drug disposition, traditionally described as tolerance.

Chronic delta-9-THC and multiple FI-DRL performance

Two rhesus monkeys were trained and stabilized on a mulitple FI 2' DRL 2' schedule with the restriction that a component would be terminated following the elapse of 5' when no reinforcer was earned. This baseline was chosen for a chronic THC study since it has the property of producing opposite effects on rate of reinforcement in the two components with increases in overall response rate, increasing the rate of reinforcement in the FI and decreasing the rate of reinforcement in DRL. This, therefore, provides a situation for examining the importance of drug effects on rate of reinforcement in the production of tolerance to the drug effect. A chronic series of drug administrations (1 mg/kg/day, three hours pre-sessions, 40 days) showed an initial increase in response rate in both components, followed by a decrease in rate in the DRL component only. Rates remained elevated throughout the entire drug series in the FI component. Initially, reinforcement rates in the DRL component went down, but they recovered to nearly baseline levels after several days. Reinforcement rates in the FI component were generally unaffected or went up, with occasional sessions with very low rates producing some variability in the data. This procedure is to be replicated with a lower dose in a second chronic series, but present data are fairly convincing, showing that the production of tolerance in a single animal performing on different schedules of reinforcement is dependent on the schedule of reinforcement.

Analysis of the contingencies underlying prevention, establishment, maintenance and elimination of self-administration of drugs in laboratory primates

As indicated on the initial protocol, the first step in the research program was an attempt to define some of the basic parameters of heroin self-administration in the baboon. To this end, considerable time was devoted to the preparation of surgical

and data analysis techniques necessary for chronic studies of administration of drugs to the baboon. The initial experiment performed with baboons allowed six animals access to heroin for 22 hours daily. Three different unit doses (dose per infusion) were used, 0.02 mg/kg, 0.10 mg/kg, and 0.50 mg/kg. These animals also had continuous access to food (0.75 g Noyes pellets) and water. The animals' cages contained three different press plates which were associated with the delivery of food (5 presses/pellet), water (1 press/2 cc drink) and drug (1 press/0.1 cc infusion). Thus, the rationale for this initial experiment was to examine the effects of unit dose of drug on acquisition of drug intake. Additionally, patterning of drug intake throughout the day, and the effects of drug intake on food and water intake were also of interest. The catheterization procedure involved the use of concentric catheters, allowing sampling of blood through the inner catheter. Thus, it was also possible to take blood samples periodically for analysis by a radioimmunoassy (RIA) procedure for heroin, morphine and its metabolites developed in the neuroendocrinology department of our division (See Work Unit 122 for details of the method).

In the initial study, we were concerned with the possibility that animals might, given free access to high unit doses of heroin, infuse enough heroin to produce severe respiratory depression or death (i.e., "overdose"). To avoid this possibility, the animals on the highest unit dose also were provided with a contingency preventing them from taking more than two infusions in any given ten-minute period. In the event that two infusions did occur in any given ten-minute period, heroin was made unavailable for the next thirty minutes. Subsequent studies have shown this precaution to be unnecessary.

Approximately six months were required to run the first squad of animals through this procedure. Presently, three more animals are being used to partially replicate and complete this experiment.

Conclusions from this initial study vary in the degree of confidence with which they can be drawn. Highest confidence can be placed in the following conclusions:

1. Intake of heroin produces an immediate and profound reduction in food intake. With high doses of heroin, there is very little tolerance to this effect, and with small doses, a greater degree of tolerance. Thus, animals exposed to chronic high levels of heroin might be expected to, and do, show signs of physical deterioration secondary to reduced food intake.

2. Patterning of food intake is altered during chronic heroin intake. Pre-drug food intake is characterized by relatively frequent bouts of eating (meals) of relatively short duration. Such eating, as does occur during chronic heroin intake, tends to be widely spaced meals of relatively long duration. This alteration of meal patterns occurs even at doses so low that food intake recovers to baseline levels.

3. Some exposure to heroin may be necessary for it to function as a reinforcer. All animals in the initial study showed some delay between initial exposure and an increase in daily infusion rate above baseline levels. In fact, the usual effect of the introduction of heroin was to effect a reduction in the baseline rate of infusions.

4. Rate of drug infusions (and food and water intake) tends to decrease during the night. However, this circadian variation in intake is considerably less pronounced for heroin than for food and water.

5. Unit dose of the drug is a powerful determinant of total drug intake within the range studied. The higher the initial unit dose, the higher the subsequent terminal intake level.

6. There is a constant ratio between the amount of heroin self-infused and the amount of circulating heroin, morphine and metabolites in the blood as determined by RIA. However, this relationship is quite damped. That is, hourly intake of heroin and blood levels do not necessarily correspond well. Average RIA scores for 24 successive hourly blood samples do, however, consistently show a level of 0.015% of the heroin intake for the same period per ml of blood.

7. The acquisition of heroin intake is quite gradual, requiring anywhere from 30 to 90 days before total daily drug intake reaches asymptotic levels.

Additional experiments are planned to examine more carefully the effect of unit dose in the determination of heroin self-administration behavior.

Withdrawal from the drug does not appear to be particularly dramatic, marked by some decrease in food intake for a few days, and by first an increase, then a decrease in drug-key responding. More elaborate studies of withdrawal are planned.

A complicating factor in the original experiment was the use of the overdose protection contingency for the high unit dose animals. This factor was found to be severely restricting the animals' intake of heroin. When the contingency was relaxed, allowing more infusions in a given ten-minute period, the level

of intake immediately increased. In subsequent manipulations of this contingency, drug intake always approached the maximum allowed with the result that the animals spent a considerable portion of the day with their drug keys inoperative. When the contingency was entirely removed, the animals' intake level stabilized. We are currently replicating the high unit dose, and have seen no evidence of a tendency to overdose as yet, even at sustained infusion rates of 4000 mg/day.

Several additional experiments have begun. As indicated above, a partial replication of the continuous access experiment is in progress. One result of the free access study is that unit dose does not seem to be particularly important in determining total daily number of infusions. Thus, as a followup to the free access study, systematic manipulations of unit dose will be made in an attempt to more clearly demonstrate the effect or lack of effect of this variable.

A second experiment is in the final stages of baseline data gathering. In this procedure, food and water access is restricted to several one-hour periods throughout the day. Animals will be required to take infusion of heroin in order to initiate these food and water access periods. Thus, they will be forced to take some drug in order to eat. This experiment will allow us to determine whether such forced exposure to the drug, and its pairing with other reinforcers, will affect the speed of acquisition of drug intake. Currently, three animals are being used in this experiment.

Two additional studies currently in progress involve the use of rhesus monkeys as subjects. In some pilot work, monkeys were implanted with indwelling jugular catheters which exited through a skull-mounted catheter restraint and protection system is being used. In the first of these studies, the effect of the effort required to obtain drugs (i.e., the number of lever presses per infusion) on drug intake is being examined. Preliminary results indicate that heroin-reinforced behavior is extremely sensitive to effort requirements with small increases in effort producing large decreases in total daily intake of drug. In a second experiment, comparisions are being made between heroin HCl and morphine HCl. Monkeys are given continuous access to heroin until daily intake has stabilized. The drug is then changed to a morphine solution equimolar to the previous heroin solution. Preliminary data indicate that such a change from heroin to morphine results in a rapid increase in frequency of infusion, in the case thus far observed, resulting in depression and death.

As an adjunct to studies currently in progress, in cooperation with the Division of Pathology of the WRAIR, several of the baboons

being used in heroin studies are also being used in a parallel study of renal morphology changes subsequent to chronic heroin intake. Kidney biopsies were performed on these animals at the same time catheters were implanted. Additional biopsies will be performed prior to and following their withdrawal from the drug, allowing some assessment of the time course of renal pathology, if any.

Two sets of experiments involving choice procedures are planned. The first of these involves the drug abuse model described in our original protocol. Briefly, this model views drug-seeking behavior as one of a number of behaviors in which the organism can engage, and between which he must choose. The experiments planned involve giving baboons a choice between working on fixed-ratio (FR) schedules for heroin, or an FR schedule for food or water. The animals will be presented, in a trial-based procedure, with two keys, one of which delivers food or water following the completion of an FR, and the other of which delivers a drug infusion. A single press on either key will turn the other key off until the FR on the chosen key has been completed. Thus, on any given trial, the animal can work for food and water, or drug, but not both. The parameters to be manipulated will be the size of the FRs, the number of trials allowed per day, and the amount of either drug or food delivered per reinforcement. In most cases, the requirements and parameters of drug reinforcement will be held constant while the requirements for the food-water choice will be varied. This will allow some assessment of the degree to which drug-reinforced behavior may be sensitive to not only the contingencies required to obtain the drug, but to the contingencies for other reinforcers as well.

We also plan to investigate the effects of restricting the daily number of choices available to the animal on the assumption that drug-food interactions that are seen when many choices per day are possible will be different from those seen when access to the choice situation is more limited. This procedure should also be quite sensitive to the effects of withdrawal from the drug. The fact that withdrawal reduces food intake and initially, at least, increases drug-key responding, suggests that a procedure requiring a choice between these two alternatives will be particularly sensitive to withdrawal from the drug.

A second class of choice procedures will also be investigated. The first set of choice experiments (described above) is specifically designed to permit changes in preference to produce parallel changes to intake of food-water [illegible]oin analogous to the dynamics of behavior that occur naturall[illegible]ose experiments, choices for heroin, for example, would [illegible]y reduce the availability

of food and water. These interactions are the focus of the first experiments. The second set of choice experiments is designed to reduce the interactions between alternatives so that a preference for one alternative will not reduce intake of the other alternatives. This will permit an assessment of the properties of the reinforcers and the causes of preference without the complication of continuously changing intake of the alternative reinforcers.

In these procedures, the animal is allowed to work on either one of two or more variable-interval schedules which are programmed to run concurrently. That is, the probability of reinforcement becoming available on either alternative schedule is controlled by time; the animal may choose to work on either alternative at any time and will occassionally obtain a reinforcer. Such schedules have several important properties for the assessment of choice behavior. First, given that a minimal amount of responding occurs to the alternatives, the relative rate of reinforcement from the alternatives remains constant across a wide range of possible relative rates of response, thus eliminating variations in intake as a function of choice. A second important property of such schedules is that they provide a continuous measure of preference between the alternatives in the form of relative rates of response to the alternatives, as well as the relative amount of time spent working on the various alternatives. Finally, these procedures allow the experimenter to vary the difficulty of changing from one alternative to another. This variable, changeover rate, has been shown to be sensitive to a variety of behavioral and pharmacologic manipulations.

Several different types of experiments with this second type of choice baseline are planned. First, as mentioned earlier, a typical effect of initial heroin infusions is to suppress drug-key responding below baseline levels. This type of response suppression has been used as an indication of punishment. Alternatively, such an effect might simply be ascribed to a reduction in general activity produced by the drug which initially overshadows the reinforcing properties of heroin. The concurrent variable-interval choice procedure provides a means of distinguishing between these two possible interpretations. Animals will be given a choice between two alternatives; responses in one alternative will produce infusions of heroin, and in the other, saline. Even though responding may be generally suppressed by the drug, the relative preference for one or the other alternatives should reflect the functional properties of the reinforcers. If initial heroin infusions are reinforcing, then heroin will be preferred; if they are punishers, saline will be preferred. In addition to this experiment on the functional properties of initial exposure to the drug, several other experiments will be carried out with

variations of this choice procedure. These will involve choice between different doses of the drug, choices between heroin and morphine, and parametric variations of the variable-interval schedules of reinforcement, as well as the difficulty of changing from one to another alternative. Preference for heroin withdrawal will also be examined within this context.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6493	74 07 01	DD DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORD'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 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 114(f)			

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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 74	6	128
FISCAL YEAR — CURRENT 75	6	143

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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) 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) 73 07 - 74 06 The study of the epidemiology of drug and alcohol abuse at a large Army post attempts to determine 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. Post-wide urine screening, individual and group interviews, questionnaires, surveys, and participant observation are the primary techniques utilized. 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. Preliminary data analysis in human subjects indicated heroin was the preferred drug for less than two percent of approximately 3,000 enlisted men E-5 and below studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug abuse prevention in military personnel

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Description

The study of the epidemiology of drug and alcohol abuse at a large Army post has been the major departmental project for the past year The purpose of this study is to determine those individual, environmental, and social factors which can be modified to decrease the likelihood of the initiation of drug abuse, disrupt its maintenance, and/or treat its consequences. In order to accomplish this task, an epidemiological model has been initiated which utilizes multiple data-gathering techniques. These techniques include post-wide urine screening, individual and group interviews, questionnaires, surveys, and participant observation. No single data-gathering technique can give an accurate assessment of the incidence and prevalence of drug usage at the garrison post. Taken together, however, the various data sources complement each other and are felt to provide a more comprehensive view of the effect of drug and alcohol abuse upon the health and performance of the Command.

Progress

Epidemiology of Drug Use

1. The study has continued over 18 months in two phases. Phase one involved a mass urine screening procedure. Over a one-month period, approximately 3200 individual urine specimens were collected from personnel at a CONUS post. In addition to urine specimens, drug usage questionnaires were collected from the respondents at the time of the initial urine screen. The urine specimens were analyzed by free radical assay technique (FRAT) and thin layer chromatography (TLC) for drug metabolites by the Division of Biochemistry, WRAIR.

2. An attempt was made to interview all of the individuals whose urines were FRAT and TLC positive for barbiturates, amphetamines, narcotics, methadone, or other chemical substances present in the urine but not analyzable by those techniques. No confirmation of the positive urines through the use of gas liquid chromatography was made since one purpose of the screen was to test the utility of the FRAT and TLC as case finding, mass screening techniques. A total of 460 interviews were conducted which were semi-structured and elicited information on standard demography, social indicators, and patterns of drug use. Included in this figure were urine negative individuals who represented a stratified cross section of the post.

3. Phase one also included the collection of mass sociometric information which was analyzed to determine the possible relationship between soldiers' friendship patterns and drug and alcohol usage on the post.

a. A number of preliminary conclusions may be drawn from the initial phases of this research. The data indicate that drug misuse, rather than being a diffuse problem equally affecting the post as a whole, was differentially distributed through the various units stationed at the post. Our convergent information-gathering techniques showed that drug use appeared to be organized on a company-by-company basis. Drugs were distributed by low-level user-dealers within each military unit and appeared to be used with one's fellows from the same company or barracks.

b. The data further indicate that the present "drug scene" involved 20% to 25% of enlisted personnel, grades E-5 and under. In early 1973 this population appeared to use a number of different drugs on an irregular but recurrent basis. Heroin was the preferred drug of use for a small minority (no more than 2% of lower ranking enlisted population). The basic drugs of choice were marijuana and hashish taken with alcohol. In addition to these agents, most other agents, i.e., hallucinogens, soporifics, amphetamines, heroin, and cocaine, were used opportunistically when available and when they could be afforded. A further dichotomy may be noted in that drug use is primarily limited to younger troops living in or assigned to barracks. Married enlisted personnel, living in quarters or off-post, are a low-risk group insofar as use of drugs other than alcohol is concerned.

4. Phase two of the study is currently in progress and is divided into four primary areas: Contact Epidemiology Studies, Unit Studies, Systems Studies, and Individual Cognitive Studies.

a. Contact Epidemiology Studies

The contact epidemiology portion of this study has described the behavior of individual soldiers and the way they organize into various groups, both on and off post. Social networks and contacts of illicit and non-illicit drug users were traced by repetitive structured and unstructured interviews. The aim of this phase of the study was to better understand the mode of transmission and the factors involved in the maintenance and acquisition of drug and alcohol use between individuals and groups.

b. Unit Studies

The aim of the unit studies section was to obtain an ethnography of company-sized units in garrison status. Enlisted field workers collected data concerning the formal organization of units, the sets of informal relations within the companies, the interactions between the formal and informal systems, the units' performance at assigned tasks, and the demographic composition of the units studied. The field workers made direct observations of these organizations while residing within the companies, and also utilized interviews and survey techniques.

c. Systems Studies

The systems studies section has been concerned with the medical rehabilitation and service systems on the study post. Section activities can be divided into four major areas:

(1) The ethnographic study of the drug treatment facility and the alcohol treatment facility has used long-term direct observations, interviews with patients and staff, and analysis of system records. From these sources, data on recruitment for treatment, the process of rehabilitation, and the behavioral outcomes of rehabilitation have been collected.

(2) Interviews have been conducted with officers and non-commissioned officers at the company level. These interviews have focused on the problems of garrison life in the post-Viet Nam era, the prevalence of drug and alcohol related problems, and the utilization of rehabilitation facilities by troops in garrison.

(3) Existing records from the Provost Marshal's Office, the Post Confinement Facility, the hospital emergency room, and the Mental Hygiene Consultation Service have been collected. Rates and trends in the use of these systems will be analyzed, with special attention to correlations with observed phenomena from the unit studies and contact epidemiology studies sections.

(4) A health diary study was initiated to collect direct data on the health problems and patterns of consultation and therapy in a random sample of soldiers. This study will describe the health behavior of troops and the organization of indigenous care systems paralleling the Army medical system.

d. Individual Cognitive Studies

The individual cognitive studies section has been involved with the development and utilization of instruments to map the cognitive organizational factors, attitudes, and behavioral modalities of individuals and groups, and the patterns of drug and alcohol use. Instruments such as the semantic differential, behavioral differential, the behavior checklist, and others are being administered to a large sample of troops.

5. Analysis of the this phase of the study is scheduled to begin in October 1974 and continue over the next 18 months.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug abuse prevention in military personnel

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Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6494	74 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
73 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	61101A	3A161101A91C	00	104
b. CONTRIBUTING	62758A	3A762758A833	00	Ch FR 104
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Drug Test Systems Development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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 74	0.1	131
FISCAL YEAR CURRENT 75	1.75	62

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NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antagonists; (U) Drug Abuse; (U) Drug Addiction, (U) Amphetamines; (U) Drug Dependence

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

23. (U) To develop a test system for evaluating the ability of drugs to antagonize amphetamine toxicity and/or dependence to identify and combat amphetamine toxicosis in military personnel.

24. (U) Development of an animal model test system capable of rapidly screening large numbers of chemical compounds for their agonistic and/or antagonistic effect on amphetamines.

25. (U) 73 07 - 74 06 Review of the literature search for techniques and models for studying amphetamine drug effects was completed. Systems reported in the literature are largely unsuitable for rapid mass screening of drugs, involving expensive animal species, long periods of training, and have measurable parameters largely subjective in nature. A search for an objective parameter for comparison of drugs was made in several species of animals. It appears that total animal activity as observed in the mouse is the most promising criterion for determination of antagonism to amphetamines. Such activity can be objectively measured by electronic means. A simple activity cage has been designed and base-line studies are being performed. Work was performed under Project 3A762758A833, Biomedical Factors in Drug Abuse, in FY74. Work will continue in FY75 under Project 3A161101A91C In-House Laboratory Independent Research. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

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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 104 Drug test systems development

Investigators
Principal: LTC Kenneth E. Kinnamon, VC
Associate: LTC Peter S. Loizeaux, VC

Review of the literature search for techniques and models for studying the modification of amphetamine drug effects was completed. Systems reported in the literature are largely unsuitable for mass screening of drugs, involving expensive animal species, long periods of training, and have measurable parameters largely subjective in nature. The search for an objective parameter produced by amphetamine which could be used for evaluation of modifying drugs was made in several species of animals. The most promising parameter appears to be total animal activity as observed in the mouse.

A simple activity cage was constructed utilizing photoelectric cells connected to a series of counters to give continuous recordings divided into half-hour periods. Each time a mouse passed through openings in a central cage barrier the photoelectric beam was broken and a count recorded. Studies were conducted to determine the influences of mouse strain, number of mice, time of day or night, amphetamine dosage, external disturbance and other environmental influences on activity. Activity of mice receiving 5 mg/kg of d-amphetamine intraperitoneally is double that of control mice, then gradually diminishes to the level of the controls in approximately 5 hours. The interval between 1 and 4 hours after amphetamine administration appears to be most suitable interval to test compounds for their ability to modify amphetamine effects. Refinement of the activity cage and baseline studies are continuing. Work will continue in FY75 under Project 3A161101A91C In-House Laboratory Independent Research.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

				DA OB 6498	74 07 01			
73 07 01	D.CHG	U	U	NA	NL			
60 MD CODES	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62758A	3A762758A833		00	105			
b. CONTRIBUTING								
c. [redacted]	CARDS 114F							

(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. DATE EFFECTIVE NA EXPIRATION
b. NUMBER
c. TYPE a. AMOUNT
d. KIND OF AWARD f. CUM AMT

18 RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS ($ Thousands)
74	2.9	158
75	2.4	178

19 RESPONSIBLE DOD ORGANIZATION
NAME Walter Reed Army Institute of Research
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20 GENERAL USE
Foreign intelligence not considered

21 PERFORMING ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME Bartos, E.M. Ph.D.
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23 KEYWORDS (U) Acetylcholinesterase, (U) Cell Culture, (U) Cell Membrane, (U) Cellular Respiration, (U) Morphine Tolerance

24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS

23. (U) To investigate the alterations of cellular metabolism underlying the development of tolerance to and dependence on drugs of abuse, an 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) 73 07 - 74 06 Continuous culture of WRL-10A mouse cells in progressively increasing concentrations of morphine resulted in the development of a cell population which after 3 months of exposure to 1.0 mM morphine remains fully viable, grows actively with a daily factorial increase of 1.65 and exhibits moderate granularity and vacuolation. Control cells cultured in the same concentration of the drug without preexposure, die within 3 weeks. Determination of the respiratory activity of the latter cells immediately upon addition of the drug revealed a progressive depression of the respiratory rate up to 35 percent during the course of a single respirometer run. In contrast, when cells preexposed to .75 mM morphine were washed free of the drug and their respiration measured before and after addition of 1.0 mM morphine the depressant effect of the drug did not exceed 15 percent. Thus, manifestations of tolerance to morphine comparable to neural tissues were exhibited by a line of connective tissue cells previously shown to contain regulated amounts of an enzyme hydrolysing acetylcholine. It was further shown that this enzyme a) is located on the outer surface of the membrane of WRL-10A cells and is missing from related cell lines and b) is active only with acetylcholine as substrate at concentrations up to 10^{-3}M, exhibiting substrate inhibition beyond this point. The enzyme, therefore, appears to be true acetylcholinesterase and, like tolerance to morphine, constitutes a common link between neural and WRL-10A cells to be further expoloited in studying mechanisms of tolerance. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 June 74.

27 APPLICABLE TO CONTRACTOR GRANT SUMMARY REPORT

DD FORM 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 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 a sizeable number of reports on the subject has 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 claimed that morphine sulfate induced a multifold increase of AChE with development of limited tolerance and total dependence in cultures of human neuroblastoma[3], while a third found that morphine sulfate increased AChE activity by only 50% and induced tolerance but no dependence in cultured chick embryo brain cells[4]. 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[5] with no greater inconsistency[6] than described above for neuronal cells. Accordingly, we undertook a study of tolerance to morphine in cell culture using L-929 mouse fibroblasts.

Progress and Results

The origin and karyology of L-929, clone WRL-10A cells and their utilization in developing a suspension culture system with well defined density dependent metabolic regulation have been described previously[7,8,9]. To determine the sensitivity of the system to morphine, 50 ml suspension cultures of WRL-10A cells were maintained at population densities below 10^6 cells through daily centrifugation

and resuspension of the appropriate number of cells in fresh media. Morphine sulfate at final concentrations ranging from 10^{-4} to 10^{-3}M was added to the cultures during media renewal and growth was estimated on the basis of cell counts performed at the beginning and the end of the renewal cycle and expressed as the daily factorial increase of the cell population.

In addition, cell viability was evaluated by the uptake of nigrosin, and four grades of cytopathology varying from slight cytoplasmic granulation to ghost cell formation were recognized and used to describe the dose-response of these cultures as previously reported (Walter Reed Army Institute of Research Annual Progress Report 1 July 72 - 30 June 73, p. 1274).

It was found that chronic exposure of the cultures to progressively increasing concentrations of morphine induced tolerance to the toxic effects of the drug and accordingly an expanded longitudinal study was undertaken in the manner shown in Fig. 1.

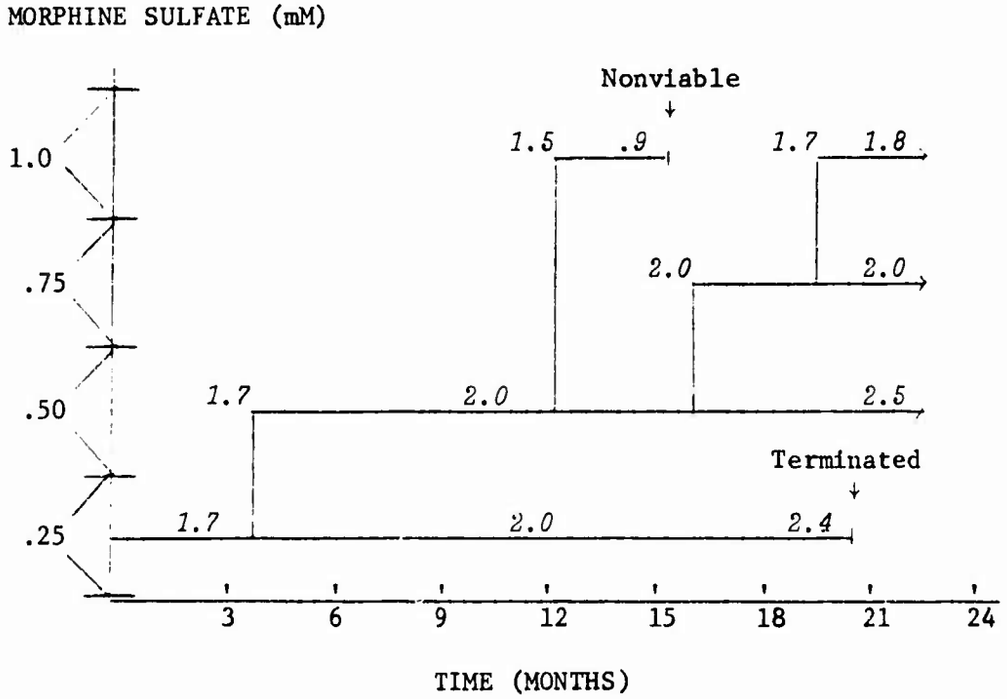

Figure 1 - Progression of exposure of cell cultures to morphine sulfate. Beginning with .25 mM,subcultures were established at progressively increasing concentrations of the drug as indicated. Arrows indicate cultures continuing at the time of the writing of this report. Figures in italics indicate mean daily factorial increase at the times shown.

To protect against accidental loss all cultures were carried in duplicate. It may be seen that in cultures exposed to .25mM morphine for 20 months the daily factorial increase of the population rose gradually and finally attained normal levels rendering these cultures indistinguishable from control populations grown in the absence of morphine. Essentially the same sequence was manifested by subcultures growing at a concentration of .5 mM morphine and, therefore, the original .25 mM cultures were considered in excess and terminated.

Additional evidence for the development of tolerance was provided by the fact that when cells grown for eight and one half months in the presence of .5 mM morphine were challenged with 1.0 mM morphine they survived for three months. As reported previously (op. cit.) and reconfirmed during the course of these experiments, survival time at 1.0 mM morphine for control cells with no previous exposure to the drug is 2 - 3 weeks. To determine whether a more gradual increase of the concentration of the drug would lead to an even longer survival at 1.0 mM morphine, cells previously cultured in the presence of .5 mM morphine for close to a year were stepped up to .75 mM and then, four months later, to 1.0 mM morphine. It may be seen that up to the time of this writing both subcultures show no decline of the daily factorial increase. Microscopically both cultures exhibit cytopathology grade II, consisting of moderate cytoplasmic granularity and slight vacuolation.

To investigate whether tolerance to the toxic effects of morphine extended to cellular respiration, as is known to occur in neural tissues[10], cells preexposed to .75 mM morphine were centrifuged, resuspended in fresh drug free media and introduced into the sample chamber of a respirometer (Yellow Springs Instruments, Inc.). A tight fitting plunger containing a Clark-type oxygen electrode was then inserted into the chamber expelling air through a small access groove. After determination of the basal respiratory rate of the cells from the slope of the declining dissolved oxygen trace, the plunger was removed and 10 λ of a stock solution of morphine sulfate calculated to yield a final concentration of 1 mM morphine added to the cells. The plunger was then reinserted into the chamber and recording of the rate of uptake of dissolved oxygen by the cells resumed until its complete exhaustion. Control cells not previously exposed to morphine were treated in exactly the same fashion. It was found that while basal respiratory rates were not different in the two types of cells and that in all cases the respiratory rate declined upon addition of morphine, the dissolved oxygen tracings of control cells exhibited two and at times three progressively decreasing slopes. As shown in Table I the final respiratory rates thus established at 34, 32 and 40 minutes, respectively, after the addition of morphine were markedly depressed. Preexposed cells, on the other hand, in two out of three cases exhibited a single slope of the dissolved oxygen tracing after morphine addition. This indicates that the slightly depressed respiratory rates, established within 1 minute after the addition of morphine,

remained constant at 3.24 and 4.07 fmoles O_2/cell/min, respectively, throughout the run. In the third case the dissolved oxygen tracing did exhibit a second slope at 28 minutes after addition of morphine, but, nevertheless, the resulting final respiratory rate was depressed by only 13 percent.

TABLE I - Effect of morphine on cellular respiration

	Control Cells	Preexp Cells[1]	Control Cells	Preexp Cells	Control Cells	Preexp Cells
Basal resp. rate (fmoles O_2/cell/min.)	4.07	3.63	4.31	4.78	3.91	4.47
Exposure to morphine in respirometer (min)[2]	34	1	32	1	40	28
Final resp. rate (fmoles O_2/cell/min.)	2.64	3.24	3.16	4.07	2.13	3.91
Depression of respiration (%)	35	15	27	11	46	13

[1]Cells preexposed to .75 mM morphine sulfate as described in the text were resuspended in drug-free medium 2 hrs prior to their introduction into the respirometer.

[2]Figures indicate time elapsing between the introduction of 1.0 mM morphine sulfate into the respirometer, as described in the text, and the establishment of the final respiratory rate.

The results discussed so far indicate that manifestations of tolerance to morphine comparable to neural tissues were exhibited by a line of cultured cells of connective tissue origin. As previously reported (op. cit.) a search as to whether these cells contain metabolic components normally found in neural tissues, revealed the presence of regulated amounts of an enzyme hydrolyzing acetylcholine(1, 2).

Since a number of different enzymes are capable of hydrolysing acetylcholine and since substrate specificity, substrate inhibition and enzyme localization are commonly used to differentiate "true" acetylcholinesterase from related enzymes, the following studies were conducted:

a) *Substrate specificity* - High density cultures ($\sim 10^7$ cells/ml) previously shown to exhibit markedly elevated enzyme activity (1) were used as donors of WRL-10A cells. Samples with the desired number of cells were centrifuged at 300 X g for 15 min. The cell pellets were subsequently washed three times in Earle's Balanced Saline (EBSS) and resuspended in 1.5 ml of phosphate buffer (50 mM potassium phosphate buffer, ph 6.8; 1 mM EDTA, potassium salt). The samples were then sonicated (Sonifier Cell Disruptor, Heat Systems Ultrasonics, Inc.) for 10 sec. and assayed as described(1) using as substrates both butyryl (1-^{14}C) choline and acetyl (1-^{14}C) choline. It was found that the amount of radioactive product formed with butyrlcholine as substrate was approximately 3% of the product formed with acetylcholine. Chromatographically the product formed using acelylcholine as substrate was indistinguishable from authentic sodium acetate added as a carrier.

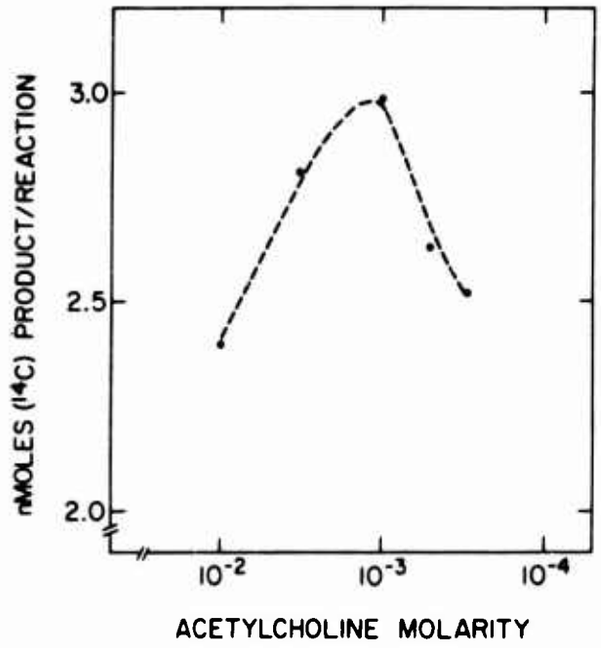

Figure 2 - Inhibition of hydrolysis of acetylcholine by sonicates of WRL-10A cells with increasing concentrations of substrate.

b) *Substrate inhibition* - Cell sonicates prepared as described above were incubated for 30 min. at 37° with five different concentrations of acetyl (1-^{14}C) choline ranging from 10^{-4} to 10^{-2}M. In Figure 2 the amount of radioactive product formed per 30 minute reaction is plotted as a function of the molar concentration of acetylcholine used in the reaction as substrate. It may be seen that above an optimal concentration of 10^{-3}M the velocity of the reaction decreases rapidly as the substrate concentration increases.

c) *Enzyme localization* - The cells were prepared as previously described with the exceptions of sonication. Instead, suspensions of whole cells in phosphate buffered saline (PBS) were incubated at 37° for 30 min. in the presence of progressively increasing concentrations of the diazonium salt of sulfanilic acid, a nonpenetrating agent known to inactivate enzymes located on the outer surface of the cell membrane but to be without effect on intracellular enzymes such as, for ex., lactate dehydrogenase (LDH). After incubation, cells to be assayed for the acetylcholine hydrolysing enzyme (AChE) were washed twice with EBSS, once with PBS, then resuspended in PBS and the whole cells assayed for AChE activity in the usual manner except that Triton X-100 was omitted and the final NaCl concentration was 0.155 M. Cells assayed for LDH activity after incubation were washed three times with EBSS, resuspended in EBSS, sonicated, centrifuged at 10,000 X g and the supernatant assayed using standard methods.

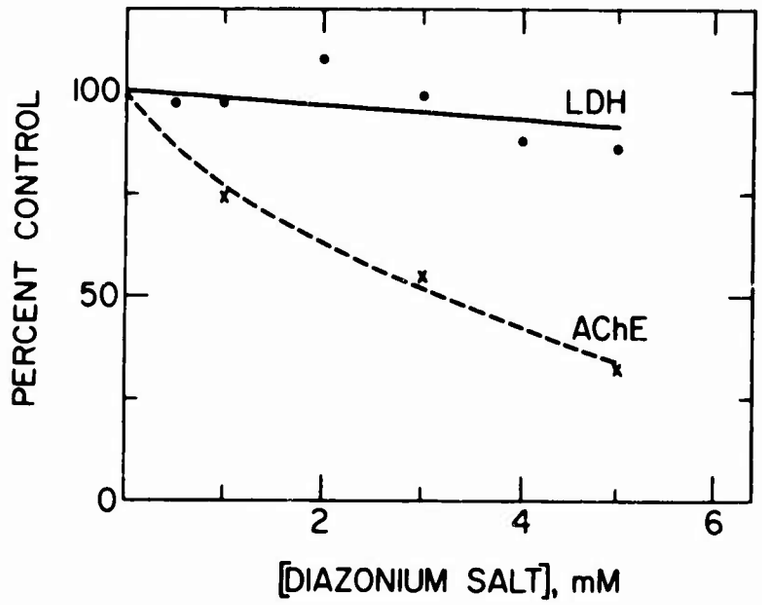

Figure 3 - Effect of increasing concentrations of the diazonium salt of sulfanilic acid on the lactate dehydrogenase (LDH) and acetylcholinesterase (AChE) activities of WRL-10A cells

In Fig. 3 the activities of the acetylcholine hydrolysing enzyme (AChE) and of lactate dehydrogenase (LDH) are plotted against the concentration of the diazonium salt of sulfanilic acid and expressed as the percent of the activities of these enzymes in control cells assayed in the same fashion except that the incubation in the presence of the diazonium salt of sulfanilic acid was ommitted. It can be seen that the activity of AChE declined by 70% as the concentration of the diazonium salt increased from zero to 5 mM. Under the same conditions

the activity of LDH was minimally affected, thus excluding the possibility that the diazonium salt inactivated the AChE enzyme by penetrating or injuring the cells.

The above results indicate that the acetylcholine hydrolysing enzyme present in WRL-10A cells exhibits the characteristics of substrate specificity, substrate inhibition and outer membrane localization, commonly associated with the presence of "true" acetylcholinesterase (EC 3.1.1.7) in neuronal and muscle cells. The question therefore arises whether the presence of this enzyme in cells of connective tissue origin is limited to the WRL-10A subline or whether it is also present in other derivatives of the parent L-929 strain of mouse fibroblasts. Accordingly, an authentic sample of the original L-929 strain obtained from the cell respository of the American Type Culture Collection and samples of two derived cell lines were grown to high density suspension cultures as described elsewhere[8].

Table II - Distribution of acetylcholinesterase in L-929 mouse fibroblasts and their derivatives

Cell lines and Sublines	Low Density Cultures ($4\text{-}8 \times 10^5$ cells/ml)	High Density Cultures ($6\text{-}10 \times 10^6$ Cells/ml)
L-929 (American Type Culture Collection)	0.053	0.020
L-929 (Microbiological Associates)	0.010	0.010
L-M (Ft. Detrick)	0.037	0.049
WRL-10A (Walter Reed)	0.050	6.0

Figures indicate specific activity of AChE in nM of product formed/mg protein/min.

In Table II it may be seen that regulated acetylcholinesterase activity, as manifested by a 100-fold increase in the high density population, is expressed only in WRL-10A cells with the parent L-929 strain and the other two sublines exhibiting only traces of activity regardless of cell density.

It is concluded therefore, that acetylcholinesterase activity like the development of tolerance to morphine discussed in the first part of this

report constitute a unique common link between neural and WRL-10A cells(3) to be further exploited in studying mechanisms of tolerance.

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 3 months of exposure to 1.0 mM morphine remains fully viable, grows actively with a daily factorial increase of 1.65 and exhibits moderate granularity and vacuolation. Control cells cultured in the same concentration of the drug without preexposure, die within 3 weeks. Determination of the respiratory activity of the latter cells immediately upon addition of the drug revealed a progressive depression of the respiratory rate up to 35 percent during the course of a single respirometer run. In contrast, when cells preexposed to .75 mM morphine were washed free of the drug and their respiration measured before and after addition of 1.0 mM morphine the depressant effect of the drug did not exceed 15 percent. Thus, manistations of tolerance to morphine comparable to neural tissues were exhibited by a line of connective tissue cells previously shown to contain regulated amounts of an enzyme hydrolysing acetylcholine. It was further shown that this enzyme a) is located on the outer surface of the membrane of WRL-10A cells and is missing from related cell lines and b) is active only with acetylcholine as substrate at concentrations up to 10^{-3}M, exhibiting substrate inhibition beyond this point. The enzyme, therefore, appears to be true acetylcholinesterase and, like tolerance to morphine, constitutes a common link between neural and WRL-10A cells to be further exploited in studying mechanisms of tolerance.

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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Publications:

1. Glinos, A.D., and Bartos, E.M.: Regulation of Acetylcholinesterase in L Cells. J. Cell Biol. 59: 110a, 1973.

2. Glinos, A.D., and Bartos, E.M.: Density dependent regulation of growth in L cell suspension cultures. III: Elevation of specific activity oa acetylcholinesterase. J. Cell. Physiol. 83: 478, 1974.

3. Bartos, E.M., and Glinos, A.D.: Properties of actylcholinesterase in L-929 sublines. Fed. Proc. 33: 478, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6499	74 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. SMSF'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	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				
XXXXXXXXXX	CARDS 114F			

11. TITLE (Precede 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 PREVIOUS: 74	2	135
FISCAL YEAR CURRENT: 75	2	159

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ASSOCIATE INVESTIGATORS

NAME: Robinson, MAJ M.

NAME: Conrad, COL M.

DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Hepatitis B antigen and antibody; (U) pulmonary functions; (U) endocarditis; (U) heroin withdrawal; (U) demographic variables

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

23. (U) Drug abuse has recently been a major problem in military populations. The objectives of these clinical and demographic studies are to obtain more information on medical problems related to drug abuse and to more clearly define demographic variables of the illicit drug using population in the military.

24. (U) Clinical studies have evaluated medical complications of drug abuse by extracting data pertinent to clinical manifestions, physical and laboratory findings and therapy. Studies for the presence of Hepatitis B antigen and antibody were carried out by radioimmune assay in two military populations, heroin and non-heroin users. Studies on the effects of heroin on pulmonary functions were done by use of an electronic spirometer using a heroin and non-heroin military population. Studies related to demographic characteristics of heroin and non-heroin using military populations studied in USARV will be carried out by comparison of the variables of the two populations by frequency analysis and cross correlation studies.

25. (U) 73 07-74 06 Hepatitis B antigen and antibody assays have been completed in 252 non-heroin using and 426 heroin using military populations. These findings are being prepared for a report. A study of the effects of heroin on pulmonary function has been completed in 123 heroin users and 58 non-heroin users. This work has been written up and is being submitted for review. The data extraction for a study of medical complications associated with drug abuse in a military population has been completed and is being prepared for reporting. The first report on 30 cases of acute endocarditis is in preparation. A study of heroin withdrawal in 320 soldiers in Vietnam has been completed. A study to compare demographic variables of 3500 heroin using and non-heroin using individuals from the military population is in progress. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 73-30 Jun 74.

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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 00 Biomedical Factors in Drug Abuse

Work Unit 106 Clinical and demographic studies of military drug abusers

Investigators.
Principal: LTC Norman W. Ream, MC
Associate: MAJ Malcolm C. Robinson, MC; MAJ Ronald R. Blanck, MC; COL Marcel E. Conrad, MC; MAJ Allen L. Ginsberg, MC; SP7 Joseph E. Fritz; Carol J. Bell

Description.

The objectives of this work unit are to complete clinical and demographic studies initiated in Vietman 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 and Results.

During this reporting period two clinical laboratory studies pertinent to heroin use by the military in Vietnam have been completed and are reported first. The status of the following studies is then reported: (1) studies of medical complications of drug abuse in military populations, (2) a study of heroin withdrawal in U.S. soldiers in Vietnam, (3) demographic studies of heroin and non-heroin using soldiers in Vietnam.

1. Prevalence of Hepatitis B Antigen and Antibody Among Heroin Users in Vietnam.

The influence of nonparenteral and parenteral routes of heroin administration on the prevalence of HBAg and HBAb were assessed in U.S. soldiers stationed in Vietnam in 1972. Sera from 252 non-heroin users and 426 heroin users were assayed. HBAg was detected by RIA (Austria-125) and specificity testing was performed by demonstrating inhibition with human HBAb and not with guinea pig serum. HBAb was measured by solid phase RIA (1). Only four of 252 control sera were HBAg positive (1.6%) and 30 were positive for HBAb (11.9%). In a group of 238 patients who smoked heroin as an admixture with tobacco (but did not utilize the intravenous route) 10 were positive for antigen (4.2%) and 48 for antibody (20.2%). With direct nasal inhalation of pure crystalline heroin, two of 67 patients were antigen positive (3.0%) and 16 had antibody (23.9%). Among 121 patients who took heroin by intravenous injection as the sole or major route, 13 were found to be antigen positive (10.7%) and 57 were antibody positive (47.2%). The prevalence of HBAg in

in heroin users exceeded that of non-users whether the primary route of administration was smoking ($p < 0.02$) or intravenous injection ($p < 0.001$). All heroin users, regardless of route of administration, had a higher prevalence of HBAb than did the control population ($p < 0.001$). Intravenous users were more likely to have both antigen and antibody than those who smoked or directly inhaled ($p < 0.001$). It appears that each route of heroin administration has a distinct associated risk of hepatitis B transmission: smallest when heroin is directly inhaled, larger when heroin-filled cigarettes are passed from user to user, and greatest when used intravenously.

2. Effects of Heroin on Pulmonary Functions.

Several investigators have described decreased vital capacity and impaired diffusing capacity in chronic heroin addicts (2, 3). The relatively high prevalence of high dose heroin use among military personnel during the recent conflict in Vietnam afforded a unique opportunity to evaluate the effects of heroin on pulmonary functions. We have previously described this heroin using population in terms of youth and freedom from the medical complications associated with heroin dependence in the United States (4). The heroin used in Vietnam was essentially pure (92-98%). The preferred routes of administration were smoking as an admixture with tobacco and direct forceful nasal inhalation of the crystalline powder. Pulmonary functions obtained in such patients should, therefore, closely reflect the specific physiological effects of heroin divorced from the complex of factors associated with intravenous use of highly adulterated drugs.

Pulmonary function data (peak flow, one of three second vital capacity, and total vital capacity) were measured with an electric spirometer and obtained from 123 heroin using soldiers admitted to a U.S. Army treatment center and from 58 healthy paramedical personnel staffing these treatment facilities. Patients were evaluated as part of the normal in-processing immediately following admission to the treatment centers. Participation in this study was voluntary, and all patients and controls gave informed consent prior to interview and pulmonary function measurement. After a minimum of three unrecorded spirometric trials, all subjects were instructed to fill lungs completely and then to exhale as rapidly, forcefully, and completely as possible through the mouthpiece of a Pulmonary Function Indicator™ (PFI*, Chemetron Corporation). This device measures flow necessary to maintain constant temperature. Values for peak flow could be read directly from a dial calibrated from 50 to 650 liters/minute. Vital capacity (FEV_1, FEV_3, VC) could be read from a scale calibrated from 0 to 7 liters. Three trials of single forced expirations were recorded for all subjects.

*Pulmonary Function Indicator (NCG), Chemetron Corp., 840 N. Michigan Ave., Chicago, Ill. 60611

Patients were significantly younger, shorter, and less heavy than controls (Table 1), but analysis of variance for pulmonary functions within the control group indicated no significant effects of age, weight, or height on the parameters of forced exhalation. Length of time served in Vietnam did not differ significantly between patients and controls nor was there any effect of tour length on pulmonary functions of either group. Since the three replications of forced exhalation did differ, with best performance (particularly in terms of peak flow) on the third trial, significant differences between pulmonary functions of patients vs. controls were assessed only on the basis of the third and last measurement.

Mean heroin intake of the patients was approximately 6 vials daily (250 mg of pure heroin per vial) for about six months. Cumulative heroin intake for the average patient could, therefore, be estimated as exceeding 250 grams. Since patients came to the drug treatment centers from all areas of Vietnam, there was considerable variation in the amount of time elapsed between the last dose of heroin and assessment of pulmonary functions. Approximately 20% of the patients had self-administered heroin in the 12-hour period prior to evaluation and 68% had been abstinent for less than 48 hours. Twenty-eight of the 123 patients reported no heroin use for periods between 72 hours and several weeks. Over 80% of the patient population smoked heroin with tobacco, and the remaining patients were evenly divided between nasal inhalation and intravenous injection as primary routes of administration. Analysis of variance revealed no significant difference in pulmonary function due to amount of heroin used, duration of use, time elapsed since last heroin dose, or route of heroin administration.

There was a significant difference in the prevalence of cigarette smoking between patients and controls with 93% of the patients being smokers vs. only 57% of the controls ($p < 0.001$). There was no difference between groups in the percent of smokers who had used cigarettes for five years or more. Controls who smoked for less than five years did not differ from non-smokers in any pulmonary function parameter. Controls who smoked for five years or more did show decreases in peak flow (16.2% less than non-smokers, $p < 0.05$) and FEV_1 (16.6% less than non-smokers, $p < 0.025$) although there were no appreciable changes in FEV_3 or vital capacity. When eight non-smoking patients were compared to 25 non-smoking controls, patients were found to have lower values for peak flow, FEV_1, FEV_3, and vital capacity (Table 2).

Marijuana used was relatively common in both patients and controls. Twenty-six of 123 patients (21.14%) and 11 of 58 controls (18.97%) reported daily use of marijuana ($p < 0.39$). However, 65% of the controls denied use of marijuana vs. only 26.8% of patients ($p < 0.001$). Smoking of marijuana had no demonstrable effect on any expiratory measure as assessed by an analysis of variance.

A substantial number of both patients (34.7%) and controls (31%) reported prior pulmonary disease (bronchitis, pneumonia, asthma, spontaneous and traumatic pneumothorax). None of the measured respiratory parameters in either group were affected by a positive history of any pulmonary disorder in the recent or more previously distant past (less than one year, less than five years, more than five years previously). Pulmonary functions of patients without any history of prior pulmonary disease differed from those of history-matches controls, the patients being shown to have significantly decreased peak flow, FEV_1, FEV_3, and vital capacity. Although the differences were slightly less marked, the overall patient and control populations differed for each of the four parameters of forced expiration (Table 3).

The amounts of heroin used by patients in this study were uniquely large, ranging from 250 mg to over 3,000 mg daily. Consumption at these levels would be physically infeasible in the United States where heroin has been regularly adulterated and purity has recently ranged between 2 and 5 percent (5). Smoking of heroin was the predominate route utilized by military personnel in Vietnam. Mo and Way (6) have estimated that pyrolysis and other loss may leave only 30% of smoked heroin available for absorption. No quantitative estimate has been made for heroin absorption by direct nasal inhalation of the powder although heroin is generally highly lipid soluble and well absorbed across other membranes. Results from this study indicate no evidence for any specific effect of route of heroin administration on pulmonary functions although such effects certainly cannot be ruled out. The facts that amount of heroin used, duration of use, and time since last dose accounted for discernible variation in expiratory patterns may support the hypothesis that the observed impairments are intrinsic to the pharmacological actions of chronic heroin use on pulmonary physiology.

Although FEV_1 and similar measurements are relatively insensitive indicators of even considerable changes in peripheral airway resistance, cigarette smoking is known to provoke acute bronchoconstriction and chronic decreases in FEV_1, maximal midexpiratory flow rate, and diffusing capacity (7, 8). Pulmonary function assessment in our control population revealed that a smoking history of five years or more was associated with significant decreases in peak flow and FEV_1 relative to non-smokers. There was no effect of cigarette smoking on pulmonary functions in patients separable from the effects of heroin use. The differences between non-smoking patients and non-smoking controls again suggest that the reduced pulmonary function values in the patients can be attributed to heroin.

The adverse effects of heroin on the lung have been attributed to the cumulative effects of the many pulmonary diseases to which the classically reported civilian heroin user is prone (2, 3).

There was no increased predilection toward pulmonary disease seen in this population of heroin users in Vietnam. Moreover, the history of prior bronchitis, pneumonia, or asthma had no apparent influence on the expiratory parameters measured in this study. Patients with or without any history of prior respiratory disorder had significantly lowered values for pulmonary functions relating to control.

Studies at the Addiction Research Center in Lexington (9) have shown that narcotics cause a continuing chronic depression of respiration despite the development of tolerance to other opiate effects. It seems likely that the chronic heroin user has persistent alveolar hypoventilation as a direct result of the pharmacological effect of the drug on respiratory control loci in the central nervous system. Additional impairment of pulmonary function may be due to persistent effects of heroin on suppression of normal intermittent deep breathing (sighing) which would also lead to decreased alveolar ventilation and atelectasis (10). Results in this study suggest that abnormalities in pulmonary functions may persist for some time following cessation of heroin use since the 18% of patients with no drug use for 72 hours to several weeks continued to demonstrate reduced values for peak flow, FEV_1, FEV_3, and vital capacity. It does not seem likely that the mechanism for these chronic changes in respiratory function would be directly attributable to any acute heroin-induced bronchoconstriction. Further study of heroin effects on the central and peripheral inputs to respiration are required to clarify the role of this drug in disturbances of normal pulmonary functioning and to define the risk, if any, of permanent pulmonary dysfunction secondary to chronic narcotic use.

3. Studies of Medical Complications of Drug Abuse.

To obtain information on types, clinical characteristics and treatment of the various medical complications of drug abuse in military populations, two studies were conducted. The first consisted of a review of the charts of all drug related admissions to three USARV Hospitals (U.S. Army Hospitals at DaNang, Long Binh and Saigon) between January 1971 to July 1972. Many of these cases were related to complications secondary to intravenous use of heroin. These data are being compiled for a chapter on Medical Aspects of Drug Abuse in the U.S. Army in Vietnam for the volume on Internal Medicine in the Vietnam conflict for the Office of the Surgeon General, Department of the Army. For the same chapter, a review of all drug induced or related deaths occurring in USARV between January 1970 to July 1972 has also been completed. A review by data extraction of all charts of admissions with medical complications related to drug abuse in military personnel admitted to five Class II U.S. Army Hospitals between January 1971

and December 1973 has been conducted and pertinent data from these cases are being compiled for a report. 247 admissions were reviewed, 30 of whom had a diagnosis of acute bacterial endocarditis. An initial report is being prepared on this group.

4. A Study of Heroin Withdrawal in U.S. Soldiers in Vietnam.

A study of demographic and drug use variables and of clinical characteristics and treatment of heroin withdrawal has been completed in 320 U.S. soldiers in Vietnam. Studied in this population were 200 who used heroin by smoking, 60 by sniffing and 60 by intravenous injection. This study is being added to the chapter on Medical Aspects of Drug Abuse in the Vietnam conflict for the Office of the Surgeon General, noted earlier.

5. Demographic Studies of Heroin and Non-Heroin Using Soldiers in Vietnam.

Demographic data taken from a questionnaire administered to 3500 enlisted men in USARV who denied heroin use and from 3500 heroin using soldiers in USARV are being compiled for comparison of these two populations. All of the data from the non-user cohort are available on a computer printout. After the data from the heroin user cohort are available, the two populations will be compared by frequency analysis and certain cross tabulations.

Table 1

Demographic Data on Controls and Heroin Users

	Controls N = 58 Mean ± SD	Patients N = 123 Mean ± SD	p*
Age (years)	25.5 ± 7.7	20.5 ± 1.9	<.001
Weight (kg)	75.4 ± 10.1	66.7 ± 7.6	<.001
Height (cm)	178.5 ± 7.5	176.2 ± 7.6	<.05
Months in Vietnam	6.6 ± 3.6	7.6 ± 5.2	NS

*t test of two independent samples

Table 2

Pulmonary Functions in Non-Smokers

	Controls N = 25 Mean ± SD	Patients N = 8 Mean ± SD	p*
Peak Flow (liters/min)	431 ± 23	397 ± 15	<.001
FEV_1 (liters)	3.5 ± 0.6	3.0 ± 0.8	<.07(NS)
FEV_3 (liters	4.0 ± 0.8	3.3 ± 0.8	<.05
Vital Capacity (liters)	4.0 ± 0.8	3.3 ± 0.8	<.05

*t test for two independent samples

Table 3

Pulmonary Functions in Controls and Heroin Users

	"Healthy"* Controls N = 46 Mean ± SD	"Healthy"* Patients N = 96 Mean ± SD	p†	Total Controls N = 58 Mean ± SD	Total Patients N = 123 Mean ± SD	p†
Peak Flow (liters/min	428 ± 24.9	398 ± 22.0	<.001	424 ± 8.1	396 ± 7.0	<.02
FEV_1	3.6 ± 0.5	3.3 ± 0.6	<.02	3.5 ± 0.6	3.3 ± 0.6	<.05
FEV_3	4.1 ± 0.7	3.6 ± 0.7	<.001	4.0 ± 0.8	3.6 ± 0.7	<.01
Vital Capacity (liters)	4.1 ± 0.7	3.6 ± 0.7	<.001	4.0 ± 0.8	3.6 ± 0.7	<.002

*In this context, "healthy" indicates no history of any pulmonary disorder.
†t test of two independent samples

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 106 Clinical and Demographic Studies of Military Drug Abusers

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6503	74 07 01	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
. NIS	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	62758A	3A762758A833	00	[redacted]
c. [redacted]	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 74	2	51
FISCAL YEAR CURRENT 75	3	78

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) 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 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. Multi-disciplinary collaborative approaches are utilized and new methods developed as required.

25.(U) 73 07-74 06 The prevalence of Hepatitis B antigen and antibody in Army health care personnel was assessed and analyzed by demographic and occupational variables. Two-years questionnaire and serologic follow-up of this cohort has begun. (This study is complementary to work described under DA OB 6513, Work Unit 176, entitled "Mechanisms of Transmission of Hepatitis Viruses"). Investigations of hepatitis outbreaks at Fort Riley, Kansas, and Camp Zama, Japan, were completed. Data reduction and analysis of the 1973 Hepatitis B antigen and antibody prevalence study at Fort Hood was completed. Enrollment of personnel newly assigned to Fort Hood into a prospective longitudinal study of Hepatitis B antigen and antibody acquisition was completed. One-year follow-up 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"). Title of this work unit in FY 74 was "Epidemiology of drug abuse in the military."

*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 108 Epidemiology of drug abuse in the military

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

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

From July 1972 to June 1974, AMEDD officers enrolled at the Academy of Health Sciences (AHS), Fort Sam Houston, Texas were administered a demographic and health experience questionnaire and had serum collected for Hepatitis B antigen and antibody determinations. Enlisted students were similarly studied from July 1973 to June 1974. A total of 7304 personnel have been studied. All individuals from this cohort remaining on active duty will be followed up, shortly before the second anniversary of their enrollment at the AHS, with an interim questionnaire and studied for the acquisition of Hepatitis B antigen or antibody. Results and discussion of this collaborative study are reported elsewhere (Project 3A161101A91C, Work Unit 105, Mechanisms of transmission of hepatitis viruses).

2. Epidemiologic Investigation of Hepatitis Outbreaks

Investigation of Hepatitis A cases among military families at Fort Riley, Kansas was initiated during May 1974. The disease was person-to-person propagated resulting in a total of 13 cases. In June, an outbreak of Hepatitis B related to intravenous drug use among enlisted personnel at USAH, Camp Zama, Japan was studied. Transmission by shared needles appeared to explain the majority of the 21 cases found. Virologic studies of materials collected during both investigations are in progress.

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

Epidemiologic investigation of an outbreak of Hepatitis B was begun in November 1972, with studies of the prevalence of antigen and antibody in medical and line units.

One-year serologic follow-up of Fort Hood personnel previously known to be antibody positive was completed. 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 were administered a demographic and health experience questionnaire and had serum collected for Hepatitis B antigen and antibody determinations. This cohort will be followed quarterly. Results and discussion of these collaborative studies are 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 0B 6505	74 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
	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. [redacted]	CARDS 114(f)			

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 — EXPIRATION

b. NUMBER: NA

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	3	106
	CURRENT 75	3	121

19. RESPONSIBLE DOD ORGANIZATION

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NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Dextrophan; (U) Levorphanol; (U) Amphetamine; (U) Morphine

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

23. (U) The objectives of this research are to study the neurophysiological localization of sites of action of drugs of abuse. These studies may provide a physiological key to treatment and maintenance of drug abusers, an excellent model for the understanding of the abuse potential of new drugs, and insight into the basic physiological events occuring during the drug and non-drug state in the military environment.

24. (U) The techniques of neurophysiology and experimental psychology are used.

25. (U) 73 07 - 74 06 Various opiate isomers were used to determine their effect on intracranial self-stimulation (ICSS). Dextrophan, the dextrorotary isomer of 3-hydroxy-n-methyl morphinan, is analgesically inactive. It had no effect on ICSS or food and water intake. Levorphanol, which is analgesically three times more potent than morphine, produces a highly variable effect on ICSS, but no effect on food or water intake. Most rats showed a considerable suppression in ICSS with levorphanol, but several animals showed the increase of ICSS after repeated injections as found with heroin. Rats with chronic monopolar electrodes in the medial forebrain bundle (MFB), ventral tegmental area (VTA), and the locus coeruleus (LC) were injected with varied doses of d-amphetamine sulfate. ICSS was most enhanced in the MFB, with diminishing effects in the VTA, and the LC. At higher doses, VTA self-stimulation predominated during the first two hours following the injection, with a delayed enhancement of MFB self-stimulation occuring at approximately four hours following the injection. LC self-stimulation was the least enhanced. Results suggest differential sensitivity to amphetamine at different anatomical loci supporting ICSS. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*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 109 Neurophysiological localization of sites of action of drugs of abuse

Investigators.
Principal: N. H. Spector, Ph.D.
Associate: CPT George F. Koob, MSC

DESCRIPTION.

This research has pursued 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 externalized using the technique of intracranial self-stimulation (ICSS).

PROGRESS.

The Effects of Dextrophan and Levorphanol on ICSS.

Previous work in our laboratories has shown that heroin causes an increase in self-stimulation while not affecting lever pressing for food or water (Koob, Spector, and Meyerhoff, 1973). Recently these studies have been expanded to include experiments designed to test the effects of the optical isomers of 3-hydroxy-n-methyl morphinan. The purpose was to further elucidate pharmacological specificity by studying optical isomers of an opiate with and without narcotic analgesic potency on self-stimulation. Dextrophan, the dextrorotary isomer of 3-hydroxy-n-methyl morphinan, which is analgesically inactive, produced no effect on self-stimulation, food or water lever pressing. Levorphanol, the levorotary isomer of 3-hydroxy-n-methyl morphinan, which is analgesically three times more potent than morphine, produced a highly variable effect on ICSS, but no effect on food and water intake. Most rats showed a considerable suppression in ICSS with levorphanol, but several animals showed the increase in ICSS after repeated injections of levorphanol. The same effect on ICSS is found with heroin administration. Work in progress should clarify whether levorphanol, a drug equipotent to heroin in its effect on analgesia, will have the same relationship to heroin in its effect on ICSS.

The Effects of Amphetamine on Concurrent ICSS in the Medial Forebrain Bundle, Ventral Tegmentum and Locus Coeruleus.

Rats were prepared with chronic monopolar electrodes aimed at three different anatomical loci that support ICSS: the medial forebrain

bundle, ventral tegmental area, and the locus coeruleus. The animals were allowed continuous access to three levers, one for each brain location. Levels of electrical current in the stimulating electrodes were adjusted to produce stable and equal daily rates of lever pressing on all three electrodes. The rats were then subjected to a series of injections of 0.5, 1.0, 2.0, and 4.0 mg/kg of d-amphetamine sulfate, administered in a random order. At 0.5 and 1.0 mg/kg, self-stimulation was most enhanced in the medial forebrain bundle, with less effect in the ventral tegmental area, followed by the locus coeruleus. At 2.0 and 4.0 mg/kg, self-stimulation in the ventral tegmental electrode predominated during the first two hours following the injection with a delayed enhancement of medial forebrain bundle self-stimulation occurring at approximately four hours following the injection. As with the lower doses of amphetamine, self-stimulation at the locus coeruleus was the least enhanced. These results suggest differential sensitivity to amphetamine at different anatomical loci supporting self-stimulation (Koob, Winger, Meyerhoff and Annau, 1974). These experiments with amphetamine were completed at the Johns Hopkins University in collaboration with Drs. Gail Winger and Zoltan Annau.

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

Literature Cited.

References:

1. Koob, G. F., Spector, N. H., and Meyerhoff, J. L.: Effect of repeated heroin administration on ad lib self-stimulation, eating and drinking. Society for Neuroscience, 1973. (Abstract)

2. Koob, G. F., Winger, G. D., Meyerhoff, J. L., and Annau, Z.: Concurrent self-stimulation in the MFB, ventral tegmentum and locus coeruleus: The effect of d-amphetamine. Society for Neuroscience, 1974. (Abstract)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 08 6507	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'TY	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
NA	D. Chg	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. //////////	Cards 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Biorhythm studies in drug abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

213400 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 — NA

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)
PRECEDING FISCAL YEAR 74	4	217
CURRENT FISCAL YEAR 75	4	233

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING 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) 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) 73 07 - 74 06 Continuous recordings of electroencephalograms, electro-oculograms, electrocardiograms, rheopneumograms, and electrogastrograms combined with blood samples and behavioral observations were obtained from heroin users undergoing abstinence and from normal controls. Computer based techniques were developed to reduce these data to manageable form. The special characteristics of the abstinence syndrome exhibited by Vietnam heroin users are being delineated. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 73 - 30 JUN 74.

*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 ØØ Biomedical Factors in Drug Abuse

Work Unit 11Ø Biorhythm studies in drug abuse

Investigators.
Principal: Frederick W. Hegge, Ph.D.
Associate: MAJ Albert J. Tahmoush, MC; CPT John G. Varni, MSC; Paul Kasper, B.S.; James Struthers, B.A.; Jeanne 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. General Background

The findings discussed below are drawn from data gathered in the Republic of Vietnam during the Spring of 1972. A group of 39 volunteers, composed of documented heroin users and normal paramedical personnel, were studied for five to seven days in a closed research ward. From this group, a core population of ten heroin users and five controls was selected for detailed analysis. The parameters electronically monitored included the electrocardiogram, electrooculogram, electroencephalogram, rheopneumogram, and electrogastrogram. Venapuncture, clinical observations, and performance tests were done three times a day and voided urine was pooled on a daily basis. Continuous records of subject activity were made by research personnel. Preliminary findings were discussed under WU 1Ø2 in the Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 72 - 3Ø JUN 73.

2. Scaling of Withdrawal Severity

Persistent field clinical reports of a mild abstinence syndrome in Vietnam heroin users were confirmed by clinical observations in the present investigation. This observation suggested a decoupling

of drug tolerance and withdrawal severity, since the patients in this study were known to consume extremely large amounts of nearly pure heroin. Such an apparent decoupling is at variance with findings of animal and human research through the years.

In an attempt to resolve this conflict, or at least to place the findings within an acceptable pharmacological framework, the standard scaling procedure for abstinence severity developed by Himmelsbach (Kolb and Himmelsbach, 1938; Quock et al., 1968) was applied to the data. This scaling procedure involves the assignment of varying point scores upon observation of certain symptoms and upon changes in measured variables, such as temperature and systolic blood pressure. The total point score is the indicant of abstinence severity.

While the Himmelsbach procedure is pragmatically useful, it should be approached with caution. The various point scores are arbitrary and may give undue weight to one factor as opposed to another. The parameter set on which the scaling is based may be less than optimal, e.g., pupillary mydriasis does not change over the period of acute symptomatology and is therefore a poor differential indicant (Robinson et al., 1974). Quantitative distinctions in symptom severity are often lacking, e.g., a normal garden variety yawn receives the same point value as the deep groaning yawn characteristic of abstinence.

Figure 1 contrasts the classical severity scores reported by Himmelsbach (Kolb and Himmelsbach, 1938) with data from the present study broken down by route of drug administration. It is apparent both that the Vietnam users did not approach the peak classical value ("Himmelsbach") at any time and that the temporal patterning of scores is markedly different. The peak score for Vietnam intravenous users ("shooters") occurred between days 3 and 4 when the classical curve (also intravenous) was approaching a minimum. Further, the "smokers" and "snorters" exhibited a virtually linear decrease over days. The observation that the day Ø score is lowest for the shooters is probably attributable to a more profound intoxication on admission for that group. The relative rankings on day Ø are inversely related to the estimated amount of the last dose, i.e., approximately 4ØØ mg for shooters, 3ØØ mg for smokers and snorters, and 5Ø-75 mg for the Himmelsbach patients.

The observation of differences in the temporal patterning between Vietnam and classical scores leads to an examination of the contributions of scale components to the total score. The most fruitful breakdown occurred when the total score was separated into two components: one where the scores were based on continuous quantitative measurement of parameters; and a second in which the scores are based on discrete counts of occurrences. The first component is comprised of measures of mydriasis, temperature, respiration, systolic blood pressure, and weight, while the second is composed of yawning, lacrimation, rhinorrhea, perspiration, tremor, piloerection, anorexia, restlessness, and emesis.

Figure 2 presents the data for these two components combined across route of administration. The upper curve for measured factors clearly resembles the curve for shooters in Fig. 1. The lower curve for counted factors resembles the classical Himmelsbach severity function. Thus, it appears that one group of withdrawal symptoms appears early in abstinence, peaking at day 1 while a second group peaks later between day 3 and day 4.

The severity of the late-appearing symptoms was higher for the Vietnam intravenous users than for the classical Himmelsbach patients. The situation was reversed in the case of the early peaking symptom set. This suggests a resolution for the conflict mentioned earlier between mild withdrawal and high tolerance. The early peaking factors are readily observable by both physician and patient and they probably are amenable to psychological modulation on the basis of expectancy, suggestion, and history. The late peaking factors seem much less observable and amenable to ready psychological modulation.

The users in this study had relatively short histories of drug taking and they had not been extensively exposed to a classical American drug culture. The field staff of the study in no way excited or exacerbated expectations of illness contingent upon abstinence. They may, in fact, have aroused and supported contrary expectations. Under this set of conditions, the increased intensity of late peaking factors is consonant with the high heroin intake levels and supports the coupling of tolerance level and abstinence severity. If this analysis proves to be correct, it suggests that a revised scale of withdrawal severity should place relatively heavier weight on late peaking factors in those cases where pharmacological questions are of central importance. Questions relating to social factors in withdrawal would be best addressed by a more precise evaluation of early peaking factors.

3. Multiple Clinical Measurements

The data generated by sampling the variables mentioned earlier, three times a day (Ø6ØØ, 1ØØØ, and 22ØØ), have been subjected to multiple analyses. The primary statistical technique employed was a split-plot analysis of variance (Kirk, 1969). The analysis is based upon a linear data model which divides total error variance into components attributable to within-groups and between-groups variables. The partitioning establishes an error term for each main effect and provides for a powerful statistical test. Other analyses employed have been more descriptive in nature with no attempt at present to establish levels of statistical significance.

When compared to rigorous laboratory work, field investigations are characterized by high variance levels. This fact of life often has the effect that differences that are observed cannot be substantiated through presentation of usual significance levels ($p<Ø.Ø5$, or $p<Ø.Ø1$).

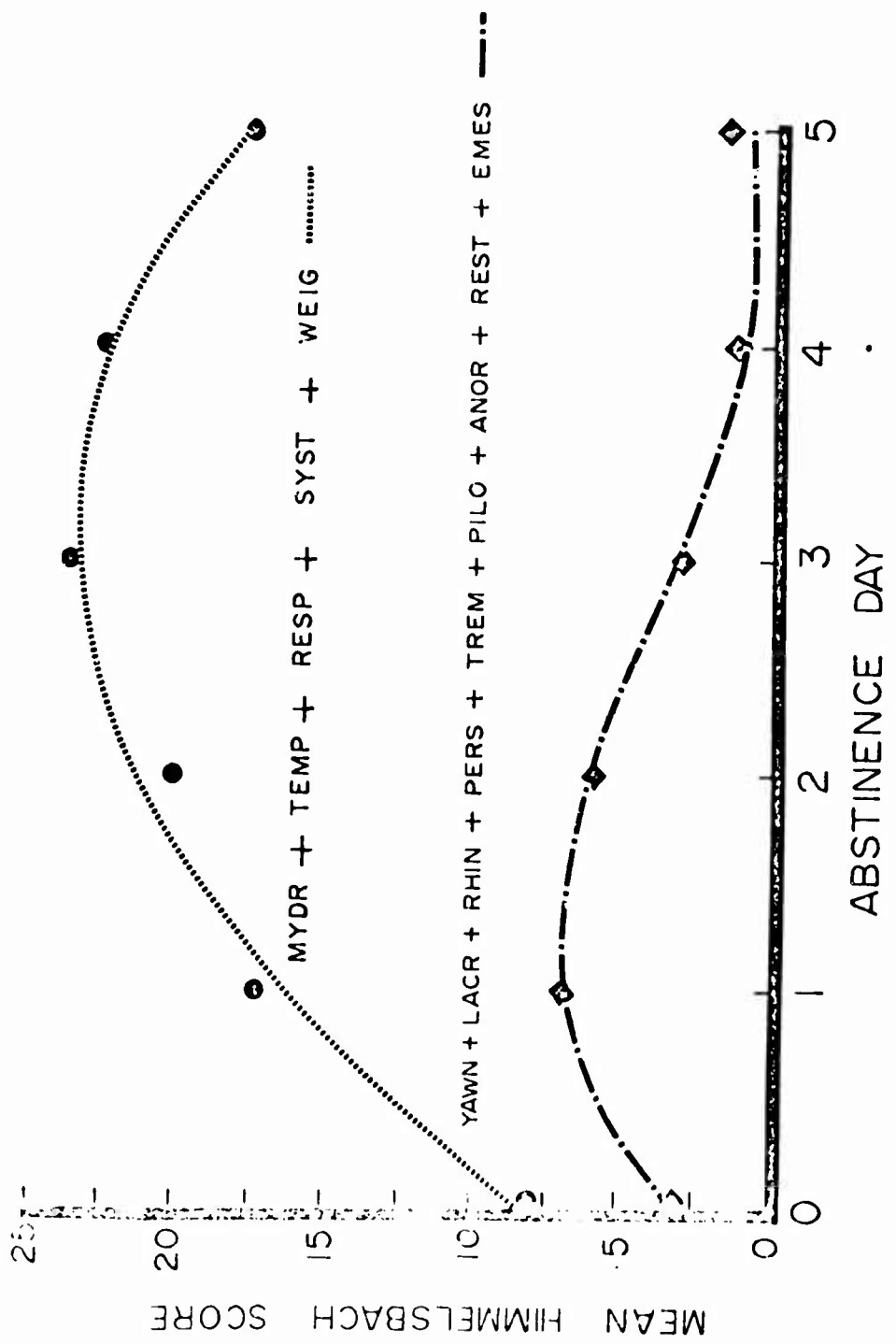

Figure 2. Viet Nam Himmelsbach scores by abstinence day grouped according to a measured/counted distinction. Curves are second order polynomials fitted according to a least squares criterion.

The strategy employed here is to describe differences and trends that, in the judgement of the investigator, are deemed to be real. Statistically unreliable observations can play an important role in directing subsequent, more refined investigation.

a. Respiration

Over the five days of observation, the users had a significantly higher respiration rate than the controls ($p<Ø.ØØ5$). The user overall mean breaths per minute (BPM) was 22.Ø. The control BPM was 15.6, very near the established norm for respiration.

For both groups, respiration rate varied as a function of time of day ($p<Ø.ØØ5$). Respiration rate was lowest at Ø6ØØ and rose to a maximum during the 22ØØ sample. Further, the interaction between groups and time of day was significant ($p<Ø.Ø5$). This interaction took the form of a greater relative change across time in the users than in the controls.

While the groups-by-days interaction did not approach statistical significance, examination of the daily mean values for users and controls is suggestive. The respiration rate of users is higher than that of controls on all observation days. The users and controls show exactly opposite trends over days. The controls show a decrease in mean BPM over the first three days followed by a return to the entry BPM level. This pattern is reasonable for a normal group entering upon a period of restricted activity and subsequently adapting to ward conditions. The users, on the other hand, show increasing mean BPMs over the first three days, peaking at day 3 and declining to entry levels at day 5. The form of the user curve resembles that for measured Himmelsbach scores presented in Figure 2.

b. Oral Temperature

The oral temperature of the users was significantly higher than that of the controls ($p<Ø.Ø1$), overall group means being 98.8°F and 98.0°F respectively. For both groups, there was a statistically significant variation in temperature as a function of time of day ($p<Ø.ØØ5$). The temperature was lowest during the Ø6ØØ sample and highest at 22ØØ, a finding entirely consistent with the well established circadian pattern of human oral temperature variation.

Both the users and the controls exhibited a uniform pattern in mean temperature over observations. Both sets of data are adequately described by straight lines of zero slope with the intercept of the users being higher than that of the controls. This finding is reminiscent of that reported for pupillary mydriasis. Neither parameter showed any tendency to return to normal values of the period of observation, i.e., the period of acute abstinence. This suggests that oral temperature, like mydriasis, may be an indicant of extended abstinence effects.

c. Radial Pulse

The pulse rate of the users was significantly elevated over that of the controls ($p<.005$). The overall mean rate for the users was 72 beats per minute (bpm) while that for the controls was 56 bpm. The pulse rates of both users and controls varied significantly as a function of time of day. The lowest rates were recorded at 0600, increasing to the highest rate during the 2200 sample.

Though neither the day main effect nor any of the interaction effects reached statistical significance, the pattern differences between groups are suggestive. Pulse rates for the controls are lowest on the first three days, rising to near 60 bpm by day 4. The day 1 value of 53 bpm is low and suggests the same pattern of inactivity-induced depression followed by adaptation described for the respiration parameter. The users show a tendency for elevated pulse rates over days 2, 3, and 4 with a downward trend on day 5. User pulse rates are elevated over control rates over all five observation days.

Pulse rate is not a parameter considered in the Himmelsbach system of scoring abstinence severity. The findings described above suggest that radial pulse rate determinations should play a role in a revised scaling model.

d. Systolic Blood Pressure

The findings with respect to systolic blood pressure (SBP) are more complex than those described for the parameters of respiration, radial pulse, and oral temperature. The SBP of the users was not significantly different from tha of the controls. The failure to find a statistically significant difference is attributable to an unexplained elevation of the control SBP to user levels on day 2 and the return of user SBP to control levels on day 5.

The SBP for both groups varied as a function of time of day ($p<.005$). SBP was lowest during the 0600 sample, rising to a maximum at 2200. The interaction effect between groups (users vs. controls) and time of day was significant ($p<.01$). The SBP of the users was elevated above control levels at 0600 and 1000 and slightly below that of the controls at 2200.

The days main effect and the days-by-group interaction effect did not reach significance due to the anomalous day 2 SBP for the controls. If day 2 is ignored, control SBPs show no trend over days. The user SBP is elevated above control level on day 1, rises to a peak on day 3 and day 4 and falls to near control level on day 5. The pattern correlates highly with that reported for respiration and the Himmelsbach scores for measured signs in Figure 2.

e. Diastolic Blood Pressure

The data on diastolic blood pressure (DBP) for users and controls are marred by great variability. Consequently, none of the main effects or interaction effects showed any statistical significance. These discouraging results are partially offset by clear trend differences that are observable in the mean data.

While the overall group means are very close, 68 mmHg for controls versus 70 mmHg for users, the patterns of daily means are strikingly different and resemble those reported for respiration and radial pulse. The mean DBP for the control group falls sharply over the first four days before returning to near the entry level. The DBP of the users is lower than that of the controls on day 1, rises to a peak value above that of controls by day 3, and falls below that of the controls on day 4. The circadian variation in DBP is more marked for users than for controls with minimum values occurring during the 1000 sample.

These data will be subjected to further analysis using variance stabilizing techniques. Diastolic blood pressure is not considered in the Himmelsbach scoring system. If variance can be stabilized and made tractable, DBP should be included in a revised scoring system.

4. General Chronobiological Considerations

In earlier discussions of individual parameters, time of day was a highly significant main effect. However, the analysis of variance employed there does not provide a finely tuned description of time of day differences between the user and the control groups. The variations in rhythm parameters between users and controls are of interest. For example, many observations of drug users suggested that their periods of peak activity occurred later in the day than was the case for non-drug users. If a phase shift of this type could be documented for heroin users, the knowledge would be useful for structuring therapeutic intervention.

Unfortunately, we were unable to observe users under stable drug taking patterns having been limited in this investigation to conditions of total abstinence. Since circadian rhythms do not shift phase rapidly, e.g., the persistence of "jet lag" after rapid time zone translocation, it was felt that drug-induced phase shifts would be observable during abstinence. In addition, it was felt that we would have the opportunity to observe further changes in rhythm parameters throughout a week of abstinence, thereby providing an additional assessment of abstinence severity.

No evidence of phase shifting in physiological functions was found, when users were compared to controls. The two groups exhibited tight temporal synchronization. Given the robustness of

biorhythm timing and the careful attempt to avoid strong rhythm synchronizers in this investigation, it seems unlikely that there were significant physiological phase differences between users and controls. While the phase shift hypothesis is not supported empirically, the notion that the abstinence might be reflected in changes in other biorhythm parameters, such as rhythm amplitude, did receive strong support.

Statistically speaking, abstinence is an unstationary period, i.e., the statistical parameters of measured variables are changing in time. Therefore, normal time series analysis techniques which depend upon stationarity assumptions were precluded from use. The ordinary fitting of sinusoidal functions to such data was an unacceptable technique. One approach to this problem was to use an equation form that permitted the peak amplitude and the mean level parameters of the sinusoid to, themselves, be functions of time. Such an equation was fitted to various data from this investigation by a hill-climbing computer program that provided the best estimate of curve parameter values according to a least squares criterion. In all cases, the curves fitted accounted for a significant proportion of the total variance. Statistical confirmation is not available at this writing.

The findings with respect to mean levels were described earlier using the analysis of variance. Here we concentrate attention on the peak amplitudes of biorhythmic variations. For systolic blood pressure and oral temperature the peak amplitudes are attenuated. The pulse, respiration and diastolic blood pressure rhythm amplitudes appear to be higher in the users than in the controls. This is not unusual in the case of diastolic blood pressure which does not exhibit a strong rhythm in the case of normal, healthy young males. However, the present finding, with respect to clinically measured pulse rate, contradicts our earlier partial analysis of individual continuous electrocardiogram records. A strong suppression of circadian rhythm amplitude was observed among users in the telemetered ECG. This apparent contradiction probably has its source in the less than optimal sampling procedure employed in the clinical assessments. The continuous data have greater statistical reliability. The analyses of the continuous respiration and gastric activity data are incomplete at this writing.

5. Metabolism Data

The urinary clearance of electrolytes and creatinine were subjected to an analysis of variance similar to that described earlier. The data are marked by high variance, and many trends apparent in the mean data plots are not supported statistically by the analysis.

Urine osmolality for the users is below that of controls on day 1, rises to a peak higher than any control value on day 3, and declines to a level below that of the controls by day 5. The

controls show a linear trend upward over the five days. The combined trends were sufficient to produce a significant main effect over days ($p<\emptyset.\emptyset1$) but not significant for the interaction described above.

The total urine volume passed by the users was lower than that passed by the controls ($p<\emptyset.\emptyset1$). A significant day effect ($p<\emptyset.\emptyset5$) and nearly significant group by day interaction ($p<\emptyset.1\emptyset$) resulted from pattern differences between users and controls. Control output decreased linearly over days in a manner characteristic of normal men subjected to enforced inactivity. User volume was well below that of controls on day 1, fell to a minimum on day 3, and rose to control levels by day 5.

In the case of urinary excretion of potassium, neither the groups nor days main effects reached significance but the interaction between them did ($p<\emptyset.\emptyset1$). The controls exhibited a linear trend upward over the five observation days. The users, on the other hand, have values half again higher than controls on day 1. These values reach a peak on day 2 and decline to a level below that of controls on day 5.

Urinary sodium excretion shows quite a different pattern with the days main effect achieving significance ($p<\emptyset.\emptyset5$). The controls vary widely from day to day, exhibiting no specific trend. The users had less than half the excretion rate of the controls on day 1. The rate of excretion for this group increased in a linear fashion over the next four days and closely approximated control values on days 4 and 5.

Both the creatinine clearance day main effect and groups by days interaction effect reached high levels of statistical significance ($p<\emptyset.\emptyset1$). The control group exhibited a highly variable trend upwards over the five observation days. Creatinine excretion by the users was comparable to that of the controls on day 1, rose to a peak well above control values on day 2, and thereafter declining to control levels on days 4 and 5.

Thus, the urine volume and the four urinary metabolites can be distinguished in terms of the timing of peak effect as in the clinical measurements. Urine volume and osmolality are late peaking factors with the highest solute concentration coinciding with the lowest volume. Sodium, potassium, and creatinine levels peak on days 1 and 2. Elevated potassium level coincides with a depressed sodium level.

6. Neuroendocrine Analyses

The responsibility for the analysis of hormone levels was shared between this work unit and WU 111. Specimens were assayed under WU 111 and the statistical analyses were conducted under this work unit. The primary purpose of this report is to place certain

aspects of the hormone data into the conceptual framework of early and late peaking abstinence effects.

Plasma growth hormone, urinary hydroxycorticosteroid (17 OHCS), plasma cortisol, and plasma leutinizing hormone (LH) levels for users all fell into the early peaking pattern of activity. In all cases, levels were highest on day 1 or day 2 and subsequently fell to near control levels by days 4 and 5.

The data for plasma cortisol were least variable and therefore most amenable to statistical analysis. In addition to the early peaking in user levels described above, interesting interactions between group by time of day ($p<Ø.Ø5$) and group by time of day by day ($p<Ø.Ø5$) occurred. The circadian rhythm in cortisol excretion is well known. Levels peak early in the morning and reach a minimum in the evening. The peak Ø6ØØ cortisol levels of users and controls were comparable. However, the differences in level between users and controls increased during the 1ØØØ and 22ØØ samples with user levels twice those of controls at 22ØØ.

The circadian rhythm for controls maintained a constant amplitude across days. The amplitude of the cortisol rhythm for users was equal to that of controls on day 2 but declined to a minimum on day 4. Thus, mean daily cortisol level appears to be an early peaking factor while the circadian disturbance is manifest late.

A different interaction pattern occurred in the circadian organization of testosterone levels. Like cortisol, testosterone is highest at Ø6ØØ and lowest at 22ØØ. Unlike cortisol, user testosterone levels were depressed well below control levels at Ø6ØØ and 1ØØØ. At 22ØØ, control and user levels were comparable. Both groups showed a disruption of activity on entry into the ward setting. The amplitude of circadian variation for users was lower than for controls over the entire observation period.

7. Performance Indices

Grip strength, simple reaction times, and two measures of hand steadiness were examined with the same statistical techniques described above. In no instance did a statistically significant difference between users and controls occur. The performances of users and controls could not be distinguished with the simple assessment battery employed.

8. Overview

The central issue to emerge from this body of investigation is the relationship between early- and late-peaking factors. The early factors appear to be those associated with "sickness" and "stress."

The peaking of late factors is indicative of functional disruption in major physiological systems. At the time the early factors are manifest, morphine equivalent levels (FRAT urine determinations) are high. By the time the late factors appear, morphine equivalent levels have fallen below 100 nanograms per milliliter and acute abstinence is over.

These findings provide no explanation for this differentiation in the patterning of abstinence symptomatology since the investigation was observational in character. A systematic investigation, using animal models, is a clear requirement if we are to understand the full import of these observations.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task ØØ Biomedical Factors in Drug Abuse

Work Unit 11Ø Biorhythm studies in drug abuse

Literature Cited.

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4. Robinson, M. G., Varni, J. G., Howe, R. C., and Hegge, F. W.: Pupillary responsivity during acute heroin withdrawal in Viet Nam. Neurol., 1974 (in press).

Publications:

1. Varni, J. G., Doerr, H. O., and Robinson, M. G.: Increased psychophysiological variability of the anisocoric individual: A neurological model. Psychophysiol. 11: 247-257, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA OB 6509	2. DATE OF SUMMARY: 74 07 01		REPORT CONTROL SYMBOL: DD-DR&E(AR)636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY: D. Change	5. SUMMARY SCTY: U	6. WORK SECURITY: U	7. REGRADING: NA	8A. DISP'N INSTR'N: NL	8B. SPECIFIC DATA-CONTRACTOR ACCESS: ☒ YES ☐ NO	9. LEVEL OF SUM: A. WORK UNIT
10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62758A	3A762758A833		00	111		
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114(f)						

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:
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	169
FISCAL YEAR CURRENT 75	1.5	94

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) 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) 73 07 - 74 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 following by a gradual recovery to basal levels if not an elevation. For a technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 111 Neuroendocrinology in drug abuse

Investigators.
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Associate: MAJ Scott E. Monroe, MC

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: As a first step in applying neuroendocrine approaches to the study of drug abuse, hormone profiles were determined in two groups of young men hospitalized during a two-week period following heroin withdrawal in Vietnam. Continuous 24-hour urine samples and three blood samples (0600, 1000, and 2200 hours) were collected daily during this period. In the second study, a group of five control subjects were similarly followed through a five-day period of hospitalization. The principal hormone analyses for both studies are now complete and it appears that neuroendocrine responses can be described with reasonable confidence, since in the two largely independent studies, conducted at different times, with different research teams, and with some differences in other conditions, the hormonal response profiles during heroin withdrawal are closely similar. Plasma cortisol levels tend to be markedly suppressed shortly after hospital admission. Thereafter, urinary and plasma corticosteroid levels are substantially elevated for the first three or four days, with a peak on the second day. Urinary epinephrine levels also rise to a peak on the second day and then tend to subside slowly into the second week. Urinary norepinephrine levels are initially low and tend to rise slightly towards normal during the first week. Plasma total thyroxine levels are initially elevated and tend

to decline slowly thereafter. Plasma testosterone levels are very low initially, but then rise to a higher level and stabilize within the first few days. Plasma LH concentrations are also initially low, and rise rapidly to levels which are slightly elevated. These levels soon stabilize in the normal range. Plasma growth hormone levels are moderately elevated and quite labile the first few days, but are relatively stable by the fifth day. Plasma insulin levels tend to be slightly low the first two or three days, but are rather stable thereafter. The definition of this profile raises a number of further questions. Preliminary inspection of clinical data in the study suggests that there is considerable similarity between the temporal course of the urinary corticosteroid response curve and clinical estimates of severity of withdrawal symptoms. At present, detailed analyses are being carried out by Dr. Hegge in a search for possible correlations between hormone responses and other clinical and autonomic response measures in this collaborative study. One aspect of the hormone profile which is particularly intriguing is the initial pattern of suppressed cortisol and testosterone levels with elevation of thyroxine levels. Since this pattern was observed within hours after the patients had heroin intake prior to hospital admission, the question arises as to whether this response pattern reflects the central action of heroin or not. The latter question is, of course, relevant to the interpretations of the hormonal changes which follow, in terms of such questions as whether they represent essentially rebound effects which might in themselves contribute to symptomatology during withdrawal, as opposed to changes related to secondary effects of heroin administration on such variables as food intake or sleep patterns, or simply secondary reflections of the discomfort and distress which attend the early phase of heroin withdrawal.

2. Organization of Neuroendocrine Response to Heroin Administration in Monkeys: In order to gain more conclusive and well-controlled data on the effects of heroin itself upon neuroendocrine balance, some pilot experiments were carried out in a chair-restrained monkey previously used only for psychoendocrine studies of initial capture and restraint. Heroin was given both by single injection (0.1 mg/kg) and by three-hour infusion (0.17 mg/kg/hr and 0.50 mg/kg/hr) through an indwelling cardiac catheter. Hormone analyses so far are available only on plasma cortisol levels, which show a consistent and gradual suppression over a three-hour period in all experiments so far to values around 3 to 4 ug%, as compared with mean values about 10 ug% at the same time of day in four control experiments in the same monkey. This suppression of cortisol levels, if confirmed in subsequent experiments, appears to coincide with the earlier observations of low cortisol levels during the initial few hours of heroin withdrawal in human subjects. Further data of the

remainder of the response profile in these monkey experiments, particularly the testosterone and thyroxine findings, should soon permit us to determine if a characteristic neuroendocrine response profile can be defined for heroin administration and if we may have a promising experimental animal model which may be useful in pursuing such issues, for example, as the identification of the neural or neurochemical substrata underlying these drug effects on neuroendocrine systems, or the possible correlations between the time course of changes in neuroendocrine balance and such behavioral variables as the time course of self-administration of heroin.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6511	74 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
	D. Change	U	U	NA	NL X	[X] 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				
XXXXXXXXXX	Cards 114(f)			

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
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	c. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	4	160
FISCAL YEAR CURRENT 75	3	168

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) Drug Abuse, (U) Neurotransmitters, (U) Self-Stimulation, (U) Cyclic Nucleotides, (U) Cerebrospinal Fluid

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 studied 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. Studies will also be done on effects of drugs on axonal flow in these pathways. 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 brain regions following acute and chronic administration of and during withdrawal from drugs of abuse. A behavioral procedure which can induce ingestion of [illegible] kg/day of ethanol or 300 mg/kg/day of sodium barbital will be used for studies of effect of chronic ingestion of these drugs. Effect of drugs will be studied on cerebrospinal fluid (csf) levels of homovanillic acid, 5-hydroxyindolacetic acid, 3-methoxy, 4-hydroxyphenylethylglycol, cAMP and CGMP, as well as on release of endogenous catecholamines.

25. (U) 73 07 - 74 06. It was demonstrated that amphetamine-induced-facilitation of ICSS persisted following lesions in locus coeruleus and ventral tegmentum which depleted norepinephrine and dopamine in specific forebrain structures. Following chronic administration of sodium barbital, marked decrease in cGMP occurred while cAMP and GABA remained unchanged. A study was completed demonstrating the desirability of microwave fixation of brain tissue prior to assay of GABA. For technical report see Walter Reed Army Institute of Research Annual Report, 1 July 73 - 30 June 74.

*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 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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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 brain and cerebrospinal fluid. 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 in brain regions.
   a. acute drug administration.
   b. chronic drug administration.
   c. abrupt withdrawal following chronic administration.
3. Cerebrospinal fluid neurotransmitters and metabolites as monitors of CNS effects of acute and chronic administration of drugs of abuse.
4. Release of endogenous catecholamine from brain.

Effects of amphetamine and other drugs of abuse on intracranial self-stimulation: neuroanatomical and neurochemical substrates.

Further work has been completed on the heroin-induced facilitation of intracranial self-stimulation (ICSS). It was demonstrated that the facilitation occurred only if the baseline response rates were maintained at a high level. When baseline response rates were low, heroin administration could produce inhibition of ICSS (Koob, Spector and Meyerhoff, 1974). To elucidate neuroanatomical-neurochemical substrates, known transmitter pathways are being lesioned

and the effect on the drug-induced changes in ICSS monitored. Lever pressing for ICSS in the medial forebrain bundle was unaffected by lesions in locus coeruleus and ventral tegmentum. Moreover, amphetamine-induced facilitation of ICSS persisted after such lesions. This suggests that neither the dorsal norepinephrine bundle nor the mesolimbic dopamine system is critical to the amphetamine-induced facilitation of ICSS. In support of such studies, a study has been completed demonstrating the time course of depletion of dopamine in three forebrain structures, olfactory tubercle, nucleus accumbens and corpus striatum, following ventral tegmental lesions. The effect was shown to be highly specific to the mesolimbic dopamine pathway. Following lesions in substantia nigra, dopamine depletion was limited to corpus striatum. These studies utilized a new (Coyle 1973) enzymatic radioisotope assay for catecholamines which has one hundred times the sensitivity of other methods. The method was also used to document the depletion of cortical norepinephrine following lesions in locus coeruleus. Further studies have examined the effect of lesions in the ventral tegmental area on the behavioral response to amphetamine. Locomotor behavior was found to be increased both spontaneously and in response to a range of doses of amphetamine.

Effects of drugs of abuse on cyclic nucleotides and neurotransmitters in brain regions.

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 adenosine3'5', monophosphate (cAMP), and cyclic guanosine3'5' 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). Within the next year, emphasis in the field will shift to cAMP/cGMP ratios, and the capacity to study both will become essential.

In two preliminary experiments, cGMP was markedly decreased in brainstem following acute heroin administration. This is significant in that there are indications that opiates impede release of acetylcholine (Belesin, 1965). GABA and GLU were unchanged. 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.

A study has been completed demonstrating a marked decrease in levels of cGMP in certain brain regions following chronic administration of barbital. Levels in brainstem, cerebellum and midbrain were decreased by 80%, 75% and 70% respectively. Levels of cAMP and GABA were unchanged. The latter is especially significant since, upon withdrawal of the drug, audiogenic seizure thresholds were reduced. GABA decrease has been reported in mice after discontinuation of chronic ethanol administration (Patel, 1973).

In conjunction with the foregoing, two studies have been completed demonstrating that the technique of using high-intensity microwave irradiation for enzyme inactivation is 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 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. Another completed study describes the development of E-field "shaping" techniques to improve the efficiency of the microwave irradiation procedures and diminish regional variability of inactivation.

Cerebrospinal fluid neurotransmitters and their metabolites as monitors of effects on brain of acute and chronic administration of drugs of abuse.

We are monitoring cerebrospinal (csf) levels of several neurotransmitter metabolites:

a. Homovanillic acid (HVA) - major metabolite of dopamine (Ashcroft, 1968).
b. Five-hydroxyindole acetic acid (5 HIAA) - major metabolite of serotonin (Ashcroft).
c. Three-methoxy, four-hydroxyphenylethylglycol (MHPG) - major metabolite of norepinephrine in brain (Schanberg, 1968; Gordon, 1971).

The csf monitoring model has the advantage that each subject can be followed through a course of acute and chronic drug administration and acute abstinence. cAMP and cGMP levels in csf will also be monitored.

We have determined the storage parameters of 5 HIAA and HVA under various experimental collecting conditions, and established a csf pool to control for interassay reliability. We have determined that at 25° C, csf values do not change over a 24-hour period and at 4° C they are unchanged after one week. Ascorbic acid is not required for

storage up to four months at -70° C. Standards in water can be stored no longer than one week. Our experiments have demonstrated that ascorbic acid interferes with the assay. This is significant in that some published methods routinely collect csf with ascorbic acid added before storage (Gordon, 1971). We have demonstrated that levels of cAMP and cGMP in csf are stable up to 24 hours at 25° C.

We are presently able to sample csf continuously, collecting hourly in automated fraction collector at 2° C, over successive 24-hour periods. As a preliminary to studies on effect of drugs of abuse on levels of monoamine metabolites in csf, the diurnal variation in levels of MHPG in csf has been studied. In a study on effects of acute amphetamine, levels were found to double within an hour of administration and remained elevated for several hours.

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 catecholamine from brain. This release is enhanced several-fold by amphetamine.

II. BEHAVIORAL BIOLOGY STUDIES OF DRUGS OF ABUSE

The drug research of this part of the Microwave Laboratory has proceeded along two basic lines. An intensive effort has been made to develop inbred strains which exhibit differential alcohol intakes with the goal of ultimately utilizing these strains in studies of differential addiction susceptibility. The second line of work, performed primarily with random bred WRC rats, has been aimed at developing behavioral techniques to systematically elaborate the effects of drugs of abuse, with primary emphasis on alcohol and barbiturates. This work has evolved into a collaborative effort between the Behavioral Biology Lab and the Neurochemistry Lab and includes the development of oral ingestion methods for producing addiction and the development of techniques by which the effects of acute and chronic drugs can be documented on a behavioral, physiological, and neurochemical level. During FY 1974, a merger between the two major lines of work began in that a few rats from the special genetic lines became available for use in basic drug experiments, providing the opportunity to begin cross comparisons of the inbred lines with one another on several types of pharmacological tests and to develop comparisons of the inbred lines with the WRC random bred rat from which inbred lines were derived.

Alcohol addiction model.

Our previous work indicated to us that increased alcohol intakes obtain if a hungry rat is offered sweetened 10% alcohol instead of plain 10% alcohol. In FY 74, a concentration of 4% sugar was determined as optimal for producing high intakes of 10% alcohol, yet allowing for body weights to be maintained at a constant level on a chronic basis by adjusting the amount of solid food in the daily feeding. Using this procedure, we have determined with a large number of WRC rats that alcohol intakes of 11 g per kilogram per day are consistently achieved in food-restricted males within only three weeks. Daily intakes of food-restricted females reach 12 g/kg within a similar period. There are several implications of the extremely high alcohol intakes we have obtained. The high intakes suggest that we are approximating a satisfactory alcohol addiction model. This is confirmed in our studies of alcohol withdrawal phenomena. Secondly, these high intakes have considerable implication relative to what we know about the rat's capacity to metabolize alcohol. An intake of 11 g/kg daily is far higher than the widely accepted 7.2 g/kg daily capacity of rats to metabolize alcohol. There are two possible explanations for this discrepancy. Rats may rapidly develop an increase in metabolic capacity under chronic alcohol exposure. A second more likely alternative is that our food-deprived rat can metabolize more than 7.2 g/kg alcohol per day at the onset. If this is the case, the accepted value for metabolic capacity is simply wrong for this rat. The question of metabolic capacity is urgent and must soon be resolved by determining the relevant blood-alcohol disappearance curves.

Physical dependence on alcohol.

Considerable effort was expended in developing a formal test with which to estimate the audiogenic seizure threshold of rats during withdrawal from alcohol. Preliminary tests revealed that food-restricted male rats maintained chronically on sweetened alcohol were maximally sensitive to high intensity sound at about 8 hours following removal of their alcohol bottles. This led to the development of a three-level sound test during which each animal receives an initial challenge at 7.5 hours into withdrawal of 95 DB. The second challenge of 100 DB is presented at 8 hours. The final challenge of 105 DB is presented at 8.5 hours. Systematic differences in the probability of running fit and seizure have been observed as a function of the loudness of sound employed and the length of time different groups of rats are maintained on the alcohol addiction procedure.

In a formal comparison, rats that had been drinking for three weeks were extremely sensitive to high intensity sounds. Of this group, 100% of the animals showed running fits and 78% exhibited clonic-tone seizures. Groups that were drinking for either two weeks or for one week showed progressively less response than the three-week group. However, all groups showed a higher frequency of response than isocaloric comparison animals, suggesting physical dependence in some of the one- and two-week animals as well as the three-week animals. Recently, we have also found that female rats show audiogenic sensitivity comparable to that seen in the males when females are subjected to similar oral addiction procedures.

Barbital addiction model.

A procedure similar to that used with ethanol has been found to be effective in producing very high rates of barbital intake in rats. A formal test of the intake procedure has been made by comparing several groups of rats. A group of food-restricted rats, which were given access to barbital laced with 4% sugar, showed the highest daily intakes (approx. 300 mg/kg). A second group, which was provided with sweetened barbital, but allowed to feed ad-lib, showed much lower intakes. The third group, which received unsweetened barbital and free access to food, exhibited the lowest intakes. Thus, sweetening barbital solutions enhances intake somewhat, but the combined procedures of sweetening the drug solution and restricting the rats' solid food to a nominal daily portion produces the highest intakes. The barbital intakes we have achieved with this simple, efficient procedure are more than sufficient in producing marked signs of physical dependence in most rats within a three-week period.

Audiogenic seizure proneness following barbital withdrawal.

Rats maintained on our three-week barbital addiction procedure have exhibited an extreme sensitivity to high intensity sounds following removal of the barbital solution bottles. Preliminary results suggested that peak sensitivity occurs at 24 to 36 hours into withdrawal. At 36 hours, the seizure threshold is only 90 DB. This is considerably below the lowest estimate of threshold we have obtained for any group of ethanol dependent rats (97 DB). Also, sensitivity to sound during withdrawal seems to follow a somewhat different time course with barbital than with ethanol. Sensitivity appears later (36 vs. 8 hours) and seems to last considerably longer. This may be either a specific barbital effect or may be a more general barbiturate effect.

Body temperature changes during barbital withdrawal.

Clinically, it is well known that the alcoholic and barbiturate addicts exhibit hyperthermia as part of their abstinence syndrome. Similar temperature changes with experimental animal subjects have heretofore not been documented. However, with two different oral ingestion procedures, we have obtained evidence that rats which have been withdrawn from chronic barbital develop hyperthermia. Colonic temperatures rise gradually through the first several days of withdrawal and then decline gradually to normal. With the most extreme procedures, temperature increases of 1.5° C above normal were achieved. Further evaluation of hyperthermia may reveal that it is a reliable signpost of the alcohol-barbiturate abstinence syndrome and that the hyperthermia shows systematic relations to other indices of withdrawal phenomena.

Morphine addiction model.

Providing food-restricted rats with sugar-sweetened morphine hydrochloride solutions resulted in substantial morphine intakes. During the last week of a three-week experiment, the rats consumed approximately 170 mg/kg daily. By contrast, animals receiving ad-lib feeding drank very little sweetened or unsweetened morphine (less than 15 mg/kg daily). During our initial tests, all food-restricted animals received a solid food allocation of 10 g per day. Morphine intakes as high as 300 mg/kg occurred when rats were given only 5 g of solid food per day for a two-week period. However, this severe restriction had to be discontinued because of rapid losses in body weight. The rats which consumed morphine at levels of 170 mg/kg per day or above had definitely become physically dependent as indicated by the severe abstinence reactions obtained upon administration of the morphine antagonist naloxone.

Alcohol effects on low rate performance.

With a low rate performance baseline (DRL), we have previously observed substantial behavioral tolerance to successive i.p. doses of alcohol administered to rats, even though the injections were spaced two weeks apart. This phenomenon frustrated our efforts to define dose-response relations for individual subjects. Therefore, a major experiment was designed to characterize both the initial response of groups of WRC rats to different doses of alcohol and to evaluate performance changes occuring with successive injections at each of the chosen dates. Initial response of the groups on different dosages indicated an inverted dose-response relation, with moderate doses producing enhanced rates and higher doses producing decreases in the bar-press rates. Tolerance was observed with 1.2, 1.6, and

2.0 g/kg. Changes with successive injections of 1.6 g/kg were interesting and complex. An initial depression of rate was followed by an overshoot of the baseline rate, followed by a gradual return toward baseline levels. This finding approximated the mirror-image of the overall dose-response curve. A dose of 2.4 g/kg completely eliminated performance. There was no apparent recovery with three successive injections. In addition, rats receiving 2.4 g/kg had developed no tolerance to the injections of 1.6 g/kg which followed. By contrast, those rats which had received a dose of 1.6 g/kg or less, for their initial three injections, showed tolerance to subsequent injections of 1.6 g/kg. These observations suggest that this behavioral index of tolerance was to a large extent a practice phenomenon, since only those animals which had performed under the intoxicating effects of alcohol showed an improvement. This finding, and the techniques used to elucidate the data, are similar to "state dependent" learning. Those animals which did not perform under drugs (2.4 g/kg group) showed no tolerance even though they had received the highest dose. If the tolerance had been primarily "neural" or "physiological," this dose should have facilitated rather than hindered tolerance.

Combined effects of alcohol and amphetamine.

We have consistently observed enhanced bar-press rates, using low-rate behavioral (DRL) baselines, when rats are injected with moderate doses of alcohol. Similar effects of amphetamine on low-rate performance are widely noted, even though alcohol and amphetamine produce many differing and often counteracting effects using other pharmacological tests. We speculated that on a functional, behavioral level, nominal doses of these two drugs might combine to produce an additive effect greater than that produced by either drug singularly. This prediction was born out. No substantial performance changes were obtained when either .8 g/kg alcohol or .25 mg/kg amphetamine were injected intraperitonally. The combination of the two drugs at these doses, given simultaneously, resulted in enhanced bar-response rates, an increase in the frequency of short inter-response times, and a concomitant decrease in the number of pellets earned during the experimental sessions. Thus, with DRL performance, the effects of alcohol and amphetamine produced an additive disruption which was greater than the comparative effect of either drug alone.

Sequential reacquisition of avoidance.

A behavioral baseline with rats which evaluates the repeated acquisition of short response sequences is being developed. Three levers are programmed to be operated in three number sequences. The subject is required to perform on one of several fixed sequences

during each of the experimental sessions. The influence of a number of procedural variables has been evaluated in order to select the procedure producing optimal performance. Within an experimental session, error levels typically decrease early in the session and stabilize, indicating that the sequence for that day has been learned. This behavioral procedure may be particularly useful in alcohol studies since practice and relearning phenomena have been implicated in many ethanol studies.

Selective breeding for alcohol intake.

Substantial progress was achieved in the development of the WRAIR inbred high-drinker and low-drinker rat strains. FY 74 was the first year that two full generations (F5 and F6) were produced from both lines. We have been able to increase the number of animals produced per generation, due to some extent through success in selectively breeding for larger litters, in addition to the selection for high and low alcohol intakes. Enough animals were produced so that animals from very small litters could be rejected from the breeding system and used for experiments in preliminary attempts to determine to what extent the two strains differ with respect to other drug related biological traits.

Pharmacological comparisons of WRAIR inbred lines.

A number of preliminary experiments suggest that the WRAIR inbred lines exhibit several differential characteristics other than their differential propensity to drink alcohol. Results from several litters indicate that the high-alcohol-drinker line also accepts barbiturate solutions more readily than the low-alcohol-drinker line. The question of whether these differences are primarily gustatory in origin or reflect a difference in neural response to intoxicating agents should receive careful attention in the future.

Results from acute alcohol and barbiturate studies do suggest that the low-alcohol drinker line is more sensitive to these types of drugs. Acute loads either of ethanol or pentabarbital seem to be more effective in producing loss of the righting reflex in low-strain rats than in the high-strain rats.

We have also observed a suggestive differential response of the two strains during withdrawal following a three-week alcohol addiction experience. The low-strain animals appear to be considerably more sensitive to audiogenic seizure than the high-strain animals, possibly suggesting a differential addiction susceptibility in the respective strains.

With each of the above observations, examination of additional animals is required in order to draw more concrete conclusions. However, the preliminary data point to genetic constitution as an important, experimentally manipulatable determinate of response and addiction to drugs of abuse.

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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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6515	74 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	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	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				
XXXXXXXXXX	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 01	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 74	4	110
FISCAL YEAR CURRENT 75	3	128

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) 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) 73 07 - 74 06 Radioimmunoassays have been developed and exploited to detect and quantitate opiate metabolites in animal models. Excretion patterns in urine of primates have been profiled. The isoenzymes of hydrolases involved in drug metabolism have been characterized in the rat. The effects of drugs and environment on the plasma isoenzyme patterns are being studied. Excretion patterns for procainamide in renal failure patients are being studied to evaluate the renal component of acetylation of drugs. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* 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 113 Metabolism of drugs of abuse

Investigators.
Principal: LTC Douglas J. Beach, MSC; John W. Diggs, Ph.D.; LTC Gale E. Demaree, MSC.
Associate: Meade R. Drumgold, B.S.; Clarence E. Emery, B.S.; SFC Lovelyn Hall; John A. Kintzios, B.S.; Nell R. Pendleton, B.S.

During FY 74, the initial year for this work unit, the basic plan of research was formulated on the basis of results of preliminary experiments. The research reported here centered on studies designed to test correlations between dependence to drugs of abuse, the metabolism of these drugs and the general metabolic status of the organism. The objectives of this work unit are (1) to evaluate metabolic parameters as a function of drug dependence in order to devise a general metabolic profile of the organism which would reflect the degree to which the organism is physically dependent on drugs of abuse, and (2) to use these biochemical parameters as indicators to elucidate the mechanisms of drug disposition in man and animals for the purpose of devising rational approaches to treating toxic and addicted drug conditions.

Two studies were initiated: (1) correlation of the degree of tolerance and dependence of rats to morphine with blood levels of morphine and with levels of some humoral substances and plasma levels of some enzymes, and (2) to assess the role of hydrolase enzymes in dependence to drugs of abuse.

Correlation of drug levels in plasma with metabolic responses following the subcutaneous implantation of morphine pellets in rats.

The plasma levels of corticosterone, insulin and growth hormone and serum levels of glucose, glutamate-oxaloacetate transaminase (SGOT), glutamate-pyruvate transaminase (SGPT), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were correlated with plasma levels of morphine during the development of tolerance following morphine pellet implantation. These values were further compared to the degree of drug dependence as judged by the severity of the behavioral responses to injections of naloxone. These studies confirmed other results that indicated that the maximum degree of dependence occurs in the rat about 72 hours after pellet implantation. This finding shows no correlation with changes in any of the humoral or metabolic parameters that were observed except blood glucose levels.

TABLE I

Plasma Levels of Corticosterone in Rats following Morphine Pellet Implantation

Hours after Implantation	Plasma Levels of Corticosterone (µg/100 ml)
1	59.5
4	54.5
8	37.5
24	24.5
48	25.5
72	28.0
72*	68.5
96	33.5

*15 minutes after the injection of naloxone to precipitate withdrawal.

TABLE II

Plasma Levels of Morphine, Glucose and Serum Levels of Some Enzymes in Rats following Morphine Pellet Implantation

Hours after Implantation	Morphine Equivalent (µg/ml)	Glucose mg%	CPK (International units per ml)	LDH	SGOT	SGPT
0	0	121	70	78	68	15
24	3.6[a]	129	462	113	82	18
48	1.2	140	140	130	83	49
72	1.0	74	59	86	87	27
96	1.5	66	57	48	61	18
120	1.6	—	--	--	--	—
144	1.4	58	126	—	58	13
168	2.2	70	102	--	58	13
192	—	121	118	214	48	27
240	—	140	30	110	39	20

[a]Morphine equivalents were estimated by Radioimmunoassay. No attempts were made to determine the materials in the plasma that were responsible for these responses in the immunoassay system.

These preliminary findings suggest several experimental avenues to be explored:

(1) Attempts will be made to determine qualitatively and quantitatively the materials present in plasma that continue to give positive values by Radioimmunoassay after 72 hours even though the animals begins to lose its dependence on morphine. The full time-course of these plasma levels must be studied until the material or materials are persistently absent from plasma samples. When identified, the pharmacological roles of these metabolites will be evaluated.

(2) The possible causal relationship between corticosterone levels and withdrawal will be evaluated.

(3) The possible causal relationships between blood sugar levels and tolerance will be assessed.

(4) Further correlations between morphine metabolism and other humoral and metabolic parameters will be made.

(5) Since chloroquine administration is known to affect morphine excretion and tolerance in rats, pretreatment with this drug will be used as experimental manipulation to evaluate the correlates between morphine metabolism and drug dependence and tolerance.

(6) Similar studies will be extended to other drugs of abuse.

Serum levels of hydrolase enzymes and their roles in drug dependence and drug tolerance.

Methods for detection and quantitation of the hydrolase enzymes, β-glucuronidase and acid phosphatase, were developed. Methods were developed to separate and identify the isoenzymes of these enzymes from kidney, liver, lung, intestine and skeletal muscle. Preliminary studies using subcellular fractionation techniques have revealed the occurrence of these enzymes in fractions other than the lysosomes. Confirmation of these findings along with refinement of these new techniques will lead to several important hypotheses to be tested regarding the possible roles of the involvement of these hydrolase enzymes in drug dependence, drug tolerance and drug metabolism.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6516	74 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
	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				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Procede 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 01	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 74	2	36
FISCAL YEAR CURRENT 75	2	76

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Drug Excretion

(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. Procede 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) 73 07 - 74 06 Chloroquine pretreatment has been demonstrated to modify the rate of excretion of morphine in rats and mice. Chloroquine pretreatment also reduced the withdrawal responses from morphine in rats and mice. Excretion patterns in urine, feces and bile are being correlated with blood levels to assess the relationship of excretion and distribution to these modified pharmacodynamic patterns. Morphine-specific binding sites in subcellular brain fractions of mice have been described. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

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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 Douglas J. Beach, MSC; LTC Gale E. Demaree, MSC; John W. Diggs, Ph.D.
Associate: Meade R. Drumgold, B.S.; Clarence E. Emery, B.S.; SFC Lovelyn L. Hall, John A. Kintzios, B.S.; Nell R. Pendleton, B.S.

During FY 74, the first year of this work unit, emphasis was placed on developing animal models for testing the effects of drug absorption, distribution, metabolism and excretion on the development of tolerance and dependence. Work during this year has focused on the effects of chloroquine to modify the kinetics of morphine and to modify the development of morphine tolerance and dependence. The objectives of these studies were to prove the animal model in order to test hypotheses concerning pharmacokinetic intervention to the development of drug tolerance and drug dependence.

Pretreatment of rats with chloroquine had been shown previously to increase the urinary excretion of morphine. These findings were verified and extended to show that chloroquine pretreatment modifies the activity of two enzyme systems involved in the metabolism of morphine. UDP glucuronyl transferase levels are increased by chloroquine pretreatment and the activity of this enzyme is suppressed by morphine. UDP glucose dehydrogenase activity, conversely, is decreased by chloroquine pretreatment and restored by morphine (Table I). These two interesting findings by themselves do not explain the effects of chloroquine to accelerate the excretion of morphine (Table II).

We have shown previously that the maximum dependence to morphine in rats induced by pellet implantation occurs at 3 days. This is based on the intensity of neurogenic behavior, the most striking of which and most easily quantified is the so-called "wet-dog shakes." As seen in Table III, chloroquine pretreatment greatly decreased the severity of Naloxone-induced withdrawal.

These data are not conclusive, but they strongly suggest that chloroquine-pretreated rats might serve as a useful model to study pharmacokinetic effects for use in treatment or prevention of morphine toxicity or dependence.

TABLE I

THE EFFECT OF CHLOROQUINE AND/OR SINGLE (ACUTE) DOSES OF MORPHINE SULFATE ON SELECTED PHYSIOLOGICAL PARAMETERS IN THE RAT

	(Mean of 18-24 Rats)					
Group	I	II	III	IV	V	VI
Body Weight						
Initial	292	291	387	296	290	296
Final	304	306	301	305	302	309
Liver Weight						
Total	13.2	12.7	13.6	14.1	12.8	13.5
gm/100 BW	4.3	4.1	4.5	4.6	4.2	4.4
Kidney Weight						
Total	2.3	2.25	2.1	2.45	2.35	2.35
gm/100 gm BW	0.75	0.73	0.70	0.80	0.78	0.76
Blood Glucose (mg%)	121	134	131	127	119	108
Protein mg/gm Liver	47.5	49.25	52.0	53.5	52.75	49.75
Glucuronyl Transferase µg Morph conjugated/ gm Liver/20 min	52.7	33.25	93.35	39.92	33.35	34.4
UDP-Glucose Dehydrogenase units/ml Sol. Fract.	22	24.1	10	18.2	21.6	21.5
Urinary Morphine Equivalents						
µg/ml				29.25[a]	182.2	93.1
µg (Total)				584	2620.0	1411

[a]Morphine levels expressed as equivalents as determined by the Free Radical Assay Technique.

Group Designations

Group I - Controls
Group II - Chloroquine, 13 mg/kg
Group III - Chloroquine, 40 mg/kg
Group IV - Morphine, 10 mg/kg
Group V - Chloroquine (13 mg/kg) + Morphine (10 mg/kg)
Group VI - Chloroquine (40 mg/kg) + Morphine (10 mg/kg).

TABLE II

URINARY MORPHINE LEVELS IN EXPERIMENTAL RATS FOLLOWING MORPHINE PELLET IMPLANTATION

Hours Following Implantation	Morphine (µg/ml Urine)	
	Untreated Controls	Chloroquine Pretreated
24 hrs	330	1460
48 hrs	470	1450
72 hrs	630	1200
96 hrs	770	650
120 hrs	860	420
144 hrs	1400	275
168 hrs	950	—
192 hrs	515	120

TABLE III

NALOXONE PRECIPITATED WITHDRAWAL SYMPTOMS EXHIBITED IN CHLOROQUINE PRETREATED RATS FOLLOWING MORPHINE PELLET IMPLANTATION

Days Implantation Morphine	CHLOROQUINE PRETREATED WET DOG SHAKES 0-15 Min	15-30 Min	30-45 Min	45-60 Min	60-75 Min	75-90 Min	90-120 Min
3 Days	4	7	2	5	3	0	0
4 Days	5	2	3	2	1	0	0
	UNTREATED CONTROLS						
3 Days	14	8	9	2	5	1	0
4 Days	11	6	8	7	2	1	0

Since both heme-iron (Cytochrome P-450) and non-heme iron (Fe^{+2}) are co-factors involved in drug metabolism, and since chloroquine has been shown to facilitate membrane transport of iron, attempts were made to prevent the effects of chloroquine by creating iron-deficiency through long-term iron deprivation in mice. Naloxone induces an urge to jump in morphine-dependent mice, which can be easily quantified. With 8 animals per group the effects of iron deficiency on chloroquine pre-treatment to reduce morphine dependence were studied. The results of this experiment (Table IV) show no evidence that dietary iron plays a role in the chloroquine action.

TABLE IV

THE EFFECT OF IRON DEFICIENT DIET ON CHLOROQUINE-INDUCED RESISTANCE TO MORPHINE DEPENDENCE

Average Number of Jumps/Mouse following Naloxone in Morphine Pellet-Implanted Mice

	Control	Iron Deficient
Chloroquine	3.2 ± 2.3[a]	3.0 ± 1.3
No Chloroquine	8.0 ± 6.0	8.3 ± 2.3

[a] Mean $\pm$ s.d. (n = 8)

Chloroquine pretreatment to decrease the effects of morphine withdrawal was also shown to be effective in blocking the adrenal cortical response to withdrawal in rats (Table V).

Considerable work remains to establish the rat and chloroquine pretreatment as working models for modifying drug kinetics to treat drug dependence. Nevertheless, the data derived to date offer considerable optimism for the feasibility of this model and its ultimate application.

TABLE V

THE EFFECT OF CHLOROQUINE PRETREATMENT ON PLASMA CORTICOSTERONE LEVELS FOLLOWING MORPHINE PELLET IMPLANTATION IN THE RAT

Hours After Pellet Implant	Plasma Corticosterone Levels	
	Controls	Chloroquine Pretreated
1	59.5[a]	44.5
4	54.5	39.5
8	37.5	31.5
24	24.5	25.5
48	25.5	23.5
72	28.0	22.5
72	68.5[b]	37.5[b]
96	33.5	23.5

[a]Given in µg%

[b]15 minutes after naloxone injection.

DISTRIBUTION:

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1 - U.S. Army Combat Developments Command

☆ U.S. GOVERNMENT PRINTING OFFICE: 1975 - 572-105

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER NA	2. GOVT ACCESSION NO. UNKNOWN	3. RECIPIENT'S CATALOG NUMBER NA
4. TITLE (and Subtitle) Research in Biological & Medical Sciences, including Biochemistry, Communicable Disease & Immunology, Internal Medicine, Nuclear Medicine, Physiology, Psychiatry, Surgery, and Veterinary Medicine		5. TYPE OF REPORT & PERIOD COVERED Annual Progress Report 1 July 1973 - 30 June 1974 6. PERFORMING ORG. REPORT NUMBER NA
7. AUTHOR(s) Listed at beginning of each work unit report		8. CONTRACT OR GRANT NUMBER(s) NA
9. PERFORMING ORGANIZATION NAME AND ADDRESS Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D. C. 20012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NA
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command Washington, D. C. 20314		12. REPORT DATE July 1974 13. NUMBER OF PAGES 1582
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

NA

18. SUPPLEMENTARY NOTES

NA

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

BIOLOGICAL SCIENCES
MEDICAL SCIENCES
BIOCHEMISTRY
COMMUNICABLE DISEASES

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.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. IMMUNOLOGY
INTERNAL MEDICINE
NUCLEAR MEDICINE
PHYSIOLOGY
PSYCHIATRY
SURGERY
VETERINARY MEDICINE