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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 1972

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

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

ANNUAL PROGRESS REPORT

1 July 1971 - 30 June 1972

VOLUME II

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SURGERY						
VETERINARY MEDICINE						

///

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.

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VOLUME II

PROJECT 3A062110A806
MILITARY PREVENTIVE MEDICINE

Task 00
Military Preventive Medicine

720-a

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(U) Global Health Data; (U) Epidemiology; (U) Geography; (U) Potential; (U) Climate; (U) Ectoparasites; (U) Infectious Diseases

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

23. (U) Unclassified health and disease data on all foreign countries is accrued, analyzed, stored and made available to approved requestors. Consultation and assistance is rendered to Medical Officers and to Global Medicine Courses. Disease potential is calculated for all geographic areas and for all diseases.

24. (U) The staff of three (2 professional and 1 administrative) makes extracts of all qualified documents relating to disease -- world wide. From these extracts, concepts are formed; and after review by world authorities on various disease entities, predicted incidence of any given malady is calculated.

25. (U) 71 07 - 72 06. The staff of three (2 professionals and 1 administrative) research for data and write extracts of documents. 13,025 of these are now in the computer and a thousand more are being processed presently. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

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720-6

Project 3A062110A806 MILITARY PREVENTIVE MEDICINE

Task 00 Military Preventive Medicine

Work Unit 030 Global health data

Investigators
Principal: COL Hinton J. Baker, MC
Associates: Ann C. Fred, M.D.; Eleanor R. Cross, M.A., and Frances K. McAllister, B.A.

1. Statement of the Problem

Any medical department operational plan requires an estimate of trauma and disease on which to base estimates of treatment and disease prevention facilities and measures. Estimates of battle injuries, vehicle accidents, cold injury, and heat injury are relatively well developed. Reliable estimates of infectious disease risks are vastly more complex and require systematization.

2. Background

Planning to cope with real disease risks was shown to be inadequate in the Lebanon landings in July 1958. The planning to cope with disease was still inadequate for overseas deployment of U.S. soldiers in Vietnam in 1965-1967.

3. Approach

A system to continually upgrade by new pertinent information the ability to describe disease risks quantitatively by specific place and specific time on the land areas of the earth. This system will be defined by explicit rules so that many disease specialists who desire to cooperate with the Armed Forces can contribute their knowledge in a form for continuing active use.

4. Results

a. A schema for disease specialists to mentally interact toward determining specific disease risks has been created.

b. A set of working rules of the system has been created and tested with data for Rocky Mountain Spotted Fever, schistosomiasis, and leptospirosis.

c. A proforma sheet has been developed to accommodate an extract of an information element of any disease.

d. A computer program has been adapted to service the system.

e. A microfiche system has been integrated to provide convenient provision of any basic document utilized by the system.

f. The system presently contains 15,000 elements of information including both extracts and concepts and making explicit the reasoning for potentials for spotted fever in Western United States and Canada, and the basis for several spot potentials on schistosomiasis and leptospirosis. Seven hundred thirty-five documents have been accessioned from which the information elements have been drawn.

5. Conclusion

The number of available biological data elements for this purpose are not as large as presumed prior to the undertaking.

The necessity to relate biological factors to physical parameters or determinants is seen to be important because of the more complete and accurate physical data for the earth's land areas.

The progress to date by 4 to 5 workers indicates that world-wide estimates of disease potentials for many diseases are feasible in a period of five years.

6. Recommendations

Encourage selected disease specialists to interact with the system and provide support for this interaction.

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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 aims of this proposed pilot project are (1) to survey a sample of retiring Army personnel for the frequency and degree of hearing impairment as recorded on separation physical examination forms, and (2) to compare the demographic characteristics available from this form for those with and those without recorded hearing impairments.

24. (U) Data will be obtained from the physical examination forms (SF 88) which are processed in PSD SGO DA. Hearing status will be evaluated from the statement of the examining physician who lists a diagnosis of hearing impairment (SF 88, Item 74) and also from the recorded results of audiometry (Item 71).

25. (U) 71 07 - 72 06 Review of 1738 separation physical examinations processed by the PSD SGO DA has shown that approximately one-third have some degree of hearing impairment (SF 88 Items 71 & 74 which is largely confined to high frequencies (4000 H_2) but includes the range of speech frequencies. No consistent pattern between hearing impairment and either age or length of service or a combination of both is evident suggesting that the causes of the impairment are not related to these variables. Further group hearing impairment among military personnel appears considerably higher than estimates of hearing impairment in general population groups as reported by USPHS National Health Survey. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

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Project 3AO62110A806 MILITARY PREVENTIVE MEDICINE

Task 00, Military Preventive Medicine

Work Unit 032, Pilot study to determine hearing impairment among Separating Army personnel

Investigators.
Principal: LTC Craig H. Llewellyn, MC
Associate: MAJ Alan S. Morrison, MC

PROBLEM: A preliminary review of separation physical examinations processed by the Office of The Surgeon General, OTSG, revealed that impairment of hearing acuity is a commonly listed separation diagnosis. Over a third of the examination forms in one small series (38 percent of 47) listed some degree of hearing impairment. Since this frequency is considerably higher than that reported for civilian populations, a pilot investigation of this apparent excess in hearing impairment was initiated.

BACKGROUND: Although the U.S. Army Medical Department pioneered in establishing hearing conservation programs during World War II, deafness and impaired hearing have continued to occur among those on active duty. The 1954 Annual Report of The Surgeon General listed 1110 cases of deafness or impaired hearing, of which only 245 were cited as existing prior to service. The average case was lost from duty for a period of more than 70 days. One hundred eighty-seven separations from active duty occurred, but this number underestimates the problem because the criteria for disability separation have been restrictive.

A more recent annual report from The Surgeon General (1959) provided information on the financial compensation associated with hearing impairment. Over a decade ago, $30 million was being paid annually by the Veterans Administration for hearing loss incurred in military service, and inflationary trends would make present costs still greater.

The need for hearing acuity above minimum levels is well recognized in military assignments, and is reflected in procurement hearing standards. However, various military activities require personnel to work where noise may reach levels capable of producing both temporary and permanent hearing deficits. While efforts have been made to control noisy environments and to monitor hearing acuity, hearing impairment continues to occur. Present Army hearing conser-

vation programs point out these dangers, but additional emphasis and further research on preventive and control measures may well be appropriate.

The specific aims of this project were: (1) to survey a sample of separating Army personnel for the frequency and degree of hearing impairment as recorded on separation medical examination forms; (2) to compare the demographic characteristics available from this form for those with and those without recorded hearing impairments.

APPROACH TO THE PROBLEM: The records reviewed in this survey were those of separating Army personnel whose separation medical records were forwarded to The Surgeon General's Office. Army regulations (AR 635-100 and AR 635-200) provide that personnel retiring for length of service or resigning for selected reasons must undergo a medical examination within six months of separation, and the results of this examination must be reviewed. However, only the examination records of personnel who are medically fit for retention on active duty are forwarded for review. Personnel who have conditions which make them candidates for disability compensation are not included in this survey, since records of such personnel are reviewed through other administrative channels. Therefore, the sample consists of separating Army personnel who have no condition which makes them medically unfit to continue military service.

During a one-month period 1738 medical records were reviewed. Each record included a standardized form for recording findings from the clinical examination (Standard Form 88). From each form the following information was obtained:

(1) military rank

(2) sex

(3) ethnic background

(4) total years of active military service

(5) results of audiometric testing for hearing acuity

(6) medical diagnosis (if any) summarizing the findings of the examination

These medical diagnoses were then classified according to the Eighth International Classification of Diseases Adapted (ICDA8). In this study hearing impairment means that diagnostic statements consistent with ICDA8 code numbers 389.0 (deafness in both ears), 389.1 (deafness in one ear and partial deafness in other), 389.2 (deafness in one ear) and 389.9 (impairment of hearing in one or both ears) were recorded as a summarizing diagnosis on the standard form.

In the results which follow 42 records were excluded from original sample of 1738. The exclusions were made because of female sex (39), unknown sex (1), unknown rank (1), and unknown ethnic background (1). The remaining 1698 records provide estimates of hearing impairment among males specific for age, rank, length of service and ethnic background.

RESULTS AND DISCUSSION: Table 1 presents the age distribution of the total sample as well as for each of 4 rank and ethnic groups. About 84 percent of the sample are white and about 28 percent are officers. The white enlisted group is more than twice as large as the next larger group; the 19 nonwhite officers comprise slightly over 1 percent of the sample. Officers have a wider age range than do enlisted personnel.

Table 2 presents information on the length of service for each rank and ethnic group. Almost 90 percent of the sample have 20 or more years of service. As with age, variation in length of service is greater among officers than among enlisted personnel. Ninety-five had less than 15 years of service, and of these 90 were white officers.

Information on the percent with impaired hearing by age for each of the 4 rank and ethnic group is presented in Table 3. When the total sample is considered, those under 35 years of age experience the lowest rate of impaired hearing (10.5 percent). At age 35 however, the rate is 35.2 percent and the proportion remains near or above that level for all older ages. One third of the total group have some measurable degree of hearing impairment.

Only the group of white officers shows a consistent increase in hearing impairment with increasing age. White officers in the 2 youngest age categories have relatively low rates of impairment, but by age 40 and older the records of about a third or more indicate evidence of hearing impairment. White enlisted men have comparatively high rates in

all age categories and have the highest overall percent impairment among these groups (39.3 percent). By contrast, nonwhite enlisted personnel have relatively low rates and the lowest total rate of impairment in the proportion impaired (22.2 percent). Neither enlisted group shows consistent change with age.

The rates for whites are higher than the rates for nonwhites for all ages except under age 35 years. The total rate for whites (35.8 percent, 513/1434) is almost 60 percent higher than the total nonwhite rate (22.5 percent, 59/262). Enlisted personnel have higher rates than officers at all ages. Overall, the enlisted rate of 35.9 percent (438/1219) is 27.8 percent greater than the officer rate (28.1 percent, 134/477).

When hearing impairment for the 4 rank and ethnic groups is considered by length of service (Table 4), no consistent pattern emerges for any group. For the total group those with less than 20 years of service have the lowest rate (19.3 percent). With 20 years of service impairment is found in about 35 percent, and the percent impaired remains near that level for all longer periods of service. When the specific groups are considered, only white officers with less than 20 years of service have a relatively low rate of impaired hearing. The other 3 groups give no evidence of spared hearing with terms of service less than 20 years. In fact nonwhite enlisted personnel have the highest rate of impairment among those with under 20 years of service.

When the combined effect of age and length of service on hearing impairment was considered, no consistent pattern relating the proportion with impaired hearing to age and length of service was demonstrated for any group. Even the white officers who showed an increasing frequency of hearing impairment with increasing age failed to demonstrate this pattern for specific durations of service. The primary observation to be made is that the frequency of impaired hearing is high among all personnel aged 35 years or older or serving 20 years of more with relatively small fluctuations from the overall average of about one-third impaired.

Hearing thresholds at 4 pure tone frequencies (500, 1000, 2000 and 4000 Hz) were also obtained from the records of the separation medical examination. The proportion of individuals with pure tone thresholds at various ranges for each of the 4 rank and ethnic groups

are presented in Table 5. High hearing thresholds for all personnel are most marked at 4000Hz. At that frequency 25.6 percent of white officers, 21.1 percent of nonwhite officers, 37.2 percent of white enlisted personnel and 22.3 percent of nonwhite enlisted personnel have thresholds of 36 decibels (db) or greater. At 500 Hz only 2.6 percent of white officers, no nonwhite officers, 5.4 percent of white enlisted personnel and 3.6 percent of nonwhite enlisted personnel have threshold levels of 36 db or greater.

High frequency hearing loss is particularly marked for white officers. At all 4 pure tone frequencies white enlisted personnel have the highest hearing thresholds among these groups.

Table 6 presents the proportions of personnel with hearing threshold levels of 36 db or more for the 4 rank and ethnic groups by age. For white officers hearing impairment is largely (but not entirely) limited to high frequency tones. This group shows an increasing proportion with impaired hearing as age increases. More than half of those age 55 or more have at least a 36 db threshold level at 4000 Hz. The total group average at 4000 Hz is 25.1 percent. Impairments at the 3 lower frequencies (which represent the important range of speech frequencies) are not so common, and the proportion impaired does not rise regularly with age as age increases.

Among the small group of 19 nonwhite officers none had a threshold level of 36 db or more at 500 and 1000 Hz at any age.

As with officers, enlisted personnel have impaired hearing which is more pronounced at 4000 Hz than at lower frequencies. Even so, 2 to 15 percent of white enlisted personnel have 36 db or higher thresholds at 500, 1000, or 2000 Hz. No consistently increasing proportion with high threshold levels for either enlisted group at any pure tone frequency is observed.

Over 37 percent of white enlisted personnel have 36 db or higher thresholds at 4000 Hz at ages 35 to 40. This is over 3 times higher than white officers of the same age. High frequency hearing impairment among white enlisted personnel is common for all age groups after 35 years of age. For white enlisted personnel the proportion with high thresholds at 500 and 1000 Hz actually drops in older age categories as it did for white officers.

Hearing impairment for the 4 groups is further considered by length of service in Table 7. The proportion of white officers with threshold levels of 36 db or more is highest at 4000 Hz, and the proportion tends to increase with increasing age. At speech frequencies the proportions with 36 db or more thresholds are much lower but by no means absent. When the thresholds of enlisted personnel are considered by length of service, no increasing pattern is observed at any pure tone frequency for either ethnic group. High proportions with 36 db or more levels are again limited to 4000 Hz.

These medical records of separating Army personnel indicate that about one third have some degree of hearing impairment. The hearing deficits are largely confined to high frequencies (4000 Hz) but also include the range of speech frequencies of some personnel as well. No consistent pattern between hearing impairment and either age or length of service or a combination of both is evident in these data. It suggests that the cause or causes of the hearing impairment are not immediately related to these variables. While considerable hearing impairment exists among these personnel, the factors of age, length of service, rank and ethnic background appear to be overshadowed by other factors not reviewed in this survey. From the information available from the separation medical records, it was not possible to identify specific military experiences of personnel in this pilot survey. It seems likely that noise-induced hearing impairment may contribute significantly to the frequent hearing deficits reported. The primary observation is that high frequency hearing loss is quite common among separating personnel.

The group hearing impairment among military personnel appears considerably higher than estimates of hearing impairment in general population groups. For example, age specific prevalence rates of hearing impairment among civilians has been reported by the USPHS National Health Survey as follows:

Age (yrs.)	Percent Reporting Impaired Hearing
Under 25	0.8
25-44	2.1
45-64	5.2
65-74	12.9
75 and over	25.6
Total	3.5

This report may underestimate total prevalence in the civilian population since interviews were limited to a sample on non-institutionalized persons self-reporting their impairments. However, results of audiometric testing performed as part of the National Health Survey also support the view that hearing impairment is considerably less than that reported for these separating Army personnel.

CONCLUSIONS: This pilot study has reviewed the separation medical records of 1696 Army male personnel to determine the frequency of hearing impairment. About one third of these records indicate some degree of hearing impairment. The impairment is largely but not exclusively confined to high frequency hearing loss. While some variations by age, length of service, rank, and ethnic background were observed among specific subgroups, these variations appear rather small in relation to the high overall proportion with evidence of impaired hearing.

RECOMMENDATIONS: The high proportion of separating Army personnel with hearing impairment deserves further investigation to identify specific causal factors: (1) In spite of the variable quality of audiometric test results recorded in the health record, valuable information can be obtained rapidly and inexpensively from these documents. Samples of routine periodic physical examinations could be surveyed to assess the effectiveness of hearing conservation programs, (2) Carefully performed audiometric testing should be carried out on selected personnel with shorter periods of service than reviewed here. Receptees should be included as well. Personnel in various occupational activities should be studied, particularly those in activities associated with high noise environments (e.g., artillery, armor and aviation), (3) In addition to audiometric surveys to estimate the prevalence of hearing impairment among various groups of Army personnel, it would be highly desirable to follow groups of selected personnel over time, particularly persons who are beginning occupational activities which will expose them to high noise environments. Prospective studies could identify those environments which are contributing to hearing impairment so that improved control efforts could be implemented.

TABLE I

AGE DISTRIBUTION BY RANK AND ETHNIC GROUP

AGE (IN YEARS)	PERCENT DISTRIBUTION				
	W.O.	NW.O.	W.E.	NW.E.	TOTALS
∠35	17.2	10.5	0.4	0.4	5.1
35-	10.5	5.3	24.2	19.3	19.6
40-	38.4	26.3	44.5	53.1	43.9
45-	17.2	26.3	14.4	12.8	15.1
50-	12.0	31.6	11.3	9.9	11.5
55 or more	4.6	-	5.2	4.1	4.8
Unknown	-	-	-	0.4	0.1
Total	100.0	100.0	100.0	100.0	100.1
	458	19	976	243	1696

TABLE 2

LENGTH OF SERVICE DISTRIBUTION (LOS) BY RANK AND ETHNIC GROUP

LOS (IN YEARS)	PERCENT DISTRIBUTION W.O.	NW.O.	WE.	NWE	TOTALS
20	22.5	15.8	5.1	8.2	10.4
20-	27.3	26.3	47.2	50.2	42.0
22-	12.0	5.3	17.2	9.9	14.6
24-	8.7	5.3	8.1	9.5	8.4
26-	7.2	10.5	8.6	6.2	7.8
28 or more	18.8	26.3	10.3	9.5	12.7
Unknown	3.5	10.5	3.5	6.6	4.0
Totals	100.0	100.0	100.0	100.1	99.9
Number	458	19	976	243	1696

TABLE 3

PERCENT WITH IMPAIRED HEARING BY AGE FOR RANK AND ETHNIC GROUPS

AGE (IN YEARS)	PERCENT IMPAIRED				
	WO.	NW.O.	W.E.	NWE	TOTAL
<35	8.9	50.0	25.0	0.0	10.5
35-	16.7	100.0	40.7	25.5	35.2
40-	32.6	40.0	36.6	22.5	33.3
45-	35.4	20.0	37.6	22.6	34.8
50-	36.4	0.0	48.2	12.5	39.0
55-	38.1	-	43.1	30.0	40.2
Unknown	-	-	-	0.0	0.0
Total	28.2	26.3	39.3	22.2	33.7
# Impaired	129	5	384	54	572
Total #	458	19	976	243	1696

TABLE 4

PERCENT WITH IMPAIRED HEARING BY LENGTH OF SERVICE (LOS) FOR RANK AND ETHNIC GROUPS

LOS (IN YEARS)	W.O.	NW.O.	WE.	NWE	TOTAL
< 20	8.7	66.7	32.0	35.0	19.3
20-	27.2	20.0	40.8	25.0	35.2
22-	32.7	0.0	36.9	25.0	34.7
24-	40.0	0.0	38.0	21.7	35.7
26-	33.3	50.0	45.8	0.0	37.6
28 or more	44.2	0 0	36.6	21.7	37.2
Unknown	18.8	50.0	38.2	18.8	29.4
Total	28.2	26.3	39.3	22.2	33.7
#	458	19	976	243	1696

PERCENT WITH HEARING THRESHOLD LEVELS WITHIN SPECIFIED RANGES AT 500, 1000, 2000 AND 4000 H2 FOR RANK AND ETHNIC GROUPS

TABLE 5a

TOTAL					
500	+5 or less	+6-	+1[illegible]-	+26-	+36-
WO	44.5	35.6	12 2	3.9	0.9
NWO	42.1	42.1	10.5	5.3	-
WE	32.1	3[illegible].7	17.4	8.3	2.4
NWE	32.5	[illegible]6.1	11.5	6.2	2.0
1000					
WO	52.0	34.5	8.5	2.4	0.9
NWO	47.4	47.4	5.3	-	-
WE	41.0	37.1	11.5	4.5	1.8
NWE	39.5	44.0	9.0	4.1	1.2
2000					
WO	50.2	30.6	10.0	3.7	1.5
NWO	63.2	15.8	5.3	-	5.3
WE	40.3	30.0	11.7	5.8	5.0
NWE	39.5	36.6	11.9	6.6	2.0
4000					
WO	23.1	24.0	15.9	11.1	7.6
NWO	21.0	31.6	15.8	10.5	-
WE	16.1	19.4	14 2	11.6	9.1
NWE	17.3	27.6	[illegible]1.8	10.3	6.6

PERCENT WITH HEARING THRESHOLD LEVELS WITHIN SPECIFIED RANGES AT 500, 1000, 2000 AND 4000 H2 FOR RANK AND ETHNIC GROUPS

TABLE 5b

TOTAL

500	+46-	+56-	+66-	+76 or more	UNK	TOTAL #
WO	1.5	-	-	0.2	1.1	458
NWO	-	-	-	-	-	19
WE	1.3	0.7	0.7	0.3	0.9	976
NWE	0.8	0.4	0.4	-	-	243
1000						
WO	0.9	-	-	0.2	0.6	458
NWO	-	-	-	-	-	19
WE	1.2	1.2	0.6	0.2	0.8	976
NWE	0.4	0.8	0.4	-	0.4	243
2000						
WO	1.1	0.6	0.2	0.2	1.7	458
NWO	5.3	5.3	-	-	-	19
WE	2.4	2.0	0.8	0.8	1.1	976
NWE	0.4	0.4	0.4	0.4	1.6	243
4000						
WO	5.3	5.3	3.9	3.5	0.6	458
NWO	10.5	5.2	5.2	-	-	19
WE	7.6	6.6	5.7	8.2	1.5	976
NWE	7.4	2.5	2.9	2.9	0.8	243

PERCENT WITH HEARING THRESHOLDS OF 36 DECIBELS OR MORE AT 4 PURE TONE FREQUENCIES (500, 1000, 2000 + 4000 Hz) BY AGE AMONG 4 RANK AND ETHNIC GROUPS

TABLE 6

Group	Pure Tone Frequency	AGE (IN YEARS) 35	35-	40-	45-	50	55+	TOTALS
White Officers	500	0	0	2.8	2.5	7.3	4.8	2.6
	1000	0	0	1.7	5.0	3.6	0	2.0
	2000	1.3	2.1	5.1	5.0	3.6	0	3.7
	4000	6.3	10.4	22.7	30.4	40.0	52.4	25.1
Nonwhite Officers	500	0	0	0	0	0	0	0
	1000	0	0	0	0	0	0	0
	2000	0	0	20.0	20.0	16.7	-	15.8
	4000	0	0	40.0	20.0	16.7	-	21.0
White Enlisted	500	0	6.8	4.4	9.2	4.5	2.0	5.5
	1000	0	4.6	4.6	8.5	5.4	2.0	5.1
	2000	0	10.1	9.9	14.2	11.8	15.7	11.0
	4000	0	37.3	31.5	46.8	44.5	45.1	37.2
Nonwhite Enlisted	500	0	0	3.1	9.7	0	20.0	3.7
	1000	0	0	3.9	6.4	0	0	2.9
	2000	0	0	3.9	9.7	0	10.0	3.7
	4000	0	14.9	22.5	32.2	25.0	20.0	22.2

PERCENT WITH HEARING THRESHOLDS OF 36 DECIBELS OR MORE
AT 4 PURE TONE FREQUENCIES (500, 1000, 2000 + 4000 Hz) BY
LENGTH OF SERVICE AMONG 4 RANK AND ETHNIC GROUPS

TABLE 7

GROUP	Pure Tone Frequency	LENGTH OF SERVICE (YEARS) 20	20-	22-	24-	26-	28+	UNK	TOTAL
White Officers	500	1.0	2.4	0	5.0	3.0	5.8	0	2.6
	1000	1.0	1.6	0	0	3.0	5.8	0	2.0
	2000	2.0	4.8	1.8	10.0	3.0	3.5	0	3.7
	4000	8.7	17.6	30.9	37.5	36.4	43.0	18.8	25.1
Nonwhite Officers	500	0	0	0	0	0	0	0	0
	1000	0	0	0	0	0	0	0	0
	2000	0	20.0	0	0	50.0	20.0	0	15.8
	4000	0	20.0	0	0	50.0	20.0	50.0	21.0
White Enlisted	500	8.0	5.6	4.2	6.3	8.4	3.0	5.9	5.5
	1000	2.0	4.1	6.0	6.3	10.8	3.0	8.8	5.1
	2000	12.0	10.4	10.1	12.6	18.1	8.9	8.8	11.0
	4000	28.0	38.0	33.3	25.6	51.8	42.6	32.4	37.2
White Enlisted	500	8.0	5.6	4.2	6.3	8.4	3.0	5.9	5.5
	1000	2.0	4.1	6.0	6.3	10.8	3.0	8.8	5.1
	2000	12.0	10.4	10.1	12.6	18.1	8.9	8.8	11.0
	4000	28.0	38.0	33.3	26.6	51.8	42.6	32.4	37.2
Nonwhite Enlisted	500	5.0	4.1	4.2	0	0	8.7	0	3.7
	1000	5.0	4.1	4.2	0	0	0	0	2.9
	2000	5.0	4.9	4.2	0	0	4.3	0	3.7
	4000	20.0	21.3	16.7	39.1	6.7	21.7	31.2	22.2

Project 3AO62110A806 MILITARY PREVENTIVE MEDICINE

Task 00, Military Preventive Medicine

Work Unit 032, Pilot study to determine hearing impairment among separating Army personnel

Literature Cited.

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PROJECT 3A062110A811
MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00
Tropical and Subtropical Military Medical Research

741-a

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	SUMMARY CONTROL SYMBOL
DA 0B 6465	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. LANG/CNTRY	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM	9. WORK UNIT
71 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO		

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	044
b. CONTRIBUTING				
c. [illegible]	CDOG 114(f)			

11. TITLE (Precede with security classification code)

(U) Virus Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 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: DA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE		g. PROFESSIONAL MAN YRS	h. FUNDS (In thousands)
FISCAL YEAR	PREVIOUS		
	72	9.0	405
	CURRENT		
	73	8.4	504

19a. RESPONSIBLE DOD ORGANIZATION

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

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

20. GENERAL USE

Foreign Intelligence Not Considered

20a. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO
ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Altstatt, COL L.B.
TELEPHONE: 984-4523
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Top, LTC F. H. Jr.
NAME: Grossman, LTC R. A. DA

21. KEYWORDS (Precede EACH with security classification code) (U)Infectious Diseases; (U)Epidemiology; (U)Virus Ecology; (U)Arbovirus; (U)Japanese Encephalitis; (U)Rabies Virus; (U)Hepatitis-Associated Antigen

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

23. (U) To define the ecology of viruses of military importance in Southeast Asia thus providing a rational basis for decisions which involve association with or control of that or a similar virus ecosystem.

24. (U) Component parts of the natural viral ecosystem (e.g. vectors, hosts, reservoirs and variables which affect these component parts (e.g. rainfall, topography, immunity) are identified and quantified through the disciplines of clinical medicine, medical entomology, epidemiology, veterinary medicine and virology.

25. (U) 71 07 - 72 06 An epidemic of Japanese encephalitis (JE) occurred in the Chiengmai valley again in 1971. Laboratory transmission studies incriminated Culex fuscocephala as a vector and the pig as an amplifying host of JEV in Thailand; dogs, cattle, and buffalo were unlikely amplifying hosts. JEV transmission was documented in the valley year round except in Jan and Feb 1971. HI tests using isolated IgM fractions of human serum improved serologic specificity and permitted diagnosis of JEV infections in Chiengmai and USARV encephalitis patients with secondary Group B arbovirus infections. Immunologic consumption of serum complement proteins was found in patients with dengue shock syndrome, which suggests that anaphylatoxins formed in vivo could mediate shock. Influenza A strains,antigenically similar to the 1968 Hong Kong strain, were isolated in Korat. Point prevalence studies of Hepatitis B antigen (HBAg) in Thai populations and a study of the consequences of transfusion of blood containing HBAg in Thai patients were undertaken. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

25. (Available to contractors upon preparer'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 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 044, Virus disease of man and animals

Investigators.

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Associate: Ananda Nisalak, M.D.; Anong Pariyanonda, M.S.; Chaninthorn Suvongse, M.D.; Charles L. Bailey, CPT, MSC; Michael W. Benenson, CPT MC; Boonchob Pongpanich, M.D.; Cholticha Kraivapan, M.S.; Debhanom Muangman, M.D.; Frank J. Dixon, M.D.; Dumrong Chiewsilp, M.D.; Hans J. Muller-Eberhard, M.D.; Howard B. Emery, M.D.; Marvin H. Firestone, MAJ MC; Dennis O. Johnsen, MAJ VC; Kwanyuen Lawhaswasdi, D.V.M.; Joe T. Marshall, Jr., Ph.D.; Natth Bhamarapravati, M.D.; Pairatana Gunakasem, M.D.; Pien Chiewanich, M.D.; Prasit Futrakul, M.D.; Pricha Singharaj, M.D.; Rapin Snitbhan, M.D.; John B. Russ, SFC; Philip K. Russell, COL, MC; Satit Sirising, D.D.S., Ph.D.; Thomas J. Smith (Deceased) COL MC; Sombodhi Bukkhavesa, M.D.; Stitaya Sirisingha, D.D.S., Ph.D.; Suchinda Uaomsakdi, M.D.; Suchitra Nimmanitya, M.D.; Suntaree Pantuwatana, R.N.; Michael J. Sullivan, CPT MSC; Robert M. Suskind, M.D.; Varanya Sangpethsong, M.S.; Milton Willhight, SFC; James E. Williams, CPT MSC; Ronald G. Wilson, M.D.

Assistant: Anan Boonkanoke, Aree Boriharnvanakett, Attaya Boonyakanist, Bunterng Dechjun, Chalam Chantrasri, Chariya Hussen, Chondun Sapavuthi, Choompun Chavacharti, Chumnong Noigamol, Inkam Inlao, Jiraporn Supavadee, Kol Mongkol Panya, Larp Punthusiri, Ming Choohong, Morakot Tunticharoen, Nantana Eikarat, Naowayubol Nutkumhang, Nathada Playooth, Nonglak Khananurak, Nongnard Sahasakmontri, Orheh Tonghee, Pucharee Nawarat, Panor Srisongkram, Phuangthong Phiungkeo, Pranom Vangnai, Prasertsri Rohitarathana, Rampa Rattanaarithikul, Ravithat Putvatana, Roypim Tiptanatoranin, Samarn Maniwongse, Somboon Maneechai, Somchai Siripatananukulchai, Sorasak Imvitaya, Suleela Seemachiboorn, Sumitda Narupiti, Supatra Chulachambok, Suthida Phongngarm, Suwanna Vithanomsat, Vandee Nongngork, Vichit Phunkitchar, Vipa Phirwuthi, Yeepu Keokarn, Yupadee Vanichakointhanes, Yuphaphga Chillanon.

The Ecology of Japanese Encephalitis Virus Infections in Chiangmai

Principal Investigators: Richard A. Grossman, LTC MC
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Associate Investigators: Debhanom Muangman, MD
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Objective: To investigate the ecology of Japanese encephalitis virus (JEV) in the Chiangmai Valley, Northern Thailand, with particular reference to aspects contributory to infection in humans.

Description: The previous two Annual Reports explained the study methodology and results of work in Chiangmai Valley from November, 1969 to March, 1971. In summary, JEV was found to be widely disseminated throughout the Valley, causing infections in pigs and man in at least 9 consecutive months (April-December, 1970). Rapid buildup in virus transmission occurred in April at the start of the rainy season and most of the human and pig infections occurred in May, June and July; 84 of the 100 encephalitis cases for 1970 occurred in these 3 months. JEV was isolated from 3 Culex species - C. tritaeniorhynchus, C. gelidus and fuscocephala and all 3 species are believed to be acting as JEV vectors. Field and laboratory evidence suggested that small wild vertebrates and domestic animals (other than pigs) are not important variables of JEV transmission.

The specific areas of interest and their objectives in work that was pursued in 1971 include the following:

(1) Epidemiology: Case ascertainment at the 3 Valley hospitals was continued throughout 1971 as was collection of climatologic data. The sentinel pig study was continued through June, 1971, to both determine whether JEV transmission to pigs occurred in the dry, winter months and to see if a similar buildup in transmission occurred in 1971

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after the start of the rainy season. The 4 village study areas were re-visited once, in November, 1971; censusing of humans and animals was again performed and blood was obtained from the people in the random sample cohorts (selected in November, 1969) who were still remaining. Sera were also obtained from the Chiangmai City school cohort.

(2) Entomology: Studies on the ecology of the vectors of JEV in Chiangmai Valley were continued during 1971. Emphasis was directed at measurement of fluctuations in population density and physiological age, determination of host preferences, and flight range and dispersal characteristics. CDC light-trap collections were made twice weekly at three sites -- villages A and B and in Chiangmai city (E). These sites are located in the northern, southern and central portions, respectively, of Chiangmai Valley. Material from light-trap collections was used for computing monthly indices of female mosquito densities as well as for determinations of physiological age and host preferences.

(3) Virology: Studies on the role of small vertebrates and domestic animals as hosts of JEV in the Chiangmai Valley continued. The role of Culex fuscocephala as a vector of JEV was examined. The epidemiology of a Simbu group arbovirus, Ingwavuma virus, isolated in the Chiangmai Valley in 1970, was explored. Further studies on the specificity of IgM antibody to JEV and dengue viruses as a means of serologic diagnosis of specific Group B arbovirus infections were undertaken. These are detailed in subsequent sections of this Annual Report.

Progress: Encephalitis occurred in 1971 in Chiangmai Valley at a remarkably similar incidence to that of 1970 (Table 1). The 99 Valley cases were admitted in the 10 consecutive months, from February to November. As in 1970, over 75% of the cases were admitted in May, June and July although the peak month (July) was both sharper and a month later in 1971. This may reflect the slower onset of rainfall in 1971. Paired sera were obtained from patients in McCormick Hospital only, but 81% of the adequately spaced serum pairs had diagnostic JEV titer rises, thus providing similar confidence in the 1971 case incidence data. Further evidence of both the widespread distribution of JEV in the Valley and the surprising agreement between the 2 years is shown in Table 2. Once again, cases occurred in each Valley district and at comparable incidence rates. One JEV isolate was obtained from the brain of a 9 year-old female admitted on 10 July 1971. Seventeen cases died in 1971, compared to 20 in 1970, and the case fatality rate was especially high (50%) under age 5 (Table 3). Both the age and sex distributions of the cases were similar in both years, although the 1971

cases were slightly younger on the average and had a smaller male preponderance. The type of HAI serologic response in the confirmed cases was also similar in both years, 72% in 1970 and 75% in 1971 having a primary response.

None of the sentinel pigs in the 4 study villages had a JEV infection in January or February, 1971 (Table 4) although this does not rule out the possibility that low-level transmission was occurring. Pig conversions occurred in one village (B) between 9-31 March, in 2 villages in April, in 3 villages in May and in all 4 villages in June when all but one susceptible pig had a JEV infection. It is attractive to believe that the very high dissemination of JEV to pigs occurring in June is directly related to the sharp peak of human cases that occurred in July (the median human case was admitted on 10 July).

One possibility for dry season maintenance of JEV appeared to exist in low level transmission among the pig population. This possibility seemed very likely because serological evidence collected during 1970 showed that there were JEV susceptible pigs present all year along with JEV vector mosquitoes. In support of this theory, one JEV isolate was obtained from a group of 25 pigs near Udorn, Thailand (see below) in December, 1972. Accordingly in January, 1972, blood samples taken from 479 pigs at the time they were butchered in Chiangmai and were inoculated intraperitoneally into weanling mice. Deaths before 14 days occurred in about 7% of the mice but viral isolates, none of them resembling JEV, were made in only three cases. Accordingly, there continues to be only indirect evidence supporting the maintenance of JEV through low level pig transmission during the dry season.

At the time the blood samples were collected from slaughter pigs statistical information was collected concerning the number of pigs butchered and their origin. Such information was thought to be of potential usefulness as an indicator of changes in pig usage in Chiangmai that might, in turn, be related to the incidence of human JEV infections. Between 1961 and 1971 the number of pigs slaughtered annually varied between 28,000 to 46,000 (figures taken from official Chiangmai Municipal Slaughterhouse records); 39,000 pigs were slaughtered in both 1970 and 1971. Pigs are usually slaughtered when they reach an acceptable size rather than age; this size is probably in excess of 100 kg. Smaller pigs are butchered but in significantly fewer numbers; they often are animals in poor health that have grown poorly. Ages of slaughter pigs usually vary from 7 months of more than a year, although a low percentage are older salvaged breeders. Younger pigs that reach slaughter size early have probably been fed a better ration. The greatest source of slaughter pigs for Chiangmai is Pitsanuloke, but animals are also purchased for

slaughter anywhere within a 200 km radius of Chiangmai and trucked in every day. Although the Chiangmai slaughterhouse is the only "official" one in the area, the animals killed there do not reflect the level of swine production in the valley because, in addition to a large number of imported pigs, there are many illegal slaughterhouses operating in the valley. It therefore would seem that slaughterhouse statistics would be of very little use in helping to interpret local epidemiologic data in the Chiangmai Valley.

There was very little population change in the study villages' sample cohorts (Table 5). Only 7 people moved away and one person died between November 1970 and November 1971. Overall there was only a 2% decrease in the residents of the random sample houses over the 2 years of observation (446 to 436) and a loss of 56 people (12.6%) from the original random sample cohort. A total of 286 people in the 4 villages who were present and had been under serologic surveillance over the 2 years submitted another serum specimen in November 1971 (about 93% participation). Blood was also obtained from 125 of the Chiangmai City schoolchildren (study area E) still at school. The JEV and dengue prevalence data are presented in Table 6 for the 3 post-epidemic specimens in November of 1969, 1970 and 1971. In those people not experiencing a group B infection, HI titers persisted well over the 2 years of observation and very few JEV titers reverted to negative despite the low proportion of infections that occurred in 3 of the 4 villages.

Two people each in Villages A, B and D had HI titer changes between 1970 and 1971 consistent with a JEV infection (2.8%). If more specimens had been collected during the year, it is probable that several more infections would have been found (only 60% of the 1969-1970 JEV infections would have been detectable if only those November sera had been collected). In Village C, a striking event occurred, as 28 villagers (39.4%) had a titer rise to one or more of the group B antigens employed (Table 7). For 16 of these infections, broadly cross-reactive titers to both JEV and dengue rose to $\geq$1:640, in all of which there was pre-existing antibody; i.e., typical secondary responses. Two people had obvious primary JEV infections with monospecific antibody rises to JEV antigen. There were no primary dengue responses. In 20 of the 28 infections the post-infection JEV titer was equal to or higher than the highest of the 4 dengue titers. None of these 28 people were hospitalized during the year. The overall sample titer changes are presented in Table 8.

Since dengue hemorrhagic fever (which was not diagnosed in 1970) did occur in Chiangmai in 1971, it is possible that this high infection incidence reflects both JEV and dengue infections having occurred in the same village

at the same general time, but it is impossible to verify this. The most unique feature of these infections is their occurrence at about the same rate in all ages from 1-39 (Table 7). This is not typical of either JEV or dengue infections. Of course, the possibility also exists that another closely related group B virus is involved. Village D, which had a similar background prevalence of JEV and dengue, and is even closer to Chiangmai City than Village C did not experience a similar phenomenon. The schoolchildren in the City (E), however, also experienced a high infection rate (17.8%). As in Village C, the large majority of these infections were secondary and the post-infection JEV titers were mostly equal to or higher than the highest dengue titer. Although there was no clear primary JEV infection, there was one monospecific primary dengue 3 titer rise.

The first rains of 1971 began in March and, as observed during 1970, were followed by a steady rise in population densities of the three vector species at village A and Chiangmai City (Fig. 1). There was an unexplained decline in the C. fuscocephala and C. tritaeniorhynchus populations in village B during April, but densities of both populations at that site rose in the following months. Peak densities for C. fuscocephala and C. tritaeniorhynchus were measured during June in Village A and Chiangmai City, while in Village B the peak for these two species occurred in July. As in 1970 the fluctuations in populations of C. fuscocephala and C. tritaeniorhynchus during 1971 were closely parallel. The patterns observed for C. gelidus populations in Villages A and B during 1971 were almost identical to those seen in 1970. In Village A the numbers of C. gelidus rose in May to reach a peak in July and declined rapidly thereafter, while in village B the C. gelidus population increased during May and June and remained elevated through September. The numbers of all three species dropped off sharply in the dry season (November-March), but the most pronounced decline occurred in the C. gelidus population. Breeding continued during the dry season, although at greatly reduced levels; newly emerged, gravid and old females were collected throughout this period.

From April through July mosquitoes collected in CDC light-traps and resting outdoors during daylight hours were compared with respect to sex-ratio and the physiologic age and insemination rates in females. Resting mosquitoes were collected from vegetation in the study sites by means of a battery-powered vacuum collector. Males and females were present in almost equal proportions in the samples from the resting populations, while the light-trap collections consisted almost entirely of females. Since the light-traps were set near animal shelters the higher proportions of females in these collections was not surprising. Several other important differences were observed in the mosquitoes obtained by the two collection methods (Tables 9 & 10). There was a larger proportion of newly

emerged females in the resting population, as indicated by the higher nulliparous rates and lower insemination rates (Table 9). On the other hand, a higher proportion of gravid females was also present in the resting population (Table 10). Between July 1971 and March 1972 ovaries of vector species were examined in an effort to determine if seasonal variations in the number of gonotrophic cycles could be detected (Table 11). For most mosquito species, the number of gonotrophic cycles a female mosquito has undergone is directly related to the number of blood meals she has taken. Ovarian dissections are thus useful in estimating the proportion of older females (i.e., theoretically, those which have had greatest opportunity to become infected) present in the population. A decline in the proportion of parous females was observed for all three species between January and March. The proportions of multiparous C. fuscocephala and C. tritaeniorhynchus were higher during July--the month of highest incidence of encephalitis cases--than during the next four months. The largest proportions of multiparous C. gelidus were observed in September and in December. This is of especial interest because in 1970 the only isolations of JEV obtained from C. gelidus were made during September, although greater numbers had been tested during previous months.

During July and August, flight range and dispersal experiments were conducted with the three vector species. Blood-fed females were collected from buffalo, cattle, and pigs, marked with Helecon luminescent pigments and released. A total of 8,832 mosquitoes, of which approximately 53% were C. fuscocephala, 20% C. tritaeniorhynchus 9% C. gelidus, and 18% other species, was released. Four marked C. fuscocephala and two C. tritaeniorhynchus were recaptured. Five of the recaptured were made within three days of the time of release at distances varying from 10 to 500 meters from the release point. One C. fuscocephala was recaptured nine days after release but only 50 meters from the release site.

Table 1

Monthly Rainfall and Encephalitis Admissions from Chiangmai Valley in 1970 and 1971

Month	Rainfall(cm)		No. Cases	
	1970	1971	1970	1971
Jan	0.0	0.0	0	0
Feb	0.2	0.0	0	1
Mar	9.2	1.7	0	2
Apr	7.0	3.3	1*	1
May	35.2	24.0	23	16
Jun	24.5	17.3	38	14
Jul	17.9	30.0	22	47
Aug	34.9	32.5	3	12
Sep	19.3	19.5	5	4
Oct	3.8	13.4	4	1
Nov	0.7	2.4	3	1
Dec	3.5	1.2	2	0
TOTAL	156.2	145.3	101	99

* Case not included elsewhere as valley address not verified.

Table 2

Incidence Rates of Encephalitis Cases in Chiangmai Valley, by District, for 1970 and 1971

District	Estimated Population	No. Cases		Incidence Rate per 100,000	
		1970	1971	1970	1971
Muang	120,000	12	17	10.0	14.2
Maerim	25,000	5	7	20.0	28.0
Sansai	45,000	8	7	17.8	15.6
Doi Saket	40,000	9	5	22.5	12.5
Sankamphaeng	50,000	7	7	14.0	14.0
Saraphi	60,000	7	8	11.7	13.3
Hang Dong	40,000	8	13	20.0	32.5
Sanpatong	80,000	10	11	12.5	13.8
Jomthong	20,000	7	7	35.0	35.0
Muang*	125,000	18	9	14.4	7.2
Pasang*	75,000	9	8	12.0	10.7
TOTAL	680,000	100	99	14.7	14.6

* Lamphun Province.

Table 3

Age and Sex Distributions of 1970 and 1971
Encephalitis Admissions and Fatalities

Age	Male				Female				Total					
	No. Cases		No. Died		No. Cases		No. Died		No. Cases		No. Died		CFR*	
	1970	1971	1970	1971	1970	1971	1970	1971	1970	1971	1970	1971	1970	1971
0-4	9	8	1	3	4	6	1	4	13	14	2	7	15.4	50.0
5-9	18	24	4	4	12	14	3	2	30	38	7	6	23.3	15.8
10-14	16	17	4	0	11	14	3	0	27	31	7	0	25.9	0.0
15-19	9	4	0	2	5	2	0	0	14	6	0	2	0.0	33.3
20-29	9	2	1	0	1	3	1	0	10	5	2	0	20.0	0.0
30-	3	2	1	1	3	3	1	1	6	5	2	2	33.3	40.0
TOTAL	64	57	11	10	36	42	9	7	100	99	20	17	20.0	17.2
Median	11.6	9.3	10.6	7.5	10.9	10.4	10.8	4.4	11.3	9.7	10.7	6.2	-	-

* Case Fatality Rate(%).

Table 4

JEV Infections in Sentinel Pigs, January - June 1971

Dates Between Bleedings	No. Pigs at Risk	JEV Infections		JEV Infections by Study Area (%)			
		No.	%	A	B	C	D
1-26 Jan	18	0	0.0	0.0	0.0	0.0	0.0
27 Jan - 8 Mar	28	0	0.0	0.0	0.0	0.0	0.0
9-31 Mar	18	2	11.1	0.0	33.3	0.0	0.0
1-26 Apr	13	3	23.1	0.0	66.7	0.0	20.0
27 Apr - 26 May	22	8	36.4	0.0	40.0	16.7	83.3
27 May - 29 Jun	15	14	93.3	100.0	66.7	100.0	100.0
TOTAL	114	27	23.7	21.4	28.6	21.4	23.3
				N=28	N=28	N=28	N=30

Table 5

Population Changes in Random Sample Households of Chiangmai Valley Study Villages, Nov 69 - Nov 71

Village	No. in Sample Houses		Pop. Change (%)	Losses		Additions	
	Nov69	Nov71		Moved Away	Died	Moved in	Born
A	89	93	+4.5	6	0	6	4
B	113	108	-4.4	18	0	8	5
C	112	117	+4.5	8	0	12	1
D	132	118	-10.6	27	2	5	5
TOTAL	446	436	-2.2	54	2	31	15

Table 6

Changes in Prevalence of JEV and Dengue HAI Antibodies* Over Two Years (Nov69 - Nov71)

Study Area	No. People Followed**	JEV Prevalence (%)			Dengue Prevalence (%)			Converting from Negative to Positive Nov70 - Nov71		% Reverting From Positive to Negative Nov70 - Nov71	
		Nov 69	Nov 70	Nov 71	Nov 69	Nov 70	Nov 71	JEV	DEN	JEV	DEN
A	69	71	74	71	19	19	17	0.0	4.3	2.9	5.8
B	63	38	32	35	19	16	16	4.8	3.2	1.6	3.2
C	71	68	72	90	77	75	83	18.3	9.8	0.0	1.4
D	83	69	65	63	61	51	53	4.8	3.6	4.8	1.2
A-D	286	62	62	65	46	41	44	7.0	4.9	2.4	2.8
E	125	78	77	85	94	96	98	10.4	3.2	2.4	1.6

* Titer ≥1:20.

** Ages 1-39 for B,C,D; everyone over age 1 for A; ages 6-8 for school E.

Table 7

Incidence of Group B Infections in Study Village (C) Between Nov70 - Nov71

Age	Male			Female			Total			People Lacking JEV and Dengue Antibody in Nov70	
	No. People	Titer Rises*		No. People	Titer Rises		No. People	Titer Rises			
		No.	%		No.	%		No.	%	No.	No. Infections
1-9	16	5	31.2	13	5	38.5	29	10	34.5	9	5
10-19	12	6	50.0	8	4	50.0	20	10	50.0	2	1
20-39	11	4	36.4	11	4	36.4	22	8	36.4	1	1
TOTAL	39	15	38.4	32	13	40.6	71	28	39.4	12	6

*$\geq$4-Fold rise in JEV and/or dengue antibody titer between Nov70 and Nov71.

Table 8

Geometric Mean Titers of JEV and Dengue HAI Antibodies, by age for Village(C)

Age	Geometric Mean Titer*					
	JEV			Dengue**		
	1969	1970	1971	1969	1970	1971
1-9	15	19	53	30	27	56
10-19	59	40	197	102	51	190
20-39	363	146	387	291	110	310
TOTAL	60	44	142	86	50	134

* Negative sera (≤1:10) called 1:5.
** Highest dengue 1-4 titer used.

Table 9. Proportions of nulliparous and inseminated females in resting and light-trap collections, Chiangmai - April - July 1971

Species	Resting			Light-trap		
	Total No. Dissected	Nulli-parous	Insemi-nated	Total No. Dissected	Nulli-parous	Insemi-nated
C. fuscocephala	761	.555	.652	1347	.366	.939
C. gelidus	140	.717	.384	714	.432	.947
C. tritaeniorynchus	422	.623	.551	1275	.328	.963

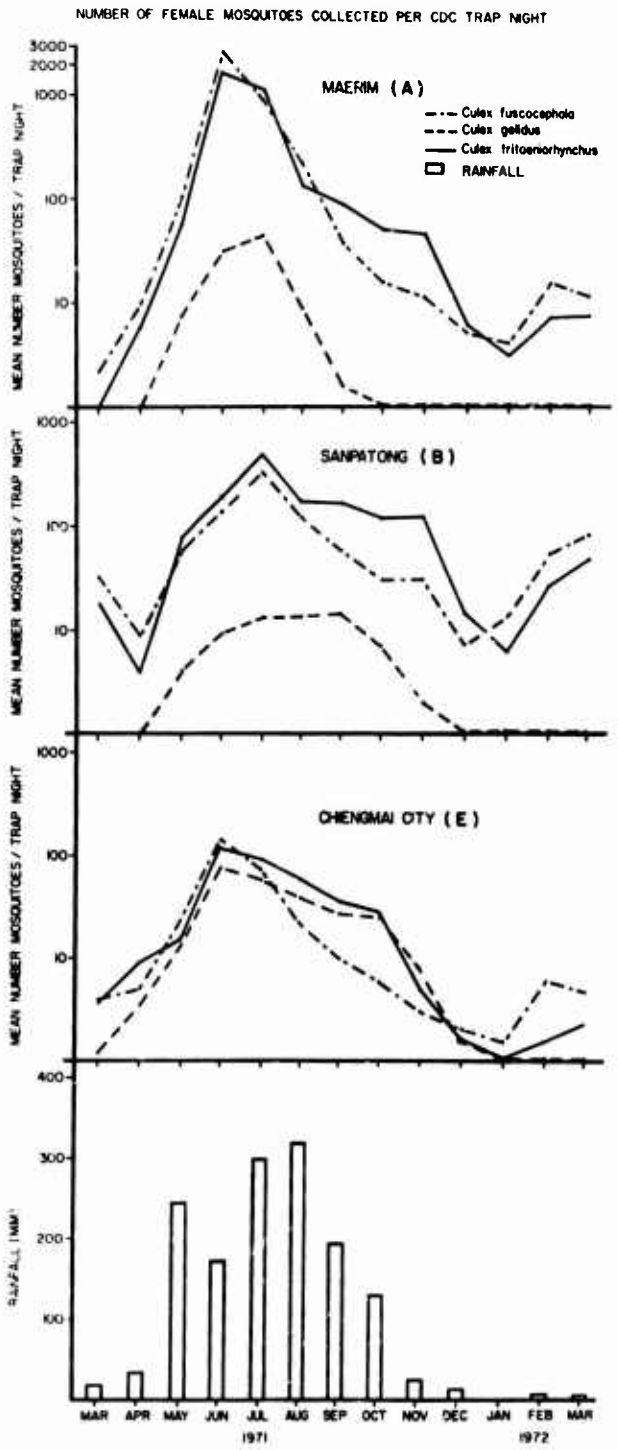
Table 10. Proportions of gravid females in resting and light-trap collections, Chiangmai-April-July 1971.

Species	Resting		Light-trap	
	Number Examined	Gravid	Number Examined	Gravid
C. fuscocephala	761	.364	16728	.083
C. gelidus	140	.244	2206	.124
C. tritaeniorhynchus	422	.176	18672	.037

Table 11. Proportions of parous, uniparous and multiparous Culex fuscocephala, gelidus and tritaeniorhynchus from light trap collections Chiangmai, July 1971-March 1972

Month	Total No. Dissected	Total Parous	Proportions uni- and multiparous: Recent Oviposition*	Uniparous	Multiparous
		C. fuscocephala			
July	379	.612	.017	.927	.056
August	0	-	-	-	-
September	129	.566	.068	.918	.014
October	127	.512	.138	.862	.000
November	241	.518	.114	.846	.040
December	495	.612	.076	.875	.049
January	842	.568	.077	.868	.055
February	1419	.428	.118	.837	.045
March	1048	.429	.077	.873	.050
		C. gelidus			
July	323	.502	.012	.907	.081
August	613	.574	.134	.847	.019
September	977	.481	.134	.743	.123
October	715	.508	.339	.634	.027
November	266	.436	.371	.586	.043
December	39	.487	.000	.895	.105
January	29	.483	.071	.929	.000
February	19	.315	.000	1.000	.000
March	24	.291	.142	.857	.001
		C. tritaeniorhynchus			
July	553	.653	.008	.898	.094
August	1909	.754	.134	.808	.058
September	2889	.577	.100	.854	.046
October	3327	.619	.129	.818	.053
November	2238	.613	.134	.829	.037
December	1304	.696	.095	.798	.107
January	426	.617	.091	.852	.057
February	805	.477	.101	.854	.045
March	784	.429	.112	.857	.031

* Females in which the number of gonotrophic cycles was not determined because ovarian tubules were stretched by recent oviposition.


NUMBER OF FEMALE MOSQUITOES COLLECTED PER CDC TRAP NIGHT
MEAN NUMBER MOSQUITOES / TRAP NIGHT
3000
2000
1000
100
10
MAERIM (A)
Culex fuscocephala
Culex gelidus
Culex tritaeniorhynchus
RAINFALL
1000
100
10
SANPATONG (B)
1000
100
10
CHIENGMAI CITY (E)
400
300
200
100
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
MAR
1971
1972

Title: A Study of Japanese Encephalitis Virus in Chiangmai Valley; The Role of Small Wild Vertebrates in Virus Transmission.

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Background: Several large field studies have provided evidence that wild birds participate in the maintenance and transmission of group B arboviruses in nature. For example Ardeid birds migrating to Japan have been implicated in re-introducing JEV into that country every year. Migrating birds probably play an important role in maintaining the transmission of St. Louis encephalitis virus in the Western Hemisphere. In Thailand Dr. Tom Yuill found that many species of birds netted in Bang Phra, Southeast Thailand, have neutralization antibody to JEV as measured in a metabolic inhibition test (SMRL Annual Report, 1968). For example 5 of 49 Passer montanus (house sparrow) had JEV neutralizing antibody in their serum, while 1 of 31 tested had antibody to Wesselsbron. Moreover Yuill inoculated 5 P. Montanus subcutaneously with JEV and measured JE viremia for 5 days in high titers of 10^5 infectious units/ml blood in one of the five brids. Other wild caught small vertebrates were also challenged with JEV, including birds (Bulbus and Pegu sparrows) mammals, (mice, roof rats, bats) and reptiles (lizards) but only the Pegu sparrow in addition to the house sparrow developed a demonstrable viremia.

The sum total of the previous work caused us to focus attention on birds, and in particular, the sparrow, as a possible important participant in JEV ecology in Chiangmai Valley of Northern Thailand. P. montanus is the most abundant bird in Chiangmai Valley, where it roosts and nests in large flocks in buildings and trees adjacent to houses and pig pens. This small bird does not migrate, and breeds nearly 10 months of the year, so that presumably virus susceptible and relatively immobile fledglings are being constantly introduced into the environment. Thus

if birds do in fact participate in an enzootic JEV cycle in Chiangmai, no bird species is as promising a host as P. montanus. We therefore elected to study this species intensively. Fortunately their low, active flight pattern made them easy to observe and to catch in mist nets.

Progress: A. Serological studies of P. montanus

In the 1970-1971 SMRL annual report we reported an evaluation of the micro culture plaque reduction neutralization test (micro-PRNT) using LLC-MK2 cell cultures. Statistical analysis revealed that the micro-PRNT was as precise and reproducible as the standard macro-PRNT. Both tests seemed more accurate measuring low-titered rather than high-titered antiserum. Representative results are shown in Table 1. These results indicated the feasibility of using the micro PRNT to measure the expected low-titered neutralizing activity of bird serum. The micro test offered the further advantage of requiring only 0.025 ml of serum per test, which was an important requirement, since in most instances no more than 0.1 ml of blood was obtained from each bird.

Birds were caught in mist nets raised in the 4 study villages, bled through jugular venipuncture, aged (immature or mature), banded, and released. Collected blood was diluted to 1.0 ml with balanced salt solution, and sent frozen to Bangkok where it was stored at -20°C.

The sera, diluted in the field to 1:10, and later in the laboratory to 1:20, were titered against JEV (Nakayama) without further dilution. The exact dilution giving 50% plaque reduction was calculated from a statistical table (supplied by the Japan National Institute of Health) which utilizes the percent plaque reduction found at the serum dilution tested in the micro-PRNT. If seronegative, each serum is expressed as <1:10, and if sero-positive, as ≥1:10. If the titer was ≥1:20 the sera was listed as positive twice, once in a ≥1:10 and again in a ≥1:20 dilution.

First a comparison was made of the percent positive serum in a large sample of immature (≤4-6 months old) and mature birds, irrespective of the date bled. Mature birds were netted in each of the 10 months of the study (June 1970 through November 1970; January 1971 through April 1971). Immature birds were sampled each month except January, February and April. These prevalence data are shown in Table 2.

Narly the same percentage of positive serum was found in mature and immature birds. Therefore it was not necessary to segregate serological data according to the age of the bird. Moreover, if specific antibody is being measured by the micro-PRNT, the data suggests that antibody is being acquired within the first 4 to 6 months after hatching,

with no serological evidence of additional JEV antigenic exposure over the next 4-5 years, the life span of a mature bird. However definitive interpretation of these results is hampered by a lack of information about the persistence of possible neutralizing antibody in birds, and the transmission of maternal antibody to offspring.

The monthly prevalence data listed in Table 3 shows that seropositive serum (≥1:10) was found in birds 9 out of the 10 months sampled. The percent seropositive serum seems to occur in a cyclic fashion characterized by 2 peaks, one peak occurring in the rainy season of July thru September, and the other in the dry season, January and February. The absolute serum titers were low; in the large majority of those few serum titering ≥1:20, the titers ranged between 1:20 and 1:40. No serum exceeded a titer of 1:80-1:100.

Seropositive birds were found in 3 of the 4 village study sites which included San Patong, Maerim, and Saraphi. Birds from Sankampaeng were seronegative, but the sample size was small, representing less than 5% of the total sera tested.

The serum obtained from 3 migratory birds, cocomantis merlinus (2) and cocomantis sonnerattii (1) (shrikes) were titered and found to be negative.

Of over 1000 birds caught, banded and released, 75 were recaptured 1 to 3 times over subsequent months. An attempt was made to demonstrate seroconversion to JEV in 71 of these birds. Fifty-nine were caught twice, 11 were netted 3 times and 1 bird was trapped 4 times. All were bled 0.1 ml after each capture and then released. The 2 or more serum samples obtained from each bird were titered by micro-PRNT at the same time.

We tested 151 sera obtained from the 71 birds. Thirty of these birds provided 42 sera which were seropositive at ≥1:10, 6 birds provided 10 sera which were also positive at ≥1:20. The percent seropositive at titers of ≥1:10 (27.8%) and ≥1:20 (6.6%) are higher than the percent positive sera obtained from single bleeding of 660 birds. Only 1 bird, an adult when first caught, converted from <1:10 in September 1970 to ≥1:20 in March 1971. The titer of the March 1971 serum in this bird was 1:25. The 29 other seropositive birds had titers that remained fixed (rise or fall of no more than 1 serum dilution) over 1 to 6 months. If we are indeed measuring antibody and regard seroconversion to JEV as a marker for JEV infection, then the birds sampled provide very little evidence of having been infected during the 10 month study period. This period covers part of the time when JEV was being actively transmitted to man and domestic animals in the villages.

Next we determined if the JEV neutralizing activity in P. montanus was specific for JEV. Serum from the January, February, March, and April bleeds (Table 3) were selected at random. Some sera were re-titered against JEV and others were run against Wesselsbron or dengue 4 virus; others were titered against 2 or 3 of the test viruses if enough serum was available. The results are shown in Table 4. It is apparent that a factor exists in bird serum that neutralizes Wesselsbron and dengue 4 virus. A striking 26.4% of serum neutralized dengue 4 at titers >1:10. A lower percentage of sera neutralized JEV and Wesselsbron. The titers against the 3 group B arboviruses were low, and no serum titer exceeded 1:80-1:100. In order to confirm the non-specific neutralizing activity of bird serum, we selected 31 sera still available from 14 JEV seropositive birds that had repeated bleedings. Each month of the study was represented in these sera, and both immature and mature birds of both sexes. Twelve of 14 birds were seropositive (≥1:10) against dengue 4, and 13 were seropositive against Wesselsbron. Two birds with fixed titers to JEV and Wesselsbron had titer rises to dengue 4. These results confirm the low titer non-specific cross-reactivity of P. montanus serum. Moreover, if we reasonably assume that dengue-4 does not infect birds, then titer rises to dengue-4 in 2 birds suggests that we are measuring either virus neutralizing factors other than antibody, or cross-reactive antibody raised against an infection by another group B arbovirus. A reasonable candidate virus would be Tembusu, a Group B arbovirus recovered in Chiangmai Valley and thought to infect birds. Attempts to plaque Tembusu for the micro-PRNT have been unsuccessful.

HAI tests on selected small wild vertebrate sera collected in Chiangmai.

A complete list of small wild vertebrates trapped and bled in Chiangmai during 1970-1971 was printed in the 1970-1971 SMRL Annual Report. A small sample including P. montanus sera, were selected for HAI testing against JEV, dengue 1-4, and Chikungunya HA antigens. The results, found in Table 5 reveal that many tree sparrow and lizard sera contained low-titered inhibitors against the 5 test group B arbovirus antigens; higher-titered inhibitors were found to Chikungunya. It is likely that these inhibitors represent acetone resistant non-specific inhibitors of HA rather than antibody; because of an inability to distinguish between these 2 alteratives, no further testing of small wild vertebrate serum was made by HAI.

B. Virus isolation attempts from Passer montanus

We attempted to isolate an infectious agent from 297 blood clots, and 136 spleens, and livers of P. montanus netted in January, February, and March. After removal from the bird, the tissues were frozen immediately

in sealed glass containers, and shipped in dry ice to Bangkok where they were stored at -90°C. The clot and organs were thawed, triturated in BAPS with mortar & pestle, centrifuged 10,000 RPM x 30 minutes, and the supernatent from the clot and organs inoculated separately IC into 6-8 suckling mice. In addition 855 Passer serum stored at -20°C and thawed inadvertently several times were injected into suckling mice. No infectious agents were isolated from the blood clots, organs, or serum.

C. Zootropism of Vector Mosquitoes for P. montanus.

The biting preference of proven mosquito vectors, C. gelidus, C. tritaeniorhynchus, C. fuscocephala for Passer montanus was examined by Drs. Joe Marshall and D.J. Gould. Sweep vaccuum collections were made near bird nests, and mosquito traps baited with live tree sparrows were placed in various habitats in order to attract vector mosquitoes. The data is summarized in Table 6. Two engorged C. gelidus were collected by sweep vacuum near bird roosts, but they did not contain ingested bird blood. No vector mosquitoes were attracted to the bird-baited traps placed in a variety of ecological sites in Chiangmai Valley. Thus we could not demonstrate attraction of JEV mosquito vectors to P. montanus. However these studies were carried out in the dry season when mosquito populations were at their nadir and therefore may not accurately reflect the biting pattern during the rainy season when vectors are abundant.

Summary of P. Montanus Study: The sum total of the serological, virological and entomological studies provides no convincing evidence that P. montanus is a maintaining or amplifying host for JEV in Chiangmai Valley. The JE virus neutralizing and HAI activity in bird serum was low-titered and cross-reactive with at least 2 other group B viruses; the neutralizing activity could represent a non-specific inhibitor of arboviruses rather than antibody. No viruses were isolated from many samples of bird tissues, and a biting preference of the 3 mosquito vectors for P. montanus could not be demonstrated.

D. Baby chickens as sentinels for JEV.

This study was the work of James E. Williams, CPT, MSC. He evaluated the suitability of baby chickens as a more convenient alternative to the pig as a sentinel for JEV in Chiangmai Valley. During the rainy season approximately 200 chicks, 3 days old, were bled, caged, and put into trees (4 feet above the ground) in the Chiangmai study villages for a period of 7 days. The chicks were bled several weeks later and their serum was titered by HAI and macro-culture PRNT. No neutralizing

antibody was found. A small number of sera were HAI positive to JEV, but converted to HAI negative (<1:20) following repeat acetone extraction. The results indicate that the vector mosquitoes were not infecting sentinel chicks.

E. Serological Study of Bats:

A small percentage of bats caught throughout the year in Japan circulate JEV in their blood, apparently store JEV in brown fat, and their serum neutralizes JEV in low titers. Bats are thus considered to be reservoir hosts for JEV in Japan. Accordingly, 54 bats collected in Chiangmai were examined for the presence of JEV neutralizing activity in their serum.

Five species of bats were collected in banana groves and roof roosts in the study villages during April, May & June, 1970. The species are Cynopterus sphinx, Rousettus leschenaulti, Epnycterus spelaea, Taphozous longimanus, and Scotophilus kuhlii. The first 3 species are fruit eaters, while the last 2 species are insectivorous. All but 7 of the 54 bats collected were fruit eaters. The bats were bled through cardiac picture and the serum shipped to SMRL. The sera was diluted 1:10 and 1:40 for the micro-PRNT against JEV, and diluted 1:10 and 1:20 for titration against dengue 4 and Wesselsbron. The absolute titers were calculated as they were for P. montanus sera; results are shown in Table 7. Of 54 sera tested, 26 (48%) were positive to JEV at a titer ≥1:10, and 14.8% & 9.3% of these sero positives titered ≥1:20 and ≥1:40, respectively. The percentage of JEV seropositive serum is almost 5 times higher for bats than P. montanus.

Bat sera did not neutralize Wesselsbron but some did neutralize dengue 4. However only 9 of 26 JEV positive (≥1:10) sera cross-reacted with dengue 4; and only 9 of 17 dengue 4 positive sera (≥1:10) neutralized JEV. Thus many sera reacted monospecifically with JEV or dengue 4. The large percentage of serum reacting specifically and at relatively high titers (≥1:40) with JEV, suggests that these bats may have been previously infected with JEV. The dengue 4 neutralizing factor most likely represents either cross-reactive antibody produced by a serologically related bat virus, or a non-specific serum inhibitor of dengue-4 plaque formation.

Viral isolation was attempted on 12 bats trapped at a large cave near Chiengdow, a town north of Chiangmai or at the field laboratory in Chiangmai City in January-February 1971. Six insectivorous bats, Scotophilus sp., were collected in the cave and 6 fruitivorous bats Cynocephalus sp from the field laboratory. Brain suspensions from the

bats were inoculated intracerebrally in suckling mice but no isolates were made. The sample tested was certainly not adequate to eliminate the involvement of bats in dry season maintenance of JEV; a more extensive survey is indicated if their role as a reservoir of JEV is to be definitely established.

Table 1. Comparison of the micro and macro PRN tests.

	Reciprocal of Antibody Titer			
	Micro-PRNT		Macro-PRNT	
JEV Antiserum	1st test	2nd test	1st test	2nd test
Monkey V141	55	40	80	28
Monkey V145	62	72	48	78
Rabbit 1	2560	1100	~10240	6400

Table 2. Serological* Results: Mature and Immature Passer montanus

		Serum titers vs. JEV	
Bird	No. Birds Tested	≥1:10 (%)	≥1:20 (%)
immature[x]	244	20 (8.2)	8 (3.3)
mature[xx]	346	36 (10.4)	10 (2.8)

* 50% plaque reduction by micro-culture PRNT
x less than 4 to 6 months old (pre-molt)
xx 6 months to 4-5 years.

Table 3. Monthly Prevalence of JEV Neutralizing Activity in Passer montanus serum.

Date Bled+	No. Birds	Serum Titer vs JEV			
		No. ≥1:10	%	No. ≥1:20	%
June 1970	43	0	0	0	0
July 1970	45*	5	11.1	4	8.9
August 1970	62xx	7	11.9	6	10.0
September 1970	72xxx	4	5.5	0	0
October 1970	46	1	2.2	0	0
November 1970	139	10	7.2	3	2.2
January 1971	95	19	20.0	6	6.3
February 1971	59	15	25.4	3	5.1
March 1971	62	3	4.8	0	0
April 1971	37	3	8.1	0	0
Total	660	67	10.2	22	3.3

+ No trapping done in December 1970 or May 1970 & 1971.
* 41 sera run by macro-plaque PRNT
xx 2 " " " " " "
xxx 27 " " " " " "

Table 4. Comparison of JEV, Wesselsbron and Dengue 4 Serum Neutralizing Titers in Mature Passer montanus

Month Bled	Serum Titer vs JEV		Wesselsbron		Dengue 4	
	≥1:10(%)	≥1:20(%)	≥1:10(%)	≥1:20(%)	≥1:10(%)	1:20(%)
Jan	15[x] (18.3)	5(6.1)	0(0)	0(0)	8(53)	2(13.3)
	82	82	15	15	15	15
Feb	14(24)	3(5.2)	8(16.6)	2(4.2)	12(25.5)	6(12.7)
	58	58	48	48	47	47
Mar[xx]	0(0)	0(0)	1(4.8)	0(0)	2(9.5)	0(0)
	50	50	21	21	21	21
April	2(5.4)	0(0)	2(11.1)	0(0)	5(27.8)	3(16.7)
	37	37	18	18	18	18
Total	26(11.5)	8(3.5)	11(10.8)	2 (2.0)	27(26.4)	11(10.9)
	227	227	102	102	101	101

(x) Total birds positive / Total birds tested

xx Includes 10 immature birds

Table 5. HAI Tests on Small Wild Vertebrate Sera, Chiengmai

Animal	No. Serum Tested	No. Sera HAI positive[x] to: D1	D2	D3	D4	JE	Chik*
1. Passer montanus (tree sparrow)	20	0	4[xx]	1	1	1	2
2. Calotes mystaccus (gingka lizard)	20	1	7[xxx]	1	3	2	5
3. Bandicota indica (bandicoot)	14	0	0	0	0	0	0
4. Rattus rattus (roof rat)	7	0	0	0	0	0	0

x titer $\geq$1:20

xx 4 of 4 sera titered 1:40

xxx 1 of 7 sera titered 1:40

* titers ranged from 1:20 to >1:160.

Table 6. Zootropism of Vector Mosquitoes: Bird Bait Trap and Sweep vacuum Mosquito Collections, Chiangmai

Location	Collection	Trap Nights	Mosq. Species	No. Mosq. Collected	No. Fed
1. Chiangmai City (Buildings)	Sweep-vac[x]	2(11-12 Feb 71)	C. quinquefasciatus	95	2
			M. uniformis	1	0
			E. luzonensis	1	0
			C. gelidus[xxx]	5	2[xxx]
2. Chiangmai City (Trees, roofs, porches)	Bait trap[xx]	7(12 Feb to 20 Mar) 29 traps/night	C. quinquefasciatus	95	89
3. Chiangmai Villages (Vegetation near houses cow & pig pens[xxxx])	Bait trap	8(14 Feb 71 to 25 Mar 71) 18 traps/night	C. quinquefasciatus	1	1
4. Chiangmai Valley (Marshes or rice fields[xxxx])	Bait trap	3(23-25 Mar 71) 7 traps/night	C. bitaeniorhynchus	17	11
			C. vishnui subgrp.	2	0
			C. nigropunctatus	2	0

x Collections made adjacent to P. montanus roosts & nests.
xx Each trap baited with 2-6 live P. montanus overnight.
xxx JEV vector; blood meal identified as bovine (1 mosq.) and non-reactive (1 mosq.) when tested against battery of antisera, including anti-bird (sparrow) serum.
xxxx preferred habitat for 3 vector mosquitoes species.

Table 7. Group B arbovirus Serology[x] in Bats: Chiangmai 1970.

Virus	Serum Titer ≥1:10 (%)	≥1:20 (%)	≥1:40 (%)
JEV	26[xx]/54 (48)	8/54 (15)	5/54 (9)
Wesselsbron	0/52 (0)	-	-
Dengue 4	17/48 (35)	3/51 (6)	0/51 (0)

x 50% plaque reduction by micro-culture PRNT

xx Total sera positive / Total sera tested

Zoonotic Aspects of JEV Infection outside the Chiangmai Valley

Principal Investigator: Dennis O. Johnsen, MAJ VC

Progress: Operators of several swine breeding farms have requested assistance from SMRL because sows in their herds seemed to be experiencing a large number of abortions, stillbirths, or pigs born to weak at birth to survive. These farms were located at diverse areas of Thailand. They were the Thai Supreme Command's Mobile Development Unit in Saiyok, Kanchanaburi Province, The Kasetsart University pig farm at Tubkwang, Saraburi Province, and a smaller catholic operated farm near the town of Nong Khai, Nong Khai Province in Northeastern Thailand. In addition to JEV, herds at these farms were checked for evidence of leptospirosis and brucellosis (reports of the bacteriologic studies are reported in the section on bacterial diseases of man and animals). A summary of the serologic results from these three areas are shown in Table 1. Although there was evidence of JEV infection at the MDU in Saiyok, it did not compare with that found at Tubkwang and Nong Khai. Virus isolation by intraperitoneal inoculation of whole blood into weanling mice was attempted at both Saiyok and Nong Khai; JEV was isolated in this manner from one yearling boar in the Nong Khai herd in December. From these findings it is apparent that JEV has a wide geographical distribution in Thailand. Further efforts to isolate JEV from sows and stillborn or aborted fetus and correlation to sequential serological examinations are required to establish with certainly that JEV is at least partly responsible for the problems swine producers in Thailand seem to be experiencing.

Table 1. JEV Serology Results of Pigs outside Chiangmai Valley.

	No. pigs examined	Median JE HI titer	Range
Saiyok	58	8.7	less than 1:10 to 1:2560
Tubkwang	171	125	less than 1:20 to 1:2560
Nong Khai	13	197	1:40 to 1:1280

Title: Experimental Infections of Thai Domestic Animals with Japanese Encephalitis Virus

Principal Investigators: Debhanom Muangman, M.D. Dr. P.H.
Dennis O. Johnsen, MAJ, VC
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Objective: The purpose of this investigation was to determine if Thai domestic dogs, water-buffalo, pigs and cattle may serve as zoonotic viral amplifers in the epidemiology of Japanese Encephalitis Virus (JEV) in Northern Thailand.

Background: Evidence that pigs are involved in the epidemiology of Japanese encephalitis has been established by studies performed in Japan. In contrast to Japan, the animal census in Chiangmai, Thailand has shown that the populations of other domestic species such as dog, water-buffalo and cattle often approach and in some cases exceed the pig population. Furthermore, pigs in Northern Thailand are born throughout the year, so there is not a larger population of susceptible pigs relative to other species present at the time of the outbreaks of Japanese encephalitis.

If transmission of Japanese encephalitis occurs when mosquitoes carry the virus from an infected animal to man, then several species of domestic animals in the Chiangmai area should be considered as being amplifying hosts in addition to the suspected pig. From data collected in 2 serological surveys of domestic animals in Chiangmai within the last year, water-buffalo, cattle, dogs, and horses all have a higher percentage of serum HI antibody to JEV than pigs. Each of these animal species produces a number of offspring that are probably susceptible to infection at the time the encephalitis season begins each year. Investigators from Japan isolated JEV from cow's blood (Otsuka S. et al., Virus 19 (6):336-339, 1969). However, in a previous study (Gould et al., SEATO Annual Progress Report, 1966 p. 42) conducted at this laboratory viremia was not detected in cattle following subcutaneous inoculation of JEV. Work by Carey (Ind. J. Med. Res., 56, 1968) confirmed Gould's finding. Other work conducted in Japan (Gresser et al., Jap. J. Exp. Med. V. 28, No. 4, 1958 p. 243-248) has shown that horses became viremic after being bitten by JE-infected mosquitoes, and this viremia was sufficient to infect feeding mosquitoes. However, horses are not kept in the villages of Chiangmai and were not studied here. To our knowledge, no investigations of JEV infections in dogs have been reported. Young

chickens experimentally inoculated with JEV apparently produce both antibody and viremia, but adult chickens bled in field studies including Chiangmai had no evidence of JEV infection.

In Chiangmai, 3 of 13 field isolates of JEV have been made from mosquitoes trapped while feeding on water-buffalo or cattle. Ten more JEV isolates were made from mosquitoes collected in light traps placed near bovines and pigs. Culex tritaeniorhynchus, a known JEV vector, and Culex fuscocephala, a potentially-important JEV vector, are present in Chiangmai area year round and preferentially feed upon the large domestic animals; they also feed upon man. The analyses of blood in the midguts of these wild-caught JE vector mosquitoes indicate their marked preference for bovine blood. The question of whether or not domestic animals other than the pig may act as important amplifying hosts for JEV must be resolved before sound methods for controlling JE epidemic disease can be formulated. Since dogs, water-buffalo, pigs and cattles are quite prevalent in the rural villages of northern Thailand, these animals were studied in the laboratory to determine their ability to develop viremia after inoculation with JEV. In addition, the serological responses of these controlled laboratory infections provide a basis for the interpretation of the HI serological patterns found in Chiangmai village animals, from whom no viruses were isolated. Representative HI antibody patterns from indigenous Chiangmai animals are shown in Table 1.

Experimental Animals: Two domestic water-buffalo (Babulus balbus); #B (female) and #D (male) approximately 11 months old, were obtained from Nakorn Pathom and Ang Thong Provinces, Thailand. These animals and those described below were housed in mosquito-proof rooms throughout the experiment. They received daily feeding and veterinary care.

Three domestic dogs (Canis familiaris); Dog #A (male) born at Vet. Med. Lab. was 11 months old; Dog #3 (male) and Dog #4 (female), 19 months old, came from Din-Daeng dog compound, Bangkok.

Two domestic pigs (Sus scrofa); Pig #226 (male), Pig #229 (female) were 2 1/2 months old when they were bought from Bang Kae District, a suburb of Bangkok.

Two cattle calves (Bos taurus) both males age 4-5 months old (#1 and #2), were born and raised at SMRL.

All of the above animals were selected because they were free of demonstrable HI and NT antibodies to JEV.

Viruses: JE virus, strain BKM-984-70, SM2, was originally isolated from Culex mosquitoes in Chiangmai. This virus strain was used in every experiment including JEV challenge. Tembusu virus, Strain BKM-4165-70, SM2, was also originally isolated from wild-caught mosquitoes in Chiangmai.

Mosquitoes: First generation (3-4 days old) laboratory-raised progeny of wild-caught Culex tritaeniorhynchus from Bangkhen District, a suburb of Bangkok, were infected by allowing them to feed on JE viremic baby chicks overnight. Each 1 day old chick had been inoculated subcutaneously with JEV (1700 PFU) 2 days previously. Blood-engorged mosquitoes were collected and maintained on 5% glucose and water for 12-13 days in the insectary at Entomology Department before transmission attempts were made. After each transmission attempt, fed mosquitoes were individually triturated and tested for virus in MK2 cell culture and suckling mice. Groups of uninfected Culex tritaeniorhynchus females were induced to feed on dogs and water buffalo on day 2, 3, 4 post infective mosquito feeding (PIMF). Engorged mosquitoes from this feeding were kept for 10 days and tested for virus as above. Attempts to transmit JEV to uninfected mosquitoes were not done in the pig and cattle experiments due to a shortage of mosquitoes.

Serological tests: Five ml of blood was drawn at various intervals after JEV challenges. The sera were tested for the presence of HI antibodies to group B arboviruses present in Thailand i.e. dengue 1-4, JEV, Tembusu, and Wesselsbron.

Virus Isolation Systems: LLC-MK2 cell culture (direct and delayed plaque method) and suckling mice were used. Serum, separated from the clotted blood, was inoculated into 2 bottles cultures of MK2 cells (0.3 ml/bottle). Heparinized whole blood was also drawn from each experimental animal at various intervals and immediately inoculated into 2 litters of 1-2 day old white, Swiss mice (8 suckling mice per litter, 0.02 ml of blood I.C./mouse). The inoculated mice were observed daily for sickness and death for a period of 21 days. Brains of sick mice were passed at least twice before the specimens were collected for virus identification. Neutralization tests were done on virus isolated from animal blood in order to confirm JEV viremia.

Progress:

Dog and Water Buffalo Study: Presumably-JE infected mosquitoes were allowed to feed on dogs and water-buffalo for 2 hours on each of 2 consecutive nights. Blood engorged mosquitoes were then collected, recorded, and individually tested for virus. Then blood was drawn from

each animal every 12 hours for 7 days and each blood specimen was tested for virus in MK-2 cell culture and suckling mice. The results are present in the first 2 columns of Table 2.

Data in Table 2 show that very low level viremia (3PFU/ml blood) was detected in Dog #A lasting less than 24 hours at 48th hour post-infective mosquito feeding. However, none of the 8 uninfected mosquitoes that fed on this dog at the time of viremia was later found to be infected. No viremia was detected in the other 2 dogs and 2 water-buffalo.

The experimental design and results of the serological studies are summarized in Tables 3,4,5.

Buffalo #B (Table 3) had only low titer serological responses to 3 consecutive inoculations with JEV. With a very high dose of JEV in the fourth challenge ($4x10^9$ PFU), HI titer to JEV rose only to 1:40 at day 7 with low titer cross-reactions to some other group B antigens. Buffalo #D (Table 4) had similar low and transient serological responses.

This weak and transient antibody response probably reflects a response to the large amount of antigen injected repeatedly rather than virus replication in vivo. The low HI titers and cross reactive antibodies in these experimentally challenged animals reproduce the serological patterns noted in animals bled in Chiangmai area (see Table 1). On this basis, it is likely that water buffalo in Chiangmai are repeatedly inoculated with JEV by vector mosquitoes. The meagre antibody responses, the absence of detectable viremia, and the inability to infect mosquito vectors support the conclusion that water-buffalo are not important amplifying hosts of JEV.

Dog #A (Table 5) which developed detectable viremia showed significant HI antibodies to JEV and dengue-4 from day 7 (PIMF) but it is impossible to tell whether antibody responses are due to infection by mosquito feeding or to JEV inoculation. Dog #3 shows similar heterologous responses to dengue 4 after mosquito and inoculation challenges. Dog #4 shows only low titered and transient antibody response after inoculation challenge. The serological patterns for the 3 dogs are similar to the serological patterns observed in Chiangmai dogs (Table 1).

JEV viremia was detected in 1 of 3 dogs bitten by JE infected mosquitoes but the viremia was brief, low titered and did not infect susceptible mosquito vectors. Therefore, it is less likely that dogs are as important JEV amplifying hosts as pigs reported below.

Pig Study: Experimental procedures employed were similar to the water-buffalo and dog experiment reported above, except that Tembusu virus rather than JEV was given as a second virus challenge to pigs initially challenged to JEV. Tembusu virus has been recovered from Chiangmai mosquito species that are known to bite pigs.

Following JEV challenge, pig #229 developed high-titered JE viremia for about 3 days (Table 2); pig #226 did not circulate detectable virus. No antibody response was detected in the non-viremic pig #226 (Table 6) but a high-titered broadly reactive antibody response occurred in the viremic pig #229, with highest titers to JEV. Following Tembusu challenge 44 days after JEV, pig #226 developed specific Tembusu antibody, while pig #229, previously sensitized to group B antigen, had an anamnestic heterospecific antibody response with highest titers to JEV. Because Tembusu viremia was not monitored, it is unknown whether Tembusu replicated in pigs. However, JEV seems to be capable of replicating vigorously in pigs.

The HI antibody patterns noted after both virus infections in Pig #229 are similar to those found in indigenous Chiangmai pigs (Table 2). On the other hand, no pig in Chiangmai had antibody patterns that resembled pig #226 after Tembusu infection. Thus there is no clear evidence that a group B arbovirus other than JEV infects pigs in Chiangmai.

Conclusions: 1) Of all the animals studied, the pig is the most likely candidate for a JEV amplifying host. It shows a vigorous antibody response indicative of virus replication in vivo and also circulates virus at high titer (10^2 PFU/ml blood) for at least 3 days.

2) Dogs are shown to be susceptible to JE infection. In contrast to the pig, their low titered HI antibody responses suggest a more limited virus replication in vivo which is also reflected by the low and transient viremia of less than 24 hours. Moreover, only 1 of 3 dogs was found to be viremic and none of the susceptible mosquitoes fed on this animal became infected. Thus, the dog would not be considered as a good amplifying host for JEV.

3) The water-buffalo seem to be poor hosts for JEV replication giving low serological responses only after repeated JEV challenges by high titred inoculums. In addition, viremia was not detected by frequent titration of the animal's blood for 7 days. None of the susceptible mosquitoes which fed on these animals became infected.

4) The serological patterns in these experimental animals resembling those noted in the Chiangmai valley for these animals provide

indirect evidence that JEV is infecting the indigenous animals in Chiangmai.

5) The preliminary results of the cattle experiment in progress shows no detectable viremia for 7 days after infective mosquito feeding. Cattle, like water-buffalo, thus appear not to be good amplyfying hosts for JEV.

Table 1. Representative HI Antibody Patterns. Indigenous Animal Sera, Chiangmai, 1970.

	Reciprocal of HI titer against						
Animal	D1	D2	D3	D4	JEV	TEMB	WESS
buffalo	10	10	10	20	80	40	20
cattle	0[1]	0	10	10	40	20	10
pig	160	160	320	640	2560	1280	1280
dog	10	20	40	320	640	160	40
horse	0	0	0	20	40	20	10
cat	0	0	0	10	80	10	0
chicken	10	20	20	40	160	640	80
duck	20	20	40	80	320	1280	80

(1) Titer 0 = <1:10; D1-4 = dengue 1-4, TEMB = Tembusu, Wess = Wesselsbron

Table 2. JEV Transmission Experiments in Dogs, Water-Buffalo, Pigs and Cattle

	Dog			Water buffalo		Pig		Cattle	
	#A	#3	#4	#B	#D	#226	#229	#1	#2
No. of presumably -JE infected mosquitoes that fed[1]	4	2	2	1	4	0	5	12	11
No. of fed mosquitoes found to be infected[2]	2	1	2	1	4	-	5	3	4
Viremia detected (hours after mosquitoes feedings)	48	-[3]	-	-	-	-	36,48, 60,72,84	-	-
Level of viremia detected (PFU/ml of blood)	3	-	-	-	-	-	approx. 100	-	-

(1) Presumably JE infected mosquitoes were 12 - 13 days post infectious blood meal

(2) Presumably-JE infected mosquitoes which became engorged after feeding on the above domestic animals were individually triturated and tested for virus immediately in MK2 cell culture and suckling mice (I.C.)

(3) No virus detected.

Table 3. HI Response of Thai Water-Buffalo #B to JEV Challenges

Days after JEV Challenge				Reciprocal of HI Titer against:						
1st	2nd	3rd	4th	D1	D2	D3	D4	JEV	Tembusu	Wess.
0[1]				0[5]	0	0	0	0	0	0
7	0[2]			0	0	0	0	0	0	0
	7			0	0	0	0	20	0	0
	14			0	0	0	0	20	10	0
	21			0	0	0	0	20	10	0
	38			0	0	0	0	10	0	0
	90	0[3]		0	0	0	0	0	0	0
		7		0	0	0	0	20	10	0
		21		0	0	0	0	20	10	0
		30	0[4]	0	0	0	0	10	10	0
			7	10	0	0	0	40	20	10
			28	10	0	0	10	20	20	10

(1) 1 JEV-infected mosquito fed on water-buffalo #B on day 0

(2) Subcutaneous (SC) inoculation with JEV (BKM-984-70, $6x10^5$ PFU).

(3) Sc. inoculation with JEV ($2.5x10^7$ PFU).

(4) Sc. inoculation with JEV ($1x10^9$ PFU at 4 sites).

(5) Titer 0 = <1:10

Table 4. HI Response of Thai Water Buffalo #D to JEV Challenges

Days after JEV challenge				Reciprocal HI Titer against						
1st	2nd	3rd	4th	D1	D2	D3	D4	JEV	Tembusu	Wess.
0[1]				0[5]	0	0	0	0	0	0
7	0[2]			0	0	0	0	0	0	0
	7			0	0	0	0	0	0	0
	14			0	0	0	0	0	0	0
	21			0	0	0	0	0	0	0
	38			0	0	0	0	10	0	0
	90	0[3]		0	0	0	0	0	0	0
		7		0	0	0	0	0	0	0
		21		0	0	0	0	0	0	0
		30	0[4]	0	0	0	0	10	0	0
			7	10	10	0	0	40	40	20
			28	0	0	0	0	40	20	20

(1) 4 JEV-infected mosquitoes fed on water buffalo #D on day 0

(2) Sc. inoculation with JEV (6×10^5 PFU).

(3) Sc. inoculation with JEV (2.5×10^7 PFU).

(4) Sc. inoculation with JEV (1×10^9 PFU at 4 sites)

(5) Titer 0 = <1:10

Table 5. HI Responses of Thai Dogs to JEV Challenges

Days after challenge		Reciprocal HI titer against						
1st	2nd	D1	D2	D3	D4	JEV	Tembusu	Wess.
Dog #A								
0[1]		0	0	0	0	0	0	0
7	0[2]	0	0	0	0	0	0	0
	7	0	0	0	40	40	10	10
	14	10	0	0	40	40	10	10
	21	10	0	0	40	40	10	10
	38	0	0	0	10	20	10	10
Dog #3								
0[1]		0[3]	0	0	0	0	0	0
7	0[2]	0	0	0	0	0	0	0
	7	10	0	10	40	160	10	10
	14	10	0	10	40	160	10	10
	21	10	0	10	40	160	10	10
	38	0	0	0	20	80	10	10
Dog #4								
0[1]		0	0	0	0	0	0	0
7	0[2]	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
	21	0	0	0	20	20	10	0
	38	0	0	0	10	10	0	0

(1) JE-infected mosquitoes fed on dog #A, #3, and #4.

(2) Sc inoculation with JEV (6×10^5 PFU/dog)

(3) Titer 0 = <1:10

Table 6. HI Responses of Thai Pigs to JEV and Tembusu Challenges

Days after challenge		Reciprocal HI titer against						
1st	2nd	D1	D2	D3	D4	JEV	Tembusu	Wess.
Pig #226								
0[1]		0[3]	0	0	0	0	0	0
7		0	0	0	0	0	0	0
14		0	0	0	0	0	0	0
21		0	0	0	0	0	0	0
44	0[2]	0	0	0	0	0	0	0
	7	0	0	0	0	0	10	0
	14	0	0	0	0	0	40	0
	21	0	0	0	0	0	20	0
	35	0	0	0	0	0	0	0
Pig #229								
0[1]		0	0	0	0	0	0	0
7		0	0	0	10	80	20	0
14		0	0	0	80	320	40	20
21		10	20	10	80	320	40	40
44	0[2]	20	20	20	40	160	40	40
	7	20	20	20	80	160	80	40
	14	40	40	40	160	640	160	80
	21	40	40	40	80	160	80	80

(1) JE-infected mosquitoes probed on Pig #226 and fed on Pig #229. JEV viremia was detected in Pig #229 from 36 to 84 hours after mosquito feeding (av. titer = 10^2 PFU/ml. blood)

(2) Sc inoculation with Tembusu virus (1.5×10^3 PFU/pig)

(3) Titer 0 = <1:10

Title: Experimental Transmission of Japanese Encephalitis Virus by Culex fuscocephala.

Principal Investigators: Debhanom Muangman, M.D., Dr. P.H.
Robert Edelman, LTC, MC
Michael J. Sullivan, CPT, MSC
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Objective: To determine if Culex fuscocephala mosquitoes can be infected by and transmit JE virus to a susceptible animal host.

Background: JEV mosquito vectors in Thailand have been previously identified as Culex tritaeniorhynchus Giles and Culex gelidus Theobald. In 1970, during a JE epidemic, JE virus was isolated from 2 pools of female Culex fuscocephala Theobald collected in Chiangmai, a northern province of Thailand. In this epidemic area, C. fuscocephala were found to be as abundant as C. tritaeniorhynchus, a known JE vector. Furthermore, their feeding and breeding habits were very similar. They also feed on a wide variety of wild and domestic animals including man. Except for 2 previous reports from Taiwan in 1958 of JEV isolations from this mosquito species, C. fuscocephala has not been implicated as a potentially important JEV vector. To our knowledge, no published study has evaluated its efficiency as a vector of JEV.

Material & Methods: JE virus strain, BKM-984-70, was isolated from a pool of 100 Culex fuscocephala females collected in Chiangmai in 1970. Thus stock virus had undergone 3 passages in suckling mice and was prepared as a 10% mouse brain suspension in bovine albumin phosphate saline (BAPS). First generations of wild-caught Culex tritaeniorhynchus and Culex fuscocephala females were allowed to feed on viremic white leghorn chicks which had been inoculated 55 hours earlier with 1,700 PFU of JEV subcutaneously. Engorged mosquitoes were then collected and maintained for transmission experiments. At selected days after the infectious blood meal, each of 8-10 presumably-infected mosquitoes from each species was induced to feed on 1 day old chicks at night. After overnight exposure, each mosquito was inspected for presence of blood in its midgut under a dissecting microscope and tested for the presence of virus in LLC-MK2 cell culture and suckling mice. Chicks which were exposed to presumably infected mosquitoes were kept for 2-3 days before their blood was drawn and tested for viremia. Thus, one group of presumably-infected mosquitoes was individually triturated and tested for virus after each transmission attempt while another group from the same cohort was similarly titrated without being allowed to feed on chicks. In addition, plague reduction neutralization tests were performed on all virus isolates from the chick sera in order to confirm JE viremia.

Results: 1) JE virus multiplication in C. tritaeniorhynchus and C. fuscocephala: Fig. 1 below shows that JE virus multiplies equally well in both mosquito species.

2) Infection Rates: In Table 1, the data demonstrates that more than 90% of both mosquito species are infected with JEV after feeding on viremic chicks and the infection remains at high level for at least 27 days.

3) Transmission Rates: In Table 2, both mosquito species show similar ability in transmitting JE virus to baby chicks. All virus isolates from chick sera were proven to be JEV by neutralization test.

4) Relationship between Mosquito Engorgement and Virus Transmission: Data in Table 3 shows that JE virus transmission could occur either by feeding until blood engorgement occurs or by feeding without engorgement (probing only).

Discussion & Conclusions: It was demonstrated that more than 90% of C. tritaeniorhynchus and C. fuscocephala became infected after ingesting very small amounts of JEV (average infecting dose = 8 PFU/mosquito). JE virus multiplied in both mosquito species to comparable, high titers (above 10^5 PFU/mosquito) within 10 days after the infectious blood meal. Once infected, both species maintained high virus titers through at least four weeks. The average transmission rates in both mosquito species were found to be similar (16% and 17%). These low transmission rates might be due to several factors, such as low concentration of JE virus initially ingested (8PFU/mosquito), somewhat low insectary temperature (25°-27°C), or the combination of first generation of wild-caught mosquitoes and JE virus of low passage. Evidence suggests that virus transmission occurred after mosquito probing as well as after feeding to repletion.

Thus, we have demonstrated a high vector efficiency of C. fuscocephala in the laboratory when compared with C. tritaeniorhynchus, a confirmed JEV vector. Together with field observations, these data provide strong evidence to incriminate Culex fuscocephala as a potentially important vector of JE virus in the northern part of Thailand.

Table 1. Number of Culex tritaeniorhynchus and Culex fuscocephala infected with JE virus at various days after the infectious blood meal

Days after the infectious blood meal	C. fuscocephala	C. tritaeniorhynchus
10	19/20*	20/20*
11	20/20	18/20
19	20/20	18/20
27	14/15	18/20
TOTAL	73/95(97%)	74/80(92%)

*No. of mosquitoes infected
Total mosquitoes tested

Table 2. Comparison of the transmission rates of JEV-infected Culex fuscocephala and Culex tritaeniorhynchus mosquitoes*

Days after the infectious blood meal	C. fusco	C. tri	% Transmission rate C. fusco	C. tri
10	1/10**	0/10**	10%	0%
11	2/10	1/10	20%	10%
19	2/10	4/10	20%	40%
27	1/8	2/10	12%	20%
TOTAL	6/38	7/40	16%***	17%***

* Each mosquito was tested for the presence of virus immediately after the transmission attempt and all were found to be infected.

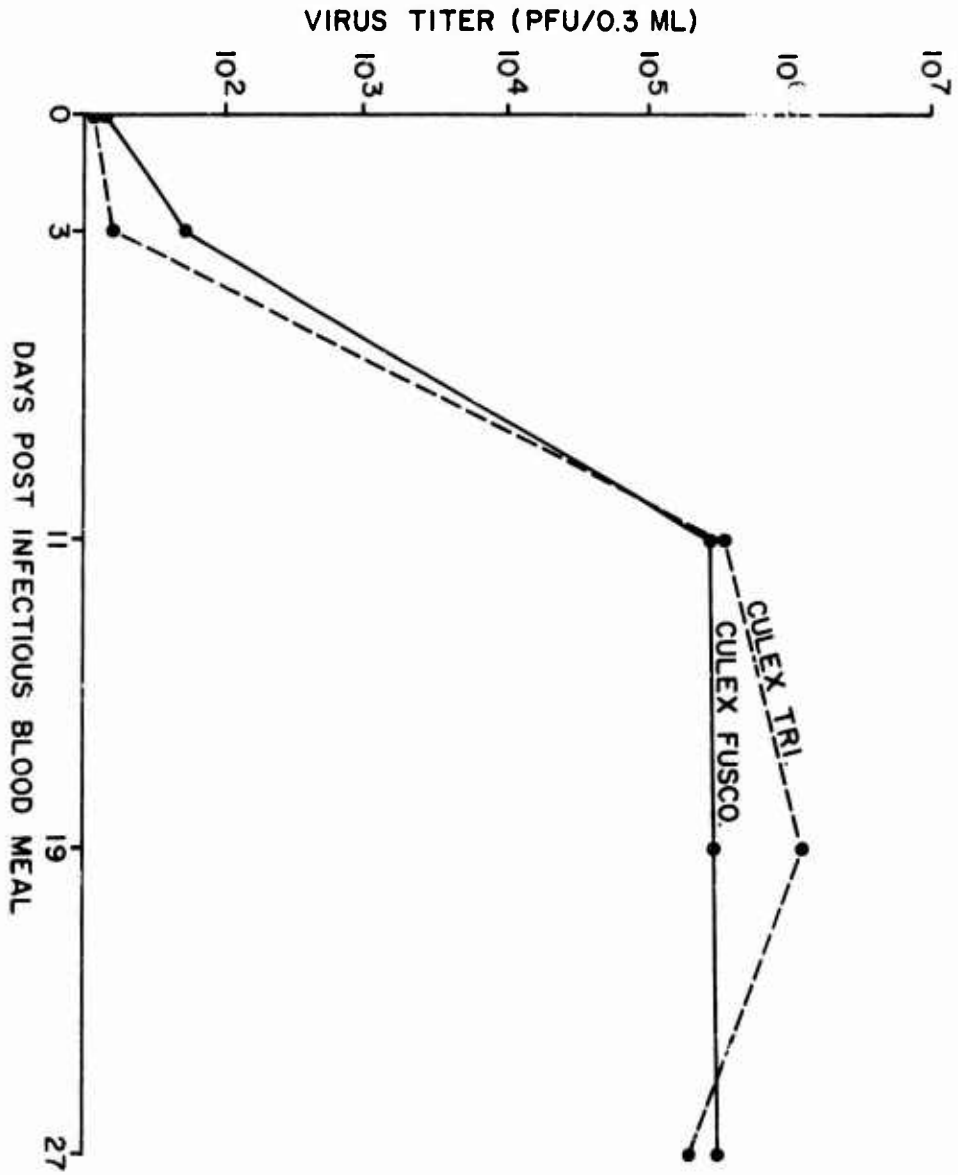
** No. of viremic chicks / total no. chicks exposed

*** Average transmission rate

Table 3. Relationship between mosquito engorgement and transmission of JE virus to 1-2 day old chicks

Virus transmission	Engorged Mosquitoes*		Unengorged Mosquitoes	
	C. fusco.	C. tri.	C. fusco.	C. tri
positive	3	5	3	2
negative	3	14	29	19
Total	6	19	32	21

* Mosquitoes with the presence of blood in the abdomen after an overnight exposure to 1-2 day old chicks.


VIRUS TITER (PFU/0.3 ML)
10^2
10^3
10^4
10^5
10^7
0
3
11
19
27
DAYS POST INFECTIOUS BLOOD MEAL
CULEX TRI.
CULEX FUSCO.

JE Virus Recovery after High Speed Centrifugation of Presumably-Infected Mosquito Pools

Principal Investigators: Debhanom Muangman, M.D., Dr.P.H.
Robert Edelman, LTC, MC
Douglas J. Gould, Ph.D.

Objective: To determine 1) whether high speed centrifugation or 2) the presence of large number of uninfected mosquitoes in a presumably-infected mosquito pool would influence virus recovery from the supernatent fluid of that triturated pool.

Background: In the Chiangmai JEV project, virus was isolated from triturated pools of various mosquito species clarified by high speed centrifugation (10,000 rpm at 4°C for 30 min). No experimental data are available which show the possible deleterious effect of such centrifugation on virus recovery. Furthermore, Yuill (SEATO Lab) had reported in the past that the presence of large number of uninfected mosquitoes in a pool might decrease the chance of virus recovery in LLC-MK2 cell culture due to virus inhibitors released by mosquito tissues. The current study was designed to elucidate the effect of centrifugation on virus recovery and to confirm that mosquito tissues are indeed inhibitory in tissue culture.

Progress: Presumably - JEV infected Culex tritaeniorhynchus (13 days post infectious blood meal) was triturated with 99 uninfected Culex tri. in 4 ml. of Bovine Albumin Phosphate Saline (BAPS) which contained penicillin, streptomycin, and Fungicidin. A total of 10 such pools were triturated. The triturated mosquito pool was divided into 2 equal portions. The first portion was centrifuged at 10,000 rpm. at 4°C for 30 min. The second portion was left in a container surrounded by wet ice in a refrigerator at 4°C for the same period of time. After that, the two supernatent fluids were collected and tested for the presence of virus in suckling mice (I.C.) and in LLC-MK2 cell culture (direct and delayed plaque method). The results are shown in Table 1.

The results demonstrate no significant difference in virus recovery in centrifuged and non-centrifuged portions of each of the 10 mosquito pools. Although MK2 cell culture and suckling mice seem to be almost equally sensitive in detecting virus, contamination (especially with fungi) of tissue culture occurs about 5 times more frequently in non-centrifuged than uncentrifuged specimens. The suckling mice were apparently not affected by this contamination. Thus centrifugation apparently does not impair recovery of JEV from infected mosquito suspensions. On the same

day of the previous experiment, 23 presumably JEV infected mosquitoes from the same infected mosquito lot were individually triturated in 1 ml. of BAPS, centrifuged, and tested for virus in MK2 cells. The results were compared with MK2 cell virus recovery from the 10 mosquito pools composed of 1 infected mosquito and 99 uninfected ones (Table 1). The results in Table 2 provide evidence that the presence of at least 99 uninfected mosquitoes in a pool of 100 mosquitoes did not interfere with the detection of virus in that pool as isolated in MK2 cell. Thus, we were unable to confirm Yuill's observation that large amounts of triturated mosquito tissues might have virus inhibitory effect in tissue culture.

Table 1. Virus Recovery from Centrifuged and Non-Centrifuged Mosquito Suspensions in Suckling Mice and LLC-MK2 Cell Culture.

Centrifuged		Non-Centrifuged	
Suckling Mice	MK2	Suckling Mice	MK2
2/10*	3/10	2/10	2/10

* No. pools positive
No. pools tested

Table 2. Infection Rates of Presumably-Infected Culex tritaeniorhynchus Triturated alone (A) or with 99 Uninfected Mosquitoes of the Same Species (B)

	Mosquito Pools-A*	Mosquito Pools - B**
No. Pools infected*** / No. Pools tested	6/23	3/10
Infection rate	26%	30%

* 1 Presumably - JE infected mosquito in 1 ml diluent
** 1 Presumably - JE infected mosquito and 99 uninfected mosquitoes in 4 ml diluent
*** JE virus isolations in MK2 cell culture.

The Sensitive Host for Recovery of Japanese Encephalitis Virus (JEV)

Principal Investigators: Pairatana Gunakasem, M.D.
Robert Edelman, LTC MC

Associate Investigator: Franklin H. Top, LTC MC

Background: In the previous Annual Report, Aedes albopictus cells (Singh line) were shown to be equally or more sensitive than LLC-MK2 cells for the growth of arboviruses recovered in Thailand. The viruses tested were the 4 serotypes of dengue, JEV, Wesselsbron, Ingwavuma and Tembusu viruses. The virus titer obtained in Aedes albopictus cells was generally higher than that in MK2 cells and the peak virus titer in A. albopictus cells appeared earlier than in MK2 cell. The present study was designed to compare A. albopictus cells and MK-2 cells as isolation systems for recovery of JEV from the brain of fatal human cases and from the brain of primates intracerebrally inoculated with various strains of JEV.

Methods: The host systems used for virus isolation were A. albopictus cells, LLC-MK2 cells and one-day old mice. The method of virus assay was plaque titration in LLC-MK2 cells.

Before inoculation, brain suspensions were serially diluted ten-fold and each dilution was inoculated into A. albopictus cells, MK2 cells and one-day old mice. The sensitivity of the 2 tissue cultures was defined as the highest dilution of brain suspension that yielded a virus titer of not less than $10^{2.0}$ in the fluid phase of the infected cell culture. In tissue culture systems half of the fluid supernatent was sampled and replaced with equal volumes of fresh medium. The period of study was 7 days. In mice, the morbidity ratio of the first brain passage was used to indicate the growth of virus. Two litters (16 mice) were used for the intracerebral inoculation of each brain dilution. The volumes of virus inoculation in tissue culture and mice were 0.3 ml and 0.02 ml respectively.

Progress: 1. Recovery of JEV from experimental monkeys: Five monkeys were inoculated intracerebrally with $7x10^{4.0}$ to $1x10^{7.0}$. PFU JEV per 0.25-0.5 ml inoculum. The passage history of the virus used in each monkey is shown in Table 1; two JEV strains were isolated from infected mosquito pools while the third strain was originally isolated from a human brain. Within 7 days after inoculation the monkeys developed intermittent fever, lethargy, paresis and collapse. The monkeys were sacrificed within 1 or 2 days after symptoms developed, the brains removed, and portions frozen for virus recovery and fixed for histopathological analysis.

Monkey y-0102 was not killed 1 to 2 days after paresis developed but rather 2 weeks later. It was anticipated that the virus titer in the brain of this monkey would be lower than the 4 other subjects and that the low titer would better test the abilities of the isolation systems to recover JEV.

The results in Table 1 show that A. albopictus cells are more sensitive than MK2 in the recovery of JEV from monkey brains, and generally detected virus at a 10-fold less dilution than obtained in MK2 cells. No virus was recovered in either cell culture in the monkey killed after 2 week of paralysis. Both cell cultures were more sensitive than suckling mice in these experiments.

2. Recovery of JEV from human brain: Suspensions of human brain from encephalitis cases were used to test the recovery of JEV by direct and delayed plaque in LLC-MK2 cells and in suckling mice; the results are shown in the last two columns of Table 2. Remaining suspensions stored at -70°C were thawed, diluted ten-fold and inoculated into A. albopictus cells, MK2 cells and one-day old mice. Agreement between initial isolation and reisolation results in MK-2 cells was found. In the reisolation, A. albopictus cells were more sensitive than MK2 cells except for specimen 48642. A. albopictus cell line is the only host that recovered JEV from brain specimen 45342 from which JEV was not isolated initially. The mouse isolation technique failed to demonstrate JEV growth of 48197 (reisolation) and 48642 (primary isolation).

These results would suggest that A. albopictus cell line may be more sensitive than MK2 for isolation of JEV from infected brain. The data would gain added significance if replicate experiment cases were performed in order to confirm the initial promising results. Suckling mice in these experiments were far less sensitive than both tissue cultures.

Table 1. The Sensitivity of A. albopictus Cells, LLC-MK2 Cells and One-day Old Mice in the Recovery of JEV from Experimental Monkey Brains.

Monkey number	JEV strains	Log_{10} dilution of brain yielding $10^{2.0}$ virus titer on day 7		Log_{10} dilution of brain causing sickness in one-day old mice	Mouse morbidity ratio: mice sick / mice tested
		LLC-MK2	A. albopictus		
y-090	BKM1096,MK2(1)	1	2	negative	0/16*
y-091	BKM1096,MK2(1)	4	5	undiluted	16/16
				1	2/16
y-097	BKM1022,MK2(1)	5	6	3	16/16
				4	3/16
y-099	40783,MK2(1)	6	6	4	16/16
				5	4/16
y-0101	40783,MK2(1)	3	3	undiluted	2/16
y-0102	40783	This monkey was killed after two weeks of paralysis. No virus was detected in cell cultures and mice showed no symptoms after inoculation			

* Undiluted brain

Table 2. The Sensitivity of A. albopictus Cells, LLC-MK2 Cells and One-day Old Mice in the Recovery of JEV from Human Brain.

Specimen number	Log_{10} dilution of brain yielding $\geq 10^{2.0}$ virus titer on day 7		Results of mouse inoculation (morbidity ratio)	Result of primary isolation**	
	LLC-MK2	A. albopictus		LLC-MK2	Mice
45342 1970*	Negative	Undiluted	Negative	Negative	Negative
48197 1971	2.0	3.0	Negative	Positive	Positive
48642 1971	3.0	3.0	undiluted 1/16 $10^{1.0}$ 0/16	Positive	Negative
48944 1971	1.0	2.0	Not done	Positive	Positive
49349 1971	1.0	2.0	Not done	Positive	Positive
48633 1971	Negative	Negative	Negative	Negative	Negative
48579 1971	Negative	Negative	Negative	Negative	Negative
48530 1971	Negative	Negative	Negative	Negative	Negative

* Year of virus isolation
** Inoculation with undiluted material

Title: Group B Arbovirus Serology: Search for Humoral Specificity.

Principal Investigators: Robert Edelman, LTC MC

Associate Investigators: Anong Pariyanonda, M.S.
Marvin H. Firestone, MAJ MC
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Suchinda Udomsakdi, M.D.

Objective: To improve serological specificity in group B arbovirus infections by isolating and titrating serum IgM antibody in individuals with dengue and Japanese encephalitis virus infections.

Background: A detailed description of this study, together with the IgM antibody serological results on 39 patients with group B arbovirus infections, was included in last year's SMRL Annual Report. Briefly we observed that the 3 standard serological tests, HI, CF, and PRNT used alone or in combination cannot clearly identify a type-specific group B arbovirus antibody in whole serum of individuals who have been previously infected with group B agents. In an attempt to improve specificity we fractionated convalescent sera obtained from Japanese encephalitis patients by sucrose density gradient centrifugation and found low-titered IgM HI antibody directed monospecifically against JEV. The IgG in these sera reacted heterospecifically in high titers with antigens common to dengue 1-4 and JEV and was responsible for the cross-reactions noted in standard serological tests.

We report here studies on the specificity and persistence of circulating IgM antibody in Japanese encephalitis patients, IgM patterns in a variety of dengue infections, and the use of IgM analysis to diagnose fevers of unknown origin.

Progress: A. Serum IgM Antibody in Japanese Encephalitis Patients, Chiangmai 1970.

The Dept. of Neuropsychiatry, SMRL, has studied the neuropsychiatric recovery of convalescing JE patients. The patients were hospitalized in Chiangmai and adjacent Lampang Valley in 1970 and were bled for group B arbovirus serology (dengue 1-4 and JEV) during hospitalization and at intervals of weeks to months over a 1 year followup period. No dengue transmission was detected in these valleys in 1970. Access to this serum has provided a unique opportunity to 1) correlate the presence or absence of detectable JEV IgM antibody with the HI and CF serological pattern

(primary or secondary infection), 2) determine in a large number of patients whether or not JEV IgM antibody cross-reacts with dengue 1-4 antigens, 3) study the persistence of circulating IgM antibody following clinical disease.

Table 1 summarizes the serologic criteria used to distinguish primary JEV infections and secondary group B arbovirus infections of unspecified virus type.

A total of 54 encephalitis patients have been studied to date; their whole serum serological patterns and IgM analyses are summarized in Table 2. The number of serum specimens analysed from each patient ranges from 1 to 6. Of 45 patients showing $\geq$4-fold rising titers, 19 had a primary JEV infection, and 18 of the 19 had JEV specific IgM antibody in 1 or more convalescent sera, thus confirming a first infection with JEV. Fifteen of 26 patients with rising titers and a secondary group B serological pattern had IgM and thus evidence of first infection with JEV. All patients with IgM antibody had recent JEV infections, because IgM was not found in the acute serum, or found in titers significantly lower than in convalescent sera. Presumably these 15 patients had been previously infected by a group B arbovirus other than JEV; this previous heterologous group B infection did not protect them against Japanese encephalitis.

Several possible reasons can be given for the failure to detect IgM in 11 of the 26 patients with serologically confirmed secondary infections. First, the high-titered IgG antibody characteristically found in secondary infection sera tends to contaminate the IgM-containing serum sucrose fractions. If IgM antibody titers are lower than contaminating IgG titers in such fractions, they will be 2-mercaptoethanol resistant, and thus falsely appear to contain no IgM. Second, timing of the convalescent serum is important, because the IgM rise may be transient and so be detectable only 10 to 28 days after the onset of illness. For example three IgM negative patients, with serums drawn on days 9, 42, and 45, may fall into this category. Thirdly, some of these 11 negative patients may have been infected with JEV before their recent JE infection and might not produce IgM after a repeat JEV infection. Fourthly, the acute heterospecific rise in whole serum titers may be caused by a group B encephalitis virus infection other than JEV. Finally it is conceivable that some individuals do not synthesize IgM antibody to JEV if they have been previously sensitized to a related group B agent. The latter 3 explanations are considered less likely than the first 2 offered. In addition, JEV-IgM antibody was present in 4 of 9 patients with fixed or falling whole serum titers indicating a recent JEV infection, although it was not possible to diagnosis a recent infection using standard serological tests.

Thirty-seven of 39 patients with IgM antibody listed in Table 2 had titers of IgM antibody reactive only against JEV. Two patients (both with secondary infections) had IgM that cross-reacted with D4 (JE-C-69) and D1, D2, and D3 (JE-M-37) (see Table 3). Thus IgM antibody produeced by 39 encephalitis patients was monospecific in 95% of cases, and in one of the exceptions with heterospecific IgM antibody (JE-C-69) the high JEV IgM titers and low D4 IgM titers strongly suggested a recent JEV infection. The unusual combination of high titered, cross-reactive and persistent IgM antibody found in the other exception, JE-M-37, remains an enigma; her IgM and whole serum patterns are compatible with a recent dengue infection (see next section on dengue infections), however no dengue transmission was documented at the time (1970) and place (rural Chiangmai valley) she was infected.

The persistence of circulating IgM antibody was determined in the 19 patients with primary and the 18 with secondary group B serological patterns (Table 2). The results, summarized in Table 4, indicate that IgM may circulate in gradually declining titers for greater than 5 months after primary and secondary infections. The IgM was detected in one patient for at least 14 months. Following sera collected later than 1 month after illness was usually drawn at 2 to 6 month intervals rather than monthly. Many primary infection patients in particular, had IgM antibody present in all serum samples, so that even longer persistence would have undoubtedly been documented with additional or more frequent sampling. The time intervals listed in Table 4 therefore represent the minimum rather than the maximum values for IgM longevity <u>in vivo</u>. The question arises whether such long term persistence of IgM is associated with persistence of the antigen, and if so, whether this antigenic persistence is clinically significant. Furthermore, will those patients with prior group B infection as evidenced by a 2^{o} serological response, be partially protected as a consequence of prior sensitization and show fewer neuropsychiatric sequelae (as compared to 1^{o} infections) during hospitalization and thereafter. Finally does a clinical difference exist between those who had detectable IgM antibody and those who do not. All of these questions are being examined now in collaboration with the department of Neuropsychiatry. There were no discernable age or sex differences between the patient groups with and without IgM antibody; approximately 1/3 were female and 2/3 were male in both groups, and ages ranged from 6 to 35 yrs in IgM negative patients and 3 to 47 years in the IgM positive patients.

C. Serum IgM antibody in inapparent JEV infections.

Approximately 31 Chiangmai villagers and urban school children studied in 1970 had serological evidence consistent with recent JE or

group B arbovirus infection. Ten persons had primary JEV infections with rising or falling JEV titers; the remaining 21 had secondary infections with rising, falling or fixed titers. 36 serum from these 31 persons were fractionated by S-DGC. Only 1 serum from a Chiangmai City school child contained trace amounts of IgM antibody to dengue 2. The 35 other serum contained no demonstrable IgM antibody. The inability to detect IgM antibody in these sera may have resulted from the 3 month interval between each bleeding, with decay of IgM antibody in the interim; alternately individuals with inapparent JEV infections may not produce IgM antibody or produce it for much more limited durations than persons with encephalitis.

In order to eliminate the latter possibility, serum obtained from 6 family members of 6 encephalitis patients was fractionated. These family members had experienced an inapparent JEV or group B infection diagnosed by rising titers in serial serums drawn prospectively over 1-3 week intervals. As shown in Table 5, 4 of the 6 subjects produced IgM antibody reactive only with JEV, thus confirming that IgM is produced in inapparent JEV infections.

C. Dengue infections.

The IgM results on 2 patients with dengue hemorrhagic fever (DHF) (2^{o} infections) and 6 patients with dengue fever (1^{o} infection) was reported in last year's Annual Report. Briefly, no IgM antibody was found in the 2 patients with DHF and in one with 1^{o} dengue fever. Five dengue fever patients did have IgM antibody in their convalescent serum, but in 2 of these individuals the IgM cross-reacted with either JEV or another dengue serotype. Four more patients admitted to Bangkok hospitals in 1971 were studied and are reported below. Three had clinically confirmed DHF (CH-404k, CH408k, CH-454m; Table 6) and 1 (RA-005k) had unconfirmed DHF (hospital record not available). These 4 children were 4 to 6 months old and all had serological patterns consistent with 1^{o}, rather than 2^{o} dengue infections. Dengue-2 virus was isolated from 1 patient who also developed severe dengue shock syndrome. Low serum complement levels were found in the 2 patients tested for C' (CH-404k, CH-408k). The IgM patterns shown in Table 6, reveal that all 4 developed IgM antibody to dengue, but unlike 95% of JE patients, the IgM antibody was heterospecific and cross-reacted with one or more of the other dengue serotypes or JEV. There is no explanation for the predominantly monospecific IgM antibody in JEV infections, and the heterospecific IgM found with more regularity in dengue. The appearance of IgM antibody in these patients confirms the serological diagnosis of a primary dengue infection and suggests further, that patients under the age of one year can develop DHF or DSS after a primary dengue infection. One of these patients (CH-404k = #26, Fig 3) is presented in greater detail in the dengue section of this Annual Report.

D. Tropical fevers caused by Group B arbovirus.

Serial serum samples were obtained from a Peace Corps volunteer in Thailand before and after he developed a dengue-like febrile illness. He had been immunized against yellow fever before coming to Thailand and had received a primary and booster course of Biken killed JEV vaccine after his arrival. As shown in Table 7, results of HI tests were consistent with a recent 2° group B infection, virus type unspecified. The cross-reactive IgM antibody in the convalescent serum suggests, more specifically, that he suffered a recent primary dengue virus infection.

MAJ Robert Howarth, 9th Med Lab, Vietnam, supplied 6 paired sera from U.S. troops in Vietnam hospitalized with fevers of unknown origin. Five of the 6 had serological evidence of a recent group B infection, with rising titers to JEV and to 3 or 4 dengue serotypes. One had high fixed titers to dengue 1-4 and JEV. These serological results together with IgM fractionation of the convalescent sera are shown in Table 8. JEV specific IgM antibody was found in 4 patients, and D1 specific antibody was detected in 1 patient. One patient was IgM negative, but his convalescent serum was drawn only 2 days after the acute phase serum. Thus IgM fractionation of convalescent serum in these FUO patients with 2° group B infections provided improved serological specificity, provided the convalescent serum is drawn at the proper time. No virus isolations are available to confirm these serological results, and the interpretation rests on serological similarity to previous virus confirmed patients together with the clinical and epidemiological data.

Summary and Conclusions: The fractionation and titration of serum IgM HAI antibody appears to be a new and useful serodiagnostic method in certain group B arbovirus infections. It is more virus specific than the standard serological tests, particularly in secondary infections, and often allows the diagnosis of a specific recent JEV or dengue infection at a time when the paired serum show only fixed elevated titers. The JEV IgM data for gibbons (this annual report) and previous human studies confirms that IgM antibody is produced after first JEV infection, apparent or inapparent, and after dengue, but not after a repeat infection with the same virus, a finding which has permitted certain seroepidemiological conclusions about group B arbovirus infections. For example, the presence of JEV-specific IgM antibody in serum of encephalitis patients showing non-specific secondary group B antibody patterns (indicative of prior group B infection) suggests that their previous group B infection was not JEV and that there was no cross-protection against the development of Japanese encephalitis in these patients.

The persistence of circulating IgM antibody for a long as 14 months

after Japanese encephalitis suggests that the virus or non-infectious viral antigens may persist in some convalescing patients beyond the acute illness. This finding is of considerable theoretical interest in view of recent work elsewhere on slow virus infections. Another example of insight gained through IgM analysis concerns the 4 infant children studied with dengue hemorrhagic fever and shock syndrome. The detection of IgM antibody in convalescent sera of these children confirms the serological diagnosis of a primary dengue infections and thus suggests that children under 1 year can develop dengue complications following a primary infection rather than after the customary 2nd dengue infections as seen in older children.

The IgM technique has not proven useful in remote infections or when the convalescent serum cannot be dated with respect to the acute illness, owing to the transitory appearance of circulating IgM in most patients. Moreover the failure to detect IgM antibody by S-DGC, particularly in secondary infections, may result from technical insensitivity of the procedure (as described above) thereby giving a false negative result in the presence of low titered IgM antibody. Consequently the successful detection of IgM antibody has considerably more meaning than the failure to detect such antibody.

Table 1. JEV Serologic Diagnostic Criteria*

Response	Serum HI Antibody Titer	
	Acute	Convalescent
primary	1. ≤1:20 to all dengue antigens.	1. ≥4 fold rise to JE antigen 2. Titer to JEV ≥4 fold titer to dengue 4 3. ≤1:80 to dengue 4 antigen
secondary	1. ≥1:40 to at least one dengue antigen.	1. ≥4-fold rise to JEV and at least one dengue antigen. 2. Titer to JEV less than 4-fold greater than titer to dengue antigens

* Serum titered against Dengue 1-4 and JEV

Table 2. JEV-specific IgM antibody in JE patients listed by serological group Chiangmai 1970.

Whole serum titer[x]	Total pts.	Number of patients in whom: IgM antibody detected		IgM not detected	
		Primary[xx]	Secondary[xxx]	Primary	Secondary
Rising	45	18	15	1	11
Fixed	6	1	1	0	4
Falling	3	0	2	0	1
Totals	54	19	18	1	16

x HI and/or CF
xx No serological evidence of previous group B arbovirus infection.
xxx Serological evidence of previous group B arbovirus infection.

Table 3. Cross-reactive IgM Antibody Patterns in Two Patients with Encephalitis

Patient	S-DGC* Fraction	HI Titre in S-DGC Fractions									
		JEV		D4		D3		D2		D1	
		C	2ME	C	2ME	C	2ME	C	2ME	C	2ME
JE-M-37											
47 yr. woman	3	32	8	16	8	16	8	16	4	32	4
6 Aug. 1970	4	64	8	16	8	>128	8	64	4	64	4
	5	32	8	16	8	16	8	16	4	16	4
Whole serum	20 July 70	160		320		320		160		160	
	6 Aug 70	5120		10240		20480		2560		10240	
JE-C-69	3	16	4	4	0	4	2	2	2	2	2
17 Aug. 70	4	32	4	8	0	4	2	2	2	2	2
	5	16	4	4	0	4	2	2	2	2	2
Whole serum	31 July 70	5120		1280		1280		1280		640	
	17 Aug 70	2560		640		1280		1280		640	

* sucrose density gradient centrifugation

Table 4. Longevity of IgM antibody in vivo after Japanese encephalitis

Whole serum antibody pattern[x]	Number of patients with IgM last detectable[xx] during month[xxx]						
	0-1	1-2	2-3	3-4	4-5	5-8	>8
Primary	11		4	3		1	
Secondary	10	4	2			1	1

x HI and/or CF
xx Values represent the minimum interval after illness
xxx After onset of illness

Table 5. Inapparent Infections (Confirmed & Questionable). Chiangmai Family Case Members - 1970. Sera fractionated by S-DGC for IgM Ab vs. D1-4, JE, Tembusu, Wesselsbron.

Pt. No./ HI pattern[xx]	Serum No.	Date	S-DGC Results	Antigens*
JE-C-6c	44585	25 May	N.T.#	
1^{o}-R	44586	9 June	N.T.	
	46037	14 June	IgM + JEV	JE, D4, T, W
JE-L-12I	44709	11 June	N.T.	
1^{o}-R	44710	23 June	IgM + JEV	JE, D4, T, W
JE-L-11a	44762	15 June	N.T.	
1^{o}-R	44763	29 June	IgM + JEV	JE, D4, T, W
JE-C-26D	44966	29 June	N.T.	
1^{o}-R	44967	13 July	IgM + JEV	JE, T, D4
	46049	19 Aug	IgM +JEV	JE, T, D4, W
JE-C-32c	45177	2 July	N.T.	
2^{o}-R	45178	17 July	IgM Neg.	JE, D1-4, T, W
	46206	21 Aug	IgM Neg.	JE, D4, T
JE-M-53d	46635	30 Sept	IgM Neg.	D4, JE, T, W
1^{o}-R	46636	12 Oct	N.T.	
	46869	12 Nov	IgM Neg.	

N.T. = not tested
* D4 = dengue 4, D1-4 = Dengue 1-4, T = Tembusu, W = Wesselsbron
xx 1^{o}-R = monospecific rising titer to JEV (see Table 1)
2^{o}-R = heterospecific rising titers to group B antigens (see Table 1)

Table 6. IgM antibody pattern in infants with dengue hemorrhagic fever & shock syndromes - 1971.

Patient	S-DGC fraction	HAI titer in S-DGC fractions									
		JEV		D4		D3		D2		D1	
		C	2ME	C	2ME	C	2ME	C	2ME	C	2ME
CH-404K	2	4	0	4	0	2	0	8	0	2	0
49133	3	8	0	16	0	8	0	16	0	4	0
D2 isolated	4	2	0	4	0	4	0	4	0	0	0
CH-408K	2	2	0	4	0	2	0	16	0	0	0
49100	3	4	0	16	0	8	0	32	2	2	0
virus neg.	4	2	0	4	0	4	0	16	2	2	0
CH-454M	2	2	0	4	0	2	0	16	0	2	0
49664	3	8	0	16	2	8	0	32	2	4	0
virus neg	4	2	0	4	2	4	0	16	2	2	0
RA-005K	2	2	4	4	2	2	0	8	0	2	0
48709	3	4	0	8	2	4	0	32	0	4	0
No isolation attempted	4	2	0	4	2	2	0	8	0	2	0

Table 7. Whole serum and IgM HAI Antibody in a Peace Corps Volunteer with a Febrile Dengue-Like Illness

S-DGC fraction	HAI titer in S-DGC fractions of convalescent serum									
	JEV		D4		D3		D2		D1	
	C	2ME	C	2ME	C	2ME	C	2ME	C	2ME
2	8	2	4	4	4	2	8	0	2	0
3	16	4	16	8	8	2	32	2	4	0
4	8	4	8	8	8	2	16	2	4	0
whole acute	<10		<10		<10		<10		<10	
serum conv.	640		2560		320		640		320	

Table 8. Whole Serum and IgM HAI Antibody in American Troops Hospitalized with Tropical Fevers. Vietnam 1970

Patient	Date serum	HAI titers in whole serum vx JEV	D4	D3	D2	D1	Chik	IgM Antibody detected[x] vs
Jackson	4/10/70	0	0	0	0	0	0	
	18/10/70	320	80	80	40	40	0	JEV
Christopherson	2/11/70	0	0	0	0	0	0	
	4/11/70	160	20	40	20	0	0	not detected
Bartsch	22/11/70	160	40	40	20	20	0	
	29/11/70	10240	1280	2560	640	320	0	JEV
Hanson	17/9/70	80	0	20	0	0	0	
	29/9/70	640	80	160	40	40	0	JEV
Shire	19/11/70	5120	5120	2560	640	1280	0	
	22/11/70	10240	1280	5120	1280	1280	0	Dengue-1
Kokanich	20/11/70	2560	320	640	160	80	0	
	2/12/70	>20480	640	1280	640	320	0	JEV

x IgM titered against D1-4 & JEV.

Title: Immunoglobulin Response and Viremia in Dengue Vaccinated Gibbons Repeatedly Challenged with Japanese Encephalitis Virus.

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Description: The background and preliminary results of this project can be found in SMRL Annual Report, 1970-1971. Briefly we have studied the humoral immunological response of gibbons previously infected with dengue viruses to repeated challenge with JEV. The purpose of the study was to attempt to improve serological specificity in secondary group B arbovirus infections. The study also provided a unique opportunity to examine whether previous dengue infections protected against JEV infection. With this report the study is concluded.

Results: First JEV Challenge.

Table 1 records the prior dengue exposure of the eight gibbons, the presence or absence of detectable JE viremia following JEV inoculation, and the antibody response as measured by the three standard serological tests. None of the gibbons evidenced clinical illness after JEV challenge. JE viremia was detected in 5 of 8 animals. Two gibbons (S-70, S-71) had preexisting JEV and dengue neutralizing (Nt) antibody on day 0, and one to one or more dengue serotypes without detectable JEV Nt antibody; 4 of these gibbons had viremia. Thus the presence of JEV or dengue Nt antibody in acute sera did not protect against JE viremia. No attempt was made to titer the amount of virus present in viremic serum.

Listed in Table 1 are the serological results in preinfection serum (day 0) and in a representative convalescent serum (day 21). The serological responses of the 8 gibbons were remarkably uniform and were characteristic of the broad serological response seen in secondary group B arbovirus infections; all showed diagnostic HAI, CF, and Nt antibody titer rises to JEV and to two or more of the 4 dengue serotypes. The JEV HAI titers were significantly high ($\geq$ 4-fold) than the highest dengue HAI titers in only 2 gibbons (S-92 S-94), and JEV CF and Nt titers in all 8 gibbons were equal to or less than the highest dengue CF and Nt titers. Thus we could not reliably identify the most recent group B infection as JEV in these dengue-vaccinated gibbons using

routine serological tests.

In an attempt to improve serological specificity for recent JEV infection, we tested for the presence and immunospecificity of IgM contained in acute and convalescent serum. The acute and convalescent whole serum from 2 gibbons was first treated with 2-ME. As seen in Table 2, the HAI titers against D1-4 and JEV were not reduced after 2-ME treatment. Thus IgM antibody could not be detected in whole serums. Nevertheless we suspected that IgM might exist, but was masked by high titered, 2-ME resistant IgG antibody in whole serum. Attempts were therefore made to isolate IgM and IgG by sucrose density gradient centrifugation (S-DGC) of serum specimens.

Representative S-DGC assay results on a convalescent serum (gibbon S-94, day 28) are listed in Table 3. Fractionation of this serum revealed that all of the IgM detectable by radial immunodiffusion was concentrated into fractions 4 and 5, whereas IgG was concentrated into fractions 7-10, and IgA into fraction 8. 2-ME sensitive HAI antibody, limited to fractions 3-5, was found to react with JEV but not dengue 1-4. In contrast HAI antibody in fractions 7-12 reacted in high titers with both JEV and dengue 1-4 antigens, was resistant to 2-ME treatment, and was principally IgG by radial immunodiffusion. The presence of JEV-monospecific IgM antibody in the convalescent serum (Table 3) and the absence of such antibody in the acute serum(day 0; not shown) provide strong serological confirmation of a recent JEV infection.

Sera of the 8 test gibbon sera obtained before and after JEV infection were fractionated and the results of these assays are tabulated for IgM in Table 4. Newly produced JEV-monospecific IgM antibody was first detected 14 and 21 days after JEV infection in 7 of 8 gibbons; it was not detected in any gibbons by day 90.

The S-DGC fractionation technique permitted us to approximate the time of appearance of IgG as well as IgM. As shown in Table 5, serum IgG HAI heterospecific antibody appeared coincident with or before IgM monospecific antibody in all of the gibbons.

Second JEV Challenge

Seven gibbons were challenged a second time with JEV 13 months after the first JEV inoculation. The purpose was to determine 1) whether a first JEV infection is associated with prevention or attenuation of a second infection as measured by viremia and serological response and 2) whether JEV monospecific IgM antibody is produced after a second JEV challenge.

Table 6 records the presence or absence of detectable viremia following the second challenge and the antibody response measured by the three standard serological tests. As noted after the first JEV challenge, none of the gibbons displayed clinical illness.

Viremia was not detected in these rechallenged animals on days 2,3,4, and 6 after infection. The high-titered JEV Nt antibody induced 13 months earlier had fallen to lower titers by day 0 of the second challenge, but in only one gibbon (S-81) had Nt antibody declined to titers of less than 1:10. In marked contrast to the antibody response noted after first JEV challenge when all gibbons demonstrated titer rises to dengue and JEV in the 3 serological tests, only one gibbon (S-51) demonstrated a broadly cross-reactive serological response after second challenge. Further comparison of antibody responses revealed attenuated titer rises with depression of titer levels in convalescent sera following second challenge. The blunting of the immune response was particularly marked against the dengue antigens.

No IgM HAI antibody was found in sera drawn 14,21 & 28 days after second challenge.

Summary. The response of primates to serial group B arbovirus infections was investigated by inoculating gibbons with live Japanese encephalitis virus (JEV) 17 to 21 months after infection with multiple dengue virus serotypes. Five of 8 gibbons developed detectable JE viremia, and all 8 animals had high-titered cross-reactive serum antibody titer rises to group B antigens, indicating JEV replication in these dengue-vaccinated gibbons. The immunoglobulin response was characterized by early production of high-titered cross-reactive IgG antibody, and later production of low-titered IgM antibody reacting monospecifically with JEV. Thirteen months later 7 of these gibbons were reinoculated with JEV. In contrast to the first JEV challenge, no viremia was detected and the serological response, including IgM antibody production, was partially or completely aborted. This study suggests that prior infection with JEV, but not dengue, can protect gibbons against JEV inoculated more than one year later. In addition, serum IgM antibody induced by JEV infection in these gibbons previously infected with dengue serotypes is shown to be serologically specific for JEV. Sucrose-density gradient fractionation of serum and measurement of IgM antibody may thus provide a more precise serological procedure with which to investigate group B arbovirus infections in previously infected populations.

Table 1. Serological response and viremia in dengue vaccinated gibbons challenged with Japanese encephalitis virus (JEV).

Gibbon / Previous virus challenge	Viremia*	Days after JEV challenge#	Reciprocal antibody titer														
			HAI					CF					Nt+				
			D1	D2	D3	D4	JEV	D1	D2	D3	D4	JEV	D1	D2	D3	D4	JEV
S-36.....	day 3	0	20	40	40	20	20	4	4	4	4	4	115	160	<40	65	<10
D-2,3,4		21	160	320	640	160	640	32	32	32	128	32	500	675	<160	350	1000
S-51.....	day 3,5	0	0**	0	0	0	0	0##	0	0	0	0	<40	50	<40	<40	<10
D-2,2		21	320	320	640	320	1280	32	32	64	32	64	450	1600	<160	650	800
S-61.....	neg	0	20	20	20	40	0	4	8	4	4	0	375	50	<40	50	<10
D-4,1,2		21	160	160	320	160	640	64	64	64	64	32	400	2600	<40	500	1000
S-70.....	day 3	0	20	40	80	40	20	16	8	4	8	4	90	200	25	70	15
D-2,3,4,2		21	160	160	320	160	640	32	128	32	128	128	250	950	100	95	1200
S-71.....	neg	0	0	20	20	20	0	4	4	4	8	0	30	160	<10	110	20
D-4,2,3		21	80	160	160	80	320	16	32	32	32	16	25	1050	90	380	500
S-81.....	neg	0	40	40	80	20	20	4	4	4	4	4	40	80	70	20	<10
D-3,2,4		21	80	80	1280	80	320	16	16	32	32	32	130	400	40	600	200
S-92.....	day 5	0	20	20	20	20	20	4	4	4	4	4	<40	60	<40	<40	<10
D-3,4,1		21	320	320	640	320	2560	32	64	256	512	128	400	2000	30	2560	3050
S-94.....	day 3	0	0	40	0	0	0	0	0	0	0	0	25	35	<40	<40	<10
D-3,4,2		21	160	160	320	160	1280	8	16	32	64	32	230	600	40	1380	2950

$ Last dengue inoculation 17-21 months before first JEV inoculation; + 50% plaque reduction neutralization test.
dengue virus serotypes 1,2,3,4 = D1,2,3,4.
** 0 = <1:20 for HAI
* Monitored 3,5 and 8 days after JEV challenge.
0 = <1:4 for CF
Each gibbon inoculated sc with $1x10^5$ pfu JEV.

Table 2. Effect of 2-mercaptoethanol (2-ME) on the HAI Antibody Titers in Whole Gibbon Serum.

Gibbon	Day after JEV inoc.	Reciprocal HAI Titer			
		Dengue-1,2,3,4		JEV	
		C[1]	2-ME[2]	C	2-ME
S-51	0	<20	<20-20	<20	20
	21	320	320-640	1280	1280
S-94	0	<20-40	<20-40	<20	<20
	21	160-320	160-320	1280	1280

(1) Serum aliquot treated with buffer.

(2) Serum aliquot treated with 2-mercaptoethanol.

Table 3. Fractionation* of convalescent serum from a dengue-vaccinated gibbon+ challenged with JEV.

Serum-Sucrose Fraction No.	Immunoglublin$ IgM	IgG	IgA	Reciprocal HAI Antibody titer JEV C#	JEV 2ME@	D4 C	D4 2ME	D3 C	D3 2ME	D2 C	D2 2ME	D1 C	D1 2ME
1	0	0	0	4	2	0	0	2	0	0	0	0	0
2	0	0	0	4	2	2	0	2	0	0	0	0	0
3	0	0	0	8	2	2	0	2	2	2	0	0	0
4	+	0	0	32	2	2	0	2	2	2	2	2	2
5	+	0	0	16	2	2	2	4	2	2	2	2	2
6	0	0	0	4	2	2	2	8	4	4	2	4	2
7	0	+	0	16	8	4	4	8	8	8	8	4	4
8	0	+	+	64	64	32	16	32	32	16	16	16	16
9	0	+	0	128	128	32	16	32	64	32	32	32	32
10	0	+	0	16	16	8	8	32	32	16	8	8	16
11	0	0	0	16	16	8	4	32	16	16	8	16	16
12	0	0	0	32	16	16	8	64	32	32	16	32	16

* Fractionation by sucrose density gradient centrifugation (S-DGC).
+ Serum obtained 28 days after JEV inoculation of gibbon S-94.
$ Presence of immunoprecipitable gibbon immunoglublin in each fraction assayed by radial immunodiffusion in agar containing anti-human IgM, IgG, or IgA; + = Ig detected, 0 = Ig not detected.
# C= aliquot of serum sucrose-fraction treated with buffer.
@2-ME = aliquot of serum-sucrose fraction treated with 2-mercaptoethanol.

Table 4. Production and persistence of JEV monospecific IgM antibody in dengue-vaccinated gibbons following JEV infection.

Gibbon	Day after JEV inoculation						
	0	7	14	21	28	56	90
S-36	0[1]	0	+[2]	+	+	+	0
S-51	0	0	0	+	+	0	
S-61	0	0	0	+	+	+	0
S-70	0	0	0	+	+	0	
S-71	0	0	0	+	0	0	
S-81	0	0	+	+	+	0	
S-92	0	0	0	0	0	0	
S-94	0	0	+	+	+	0	0

(1) 0 = IgM JEV-specific antibody not detected in the serum drawn on that day.

(2) + = IgM JEV-specific antibody detected in the serum drawn on that day.

Table 5. Appearance of IgG and IgM HAI antibody in the serum of dengue-vaccinated gibbons following JEV inoculation.

Gibbon	Day Ig first detected[1]	
	IgG[2]	IgM[3]
S-36	14	14
S-51	14	21
S-61	14	21
S-70	14	21
S-71	7	21
S-81	14	14
S-92	7	not detected
S-94	7	14

(1) Data obtained from HAI tests of S-DGC fractions of serum drawn on days 0,7,14,21.

(2) IgG antibody reactive against JEV and Dengue 1-4 antigens.

(3) IgM data summarized from Table 4.

Table 6. Serological response and viremia in dengue-vaccinated gibbons challenged a second time with JEV.

Gibbon previous virus challenge	Viremia*	Days after JEV rechallenge#	Reciprocal antibody titer												
			HAI					CF				Nt[xx]			
			D1	D2	D3	D4	JEV	D1	D2	D3	D4	JEV	D2	D3	D4	JEV
S-36		0	80	160	40	40	160	0[##]	4	4	4	0	80	0[$]	45	20
D-2,3,4,JEV+	Neg	14	40	80	80	40	1280	0	8	4	4	4	-	-	-	-
		28	40	80	40	40	320	0	8	4	8	4	75	20	60	300
S-51		0	20	40	20	20	80	0	0	0	0	0	35	10	45	30
D-2,2,JEV	Neg	14	40	80	80	80	2560	0	4	4	8	32	-	-	-	-
		28	40	40	40	40	1280	0	4	4	4	32	100	10	20	3300
S-61		0	80	80	80	160	320	4	8	8	16	4	40	0	125	365
D-4,1,2,JEV	Neg	14	40	40	40	160	320	8	8	8	16	4	-	-	-	-
		28	40	40	40	80	320	8	8	8	16	4	125	60	80	530
S-70		0	40	40	80	80	160	8	16	8	8	0	120	25	215	45
D-2,3,4,2,JEV	Neg	14	40	40	80	80	640	8	16	8	8	8	-	-	-	-
		28	40	80	80	80	640	8	16	8	16	8	60	30	30	30
S-81		0	20	20	40	10	80	0	0	0	0	0	150	50	-	0
D-3, 2,4, JEV	Neg	14	20	20	40	20	320	0	0	0	4	4	-	-	-	-
		28	20	20	40	20	320	0	0	4	4	4	45	20	0	90
S-92		0	40	40	80	80	320	0	8	4	4	4	70	35	20	25
D-3,4,1,JEV	Neg	14	40	40	80	40	320	0	8	8	8	8	-	-	-	-
		28	40	80	80	80	320	0	8	8	8	8	20	10	20	200
S-94		0	10	10	20	20	40	0	0	0	0	0	20	20	50	45
D-3,4,2,JEV	Neg	14	40	40	80	40	1280	0	4	0	8	64	-	-	-	-
		28	40	40	80	40	1280	0	4	4	8	32	20	15	10	13200

* Monitored 2,3,4 and 6 days after JEV rechallenge.
\# Each gibbon inoculated sc with $2x10^4$pfu JEV.
xx 50% plaque reduction neutralizat ion test; D1 not tested.
+ First JEV inoculation 13 months before 2nd inoculation.
[##] 0 = <1:4 for CF
[$] 0 = <1:10 for PRNT

Title: Evaluation of the Antigenic Potency of Biken JEV Vaccine in Adults

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Objective: To determine the antigenic potency of the killed Japanese encephalitis vaccine (Biken purified mouse brain) in a group of young adult Peace Corps Volunteers (PCV's) in Thailand.

Introduction: The background and experimental design of this collaborative study between the U.S. Peace Corps in Thailand and SMRL was covered in last year's Annual Report.

Progress: Three different lots of vaccine (A,B,C) have now been given to 3 groups of volunteers, one lot per group. The experimental design is outlined in Table 1. Serological tests have been performed on most of the serum up to and including the 7-12 week post-booster serum. The results of the HAI and PRNT tests against JEV (Nakayama strain) are summarized in Tables 2-5. The average conversion rate in volunteers without detectable JEV antibody in preimmunization serum determined 4-6 weeks following primary immunization for the 3 vaccine lots was 20% by HAI and 17% by PRNT. The total percentage of PCV that had converted after 3 immunizations, including booster, was 31% by HAI and 22% by PRNT.

The results indicate that this killed JEV vaccine lacks antigenic potency in American adults.

A question can be raised concerning the sensitivity of the serological tests used to measure serum antibody. An insensitive test would naturally lead to the conclusion that the vaccine lacked antigenic potency. An attempt was therefore made to determine the ability of the HAI and PRNT to measure antibody in serum obtained from 3 individuals with primary inapparent JEV infections. The serum donors were 3 healthy Chiangmai Valley villagers who had no serological evidence of prior group B arbovirus infection. Their lack of prior exposure to JEV was confirmed by the detection of IgM antibody to JEV in their convalescent phase serum. As seen in Table 6, the HAI and PRNT titer levels were similar in the convalescent serum of all 3 subjects, and it is clear that the

PRNT can detect antibody rises after inapparent natural infections. Moreover titers in 2 individuals are reasonably high (1:40) using the 2 serological tests.

The collection of 7 month post booster serum is complete but the 12 month collection is pending.

Mouse Potency Test.

The antigenic potency of the Biken vaccine (lot B & C) was measured further by a standard mouse potency test. The Japanese NIH method was employed. Groups of mice were inoculated IP every other day for a total of 4 injections with serial dilutions of vaccine. Seven days after the last inoculation of vaccine, the mice were challenged intracerebrally with JEV (strain BKM-984-70), 150-200 mouse LD_{50}/ .03 ml/mouse. Control groups of mice given I.P. saline were similarly challenged. The standard dose of vaccine affording 50% survival (the minimal immunizing dose (MID)) should be 0.02 cc of vaccine or less. This dose is nearly equal to the 1/32 dilution of vaccine used in this test. The results revealed that the MID for vaccine lots B & C were both 0.0625 cc which corresponds to a vaccine dilution of 1/8. Thus two lots of Biken vaccine used in this study are but 1/3 to 1/4 the minimal acceptable potency for a JEV vaccine. If the 7 and 12 month antibody titers show no striking improvement over the low conversion rate in earlier sera, then the vaccine tested may not be suitable for use in American adults.

Table 1. Experimental Design Biken Vaccine Potency Test in PCV.

Immunization	Bleeding	Time following onset	Vaccine lot
1st dose	acute	0 days	A,B,C
2nd dose		7 days	A,B,C
Booster dose	Post 1° immunization	3-6 weeks	A,B,C
	Post booster	7-12 weeks	A,B,C
	Post booster	7 months	A,C
	Post booster	12 months	C

Table 2. Biken JEV Vaccine Study: HAI Test Results after primary immunization.

PCV group	Vaccine lot	No. studied	No. converted[x]	% converted
00-32	A	107	18	17
33	B	11	1	10
34-35	C	91	23	25
	Totals	209	42	20%

x Acute serum titer = $<$1:10

Convalescent serum titer = $\geq$1:20 (3-6 weeks)

Table 3. Biken JEV Vaccine Study: PRNT Results after Primary Immunization

PCV group	Vaccine lot	No. studied	No. converted (x)	% converted
00-32	A	57	9	15
33	B	11	2	20
34*	C	40	7	15
	Totals	108	18	17%

(x) Acute serum = <1:10

Convalescent serum titer = >1:10 (3-6 weeks)

* Group 35 sera not yet titered

Table 4. Biken JEV Vaccine Study: HAI Test Results after Booster immunization.

PCV group	Vaccine lot	No. studied	No. converted (x)	% converted
00-32	A	50	13	26
33	B	11	1	10
34-35	C	81	31	38
	Totals	142	45	31%

(x) Acute serum titer = <1:10

Convalescent serum titer = ≥1:20 (7-12 weeks)

Table 5. Biken JEV Vaccine Study: PRNT Results after Booster Immunization

PCV group	Vaccine lot	No. studied	No. converted (x)	% converted
00-32	A	31	5	16
33	B	11	3	30
34*	C	39	10	24
	Totals	81	18	22%

(x) Acute serum titer = <1:10

Convalescent serum titer = >1:10 (7-12 weeks)

* Group 35 sera not yet titered

Table 6. Serological Test in Primary* Inapparent JEV Infection

Villager	Serum no.	Date	Reciprocal Antibody titer vs JEV HAI	PRNT
JE-C-6c	44586	9 June 70	0x	0x
	46037	14 August 70	80	60
JE-L-12I	44709	11 June 70	0	0
	44710	23 June 70	40	20
JE-L-11a	44762	15 June 70	0	0
	44763	29 June 70	80	105

* Primary infection confirmed by detection of IgM HAI Antibody to JEV in the convalescent phase serum.

x 0 = <1:10

Isolation of Ingwavuma Virus and Study of Ingwavuma Antibody Prevalence

in the Chiangmai Valley

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Purpose: To define the role of Ingwavuma virus in infection of man and animals in the Chiangmai Valley.

Background: A virus (BKM 705-70) was isolated from a pool of 10 Culex vishnui mosquitoes aspirated from a pig in the Chiangmai Valley on 11 May 1970. The virus was identified as a member of the Simbu group of arboviruses, Ingwavuma virus by the Department of Virus Diseases WRAIR, and Dr. Robert E. Shope, Yale Arbovirus Research Unit. This strain represents the first isolation of a Simbu group virus in Thailand and the first isolation of Ingwavuma virus from a possible insect vector. Little is known of the role of Simbu group arboviruses and specifically Ingwavuma virus in human infection and disease. Further, potential vertebrate hosts of Ingwavuma virus, previously isolated only from birds in South Africa, India, and Egypt, remain unidentified. The isolation of Ingwavuma virus during the JEV study in the Chiangmai Valley during which sequential bleedings of Chiangmai villagers and schoolchildren and random bleedings from domestic animals were obtained, permitted a retrospective search for Ingwavuma antibody in human and domestic animals in the valley.

This report describes the establishment and validity of a macro and micro plague reduction neutralization test (PRNT) for Ingwavuma antibody and describes the prevalence of Ingwavuma antibody in the Valley based upon the micro-PRNT.

Methods: Technique for isolation of viruses from mosquito pools has been described in the 1971 Annual Report. Technique for Ingwavuma macro and micro PRNT are identical to those described for JEV PRNTs in the 1971 Annual Report. LLC-MK-2 cells were utilized in both tests. In order to test the validity of a micro-PRNT as an antibody assay, 2 rabbit hyperimmune Ingwavuma antisera were assayed by the micro-PRNT and standard macro-PRNT. Serum A was undiluted BKM 705-70 antiserum and serum B an unknown dilution of serum A. Both sera were diluted to

1:10 and heated at 56°C for 30 minutes prior to use. Serial 2-fold dilutions of serum A and B were made for both tests. Three replicate bottles were used for each serum dilution and the virus control in the macro-PRNT. Six replicate wells were used for each serum dilution and the virus control in the micro-PRNT.

In both tests, the arithmetic mean of the 6 (micro) wells or 3 (macro) bottles of control virus was used to calculate the percentage reduction for each individual well or bottle in a test. The serum dilutions used were transformed to logarithms and the percent plague reduction to corresponding probit values. The 50% effective dose (ED_{50}, serum dilution which caused a 50% reduction in the number of plagues); 95% confidence limits of the ED_{50}; relative potency (RP) of serum in each of the two PRNT tests; and the 95% confidence limits of the RP were calculated by the method for a parallel line, graded response bioassay.

Human sera were collected from 4 study villages and an urban Chiangmai school during November 1970. Sera from chickens, ducks, cattle, buffaloes, pigs, and dogs from the 4 study villages was collected in November or July 1970. All human and animal sera tested were diluted to 1:10 and heated at 56°C for 30 minutes. A 1:20 dilution of serum was used to screen for Ingwavuma antibody in the micro-PRNT.

Progress: In addition to BKM 705-70, 10 additional pools of 4 mosquito species (Culex vishnui, fuscocephala, tritaeniorhynchus, and Aedes linneatopennis) yielded Simbu group agents (which have not yet been tested for identity to Ingwavuma virus). All 10 pools were obtained from biting collections on cattle and buffalo at San Sae obtained on 22 May 1970. Since both unengorged and engorged mosquitoes were contained in these pools, these isolations may have contained engorged mosquitoes which fed on the same viremic host. No evidence of a possible vector role for these 4 species was thus obtained. The virus was reisolated from positive pools of all 4 mosquito species.

In order to utilize a micro-PRNT as an antibody assay in seroepidemiologic studies subsequently described, it was important to determine whether this assay measured Ingwavuma antibody similarly to the standard macro-PRNT. Therefore a standard undiluted hyperimmune rabbit Ingwavuma antiserum (serum A) and an unknown dilution of this antiserum (serum B) were used as described in methods section to compare the similarity of both tests in measuring antibody. Results of these tests are shown in Table 1 (serum A) and Table 2 (serum B). In both tests using both sera, at least 4 responses were obtained between 15-85% plague reduction and plague reduction responses bracketed 50% plague reduction. Control plague counts averaged 32 in the macro and 15 in the micro-PRNT.

Statistical analysis of these tests by the method for a parallel line, graded response bioassay are shown in Table 3. With serum A, the mean probit response and regression lines (Y intercepts) are in close agreement, and the neutralizing slopes of both tests were parallel. The relative potency of serum A (89%) is close to the expected 100%. Since the error variance in this comparison is quite small resulting in narrow confidence limits, the 95% confidence limits of the relative potency do not overlap 100%. The ED_{50} of serum A was 501 by macro-PR and 564 by micro-PRNT. From a biologic standpoint this difference is small, and only because of small error variance of the test and the closeness of the mean probit responses to 5 it is a significant difference.

With serum B, the mean probit responses and regression lines are in close agreement and the neutralization slopes of both tests nearly parallel. The 95% confidence limits of the relative potency clearly overlap 100% and the ED_{50} of serum B by both tests remarkably close.

Neutralization slopes of the 2 sera by both PRNT tests are illustrated in figures 1 and 2. In both figures the mean points at each dilution are quite close to the estimated regression line, evidence of the small error in these tests. The closeness of the mean plague reduction points of each PRNT for each serum indicate a similar slope of neutralization for each test and are compatible with the hypothesis that both tests are measuring identical antibodies in the same sera.

These results indicated that the micro-PRNT indeed will reproduce virtually identical results to the standard macro-PRNT test, providing at least 14 plagues are obtained in the control wells, at least 3 responses are obtained between 15-85% plague reduction, and the responses bracket 50% plague reduction.

Sera were initially tested at a 1:20 dilution in the micro-PRNT. Sera showing equal or greater than 50% plague reduction were considered to contain antibody to Ingwavuma virus. Representative positive sera were further examined for antibody titers.

The results of testing serum obtained from 490 residents of the Chiangmai Valley in November 1970 (6 months after the initial isolation of the virus) is shown in Table 4. None of the 490 inhabitants tested possessed Ingwavuma antibody at a 1:20 serum dilution.

The prevalence of Ingwavuma antibody in various domestic animal species indigenous to the 4 valley villages studied is shown in Table 5. The prevalence of antibody in cattle, water buffalo, chickens and ducks

was low. Further, the titers in animals considered positive (equal or greater than 50% plague reduction) did not equal 1:40 in the 2 cattle, 4 buffaloes, 3 chickens, or 4 ducks tested.

In contrast, the prevalence of Ingwavuma antibody in dogs and pigs in the 4 study villages was higher. Most of the dog and pig sera found positive for antibody gave 100% plague reduction at the 1:20 screening dilution. Representative sera from 11 dogs and 16 pigs all showed antibody titers of $\geq$1:160 upon further testing.

Sequential bleedings of domestic animals in the Chiangmai Valley was not reproducibly accomplished during the 1970 study; consequently serum from serologically positive domestic animals obtained prior to November 1970 was not available to test. Thus it is not known whether some of these animals developed Ingwavuma antibody during 1970 when the virus was isolated in the Valley.

To measure the force of JEV infection in the Valley during the 1970 season, sentinel pigs (which lacked antibody to JEV) were purchased from farms in Nakorn Panom and Saraburi and placed in the four study villages. Four of these sentinel pigs bled after residence in the Valley were found to have Ingwavuma antibody in sera obtained in November 1970. Serum obtained in April 1970 (one pig) and July 1970 (3 pigs) was tested for Ingwavuma antibody to determine if Ingwavuma infection occurred while these animals were quartered in Chiangmai Valley. All 4 sentinel pigs had Ingwavuma antibody in sera botained before shipment to the Valley.

These findings for Ingwavuma virus in Thailand are similar to those found for other arboviruses of the Simbu group. Many of these viruses have been isolated from Culex species, and antibody prevalence studies have shown that large domestic animals (cattle, goats, or pigs) are important hosts. With most Simbu group viruses, man appears to be a rare and probably incidental host. We found no evidence of human Ingwavuma infection in Chiangmai Valley.

Table 1. Comparison of macro with micro-PRNT (serum A)

Dilution[a]	Macro-PRNT			Micro-PRNT		
	No. of plaque[b]	Average	% plaque reduction	No. of plaque[c]	Average	% plaque reduction
20	0,0,0	0	100	0,0,0,0,0,0	0	100
40	0,0,0	0	100	0,0,0,0,0,0	0	100
80	1,0,2	1	97.2	2,0,0,0,2,0	0.67	95.6
160	6,4,9	6	81.25	3,2,2,1,3,3	2.33	84.2
320	9,13,14	12	62.5	5,6,6,5,5,5	5.33	65.4
640	20,17,16	18	43.7	8,9,9,9,8,8	8,17	49.4
1280	25,26,23	25	21.9	13,10,11,12,10,11	11.11	27.9
2560	28,29,31	29	9.4	15,16,16,16,15,15	15.5	0

a
Reciprocal of serum dilution

b
Control plaque count in macro-PRNT 34,34,28 - average 32

c
Control plaque count in micro-PRNT 15,16,15,16,15,15 - average 15.4

Table 2 Comparison of macro with micro-PRNT (serum B)

Dilution[a]	Macro-PRNT			Micro-PRNT		
	No. of plaque[b]	Average	% plaque reduction	No. of plaque[c]	Average	% plaque reduction
10	0,0,0	0	100	0,0,0,0,0,0	0	100
20	1,0,0	0	100	0,0,1,1,0,1	0.5	96.8
40	2,2,4	3	90.6	6,2,1,4,2,2	2.83	81.7
80	5,6,7	6	81.25	6,4,6,6,4,4	5	67.5
160	15,18,15	16	50.0	3,6,8,8,7,8	6.67	56.7
320	20,22,20	21	34.4	12,9,9,10,9,8	9.5	38.3
640	25,26,25	25	21.9	13,11,10,12,13,10	11.5	25.3
1280	29,30,29	29	9.4	16,15,14,14,14,16	15	0

a
Reciprocal of serum dilution

b
Control plaque count in macro-PRNT 30,34,32 - average 32

c
Control plaque count in micro-PRNT 15,15,15,16,15,16 - average 15.4

Table 3. Summary of Ingwavuma virus PRNT analysis comparison of macro-with micro-PRNT

	Serum A		Serum B	
	Macro	Micro	Macro	Micro
Dilutions Used	160-1280	160-1280	80-640	80-640
Mean Probit Response	5.08	5.18	4.93	4.90
Parallelism Validity	Valid		Borderline	
Curvature Validity	Valid		Valid	
Error Variance	.039		.060	
Combined Slope	-1.8248		-1.7965	
Y Intercept	9.927	10.021	9.067	9.042
Relative Potency (%)	89		103	
95% CL Rel. Potency (%)	86-92		98-109	
ED_{50}	501	564	183	178
95% CL ED_{50}	486-516	552-567	176-192	173-183

Table 4. Antibody to Ingwavuma virus in the villagers and school children in Chiangmai valley

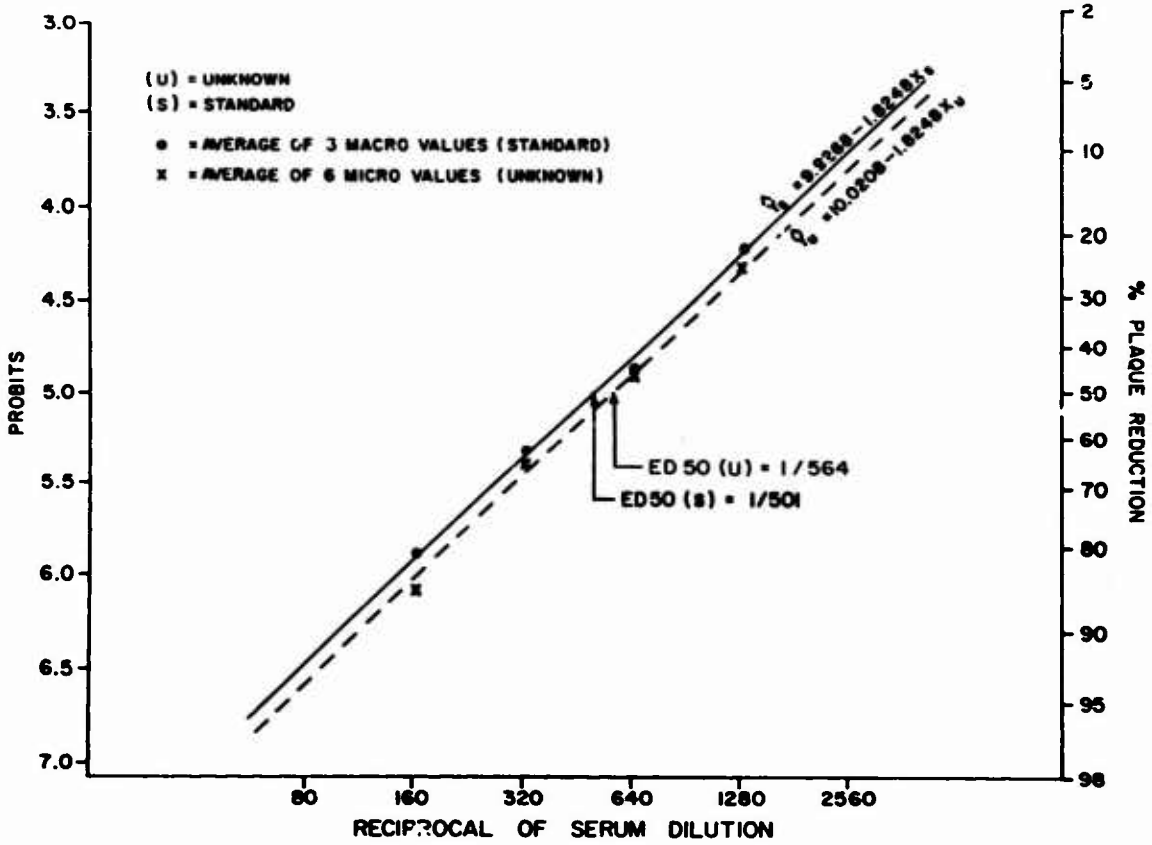
Study area	No. studied	No. with Ingwavuma Antibody	Prevalence rate (%)
(A) - Maerim	81	0	0
(B) - Sanpatong	79	0	0
(C) - Sankampang	81	0	0
(D) - Sarapee	91	0	0
(E) - Paug Chang School	158	0	0
Total	490	0	0

Table 5. Antibody to Ingwavuma virus in the animal sera in Chiangmai valley

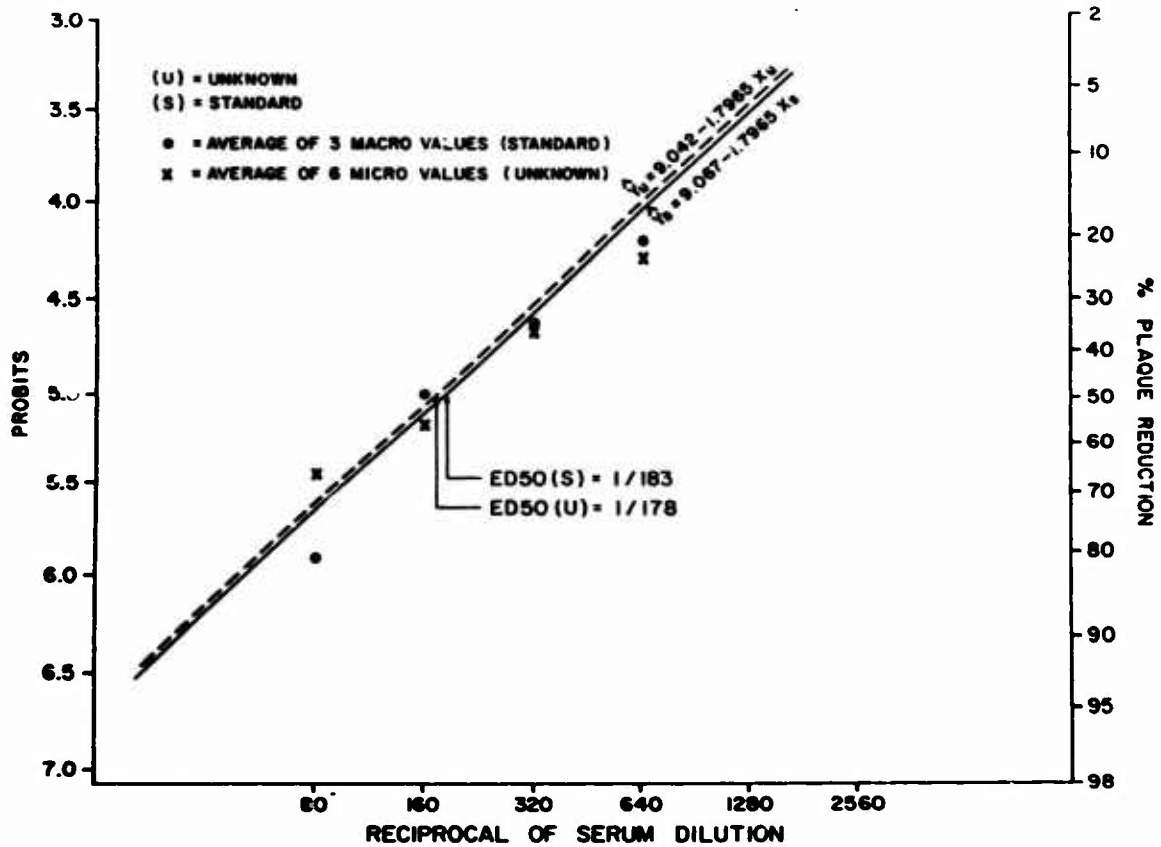
Species[a]	No. studied	No. with Ingwavuma antibody	Prevalence rate (%)
Bovine	50	2	4
Buffalo	50	4	8
Chicken	50	3	6
Duck	55	4	7.3
Dog	50	11	22
Pig (Local)	26	7	26.9
(Sentinel)	24	11	45.8

[a] Collected from area (A) through (D)

INGWAVUMA VIRUS ANTIBODY; COMPARISON OF MACRO (s) AND MICRO (u) PLAQUE REDUCTION NEUTRALIZATION TESTS SERUM A.



INGWAVAMA VIRUS ANTIBODY; COMPARISON OF MACRO (S) AND MICRO (U) PLAQUE REDUCTION NEUTRALIZATION TESTS SERUM B.



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Pathogenesis of Dengue Shock Syndrome

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Purpose: To determine if dengue shock syndrome is due to immunopathologic processes.

Background: Dengue hemorrhagic fever (DHF) can be defined as a febrile illness due to dengue infection with associated hemorrhagic manifestations. The dengue shock syndrome (DSS) is considered to be a severe form of DHF differentiated by shock as manifested by a pulse pressure of less than 20 mm Hg or a fall in systolic blood pressure below 90 mm Hg.

The course of a patient with DSS typically consists of an early febrile phase lasting 3 to 6 days followed by a shock phase of a few hours to three days. The onset of shock is often preceded by lethargy and abdominal pain and is usually abrupt. Prior to the onset of shock, the hematocrit rises and the concentration of serum proteins falls. The decreased plasma volumn is apparently due to a sudden increase in vascular permeability with resultant loss of plasma from the intravascular compartment. In most cases, early and effective replacement of plasma volume by infusion of colloids results in a favorable outcome.

All 4 dengue serotypes have been associated with DSS. The presence of 2 or more dengue serotypes and rapid dengue transmission in a locality appear to be the basic epidemiologic conditions essential to the occurrence of DHF and DSS. Epidemiologic evidence indicates that DSS is associated only with secondary dengue infections which result in an anamnestic antibody response with very high titers of effective complement fixing IgG antibody appearing early in the course of infection when virus or viral antigens may also be present in vivo. A marked depression of Bl c/a

levels has been documented during the shock phase of illness, suggesting that the third component of complement may be consumed in DSS. These observations lead to the hypothesis that DSS may be immunologically mediated and that physiologically active products produced in vivo from activation of the complement system such as the anaphylatoxins, C3a and C5a, may be the means by which increased vascular permeability and shock are produced.

To test this hypothesis, a joint study sponsored by the WHO was undertaken in Bangkok from Jul-Oct 1971. Briefly an attempt was made to study at least 20 well characterized patients with DSS for

1. immunohistochemical evidence of immune complexes in biopsy material and peripheral blood leukocytes

2. circulating immune complexes

3. immunologically induced consumption of complement components

4. evaluation of the coagulation and fibrinolytic systems.

This report will concentrate principally upon the virologic data obtained at SMRL and the complement data obtained at Scripps Institute. Data obtained from the other collaborating laboratories is not detailed but used only to emphasize major findings.

Methods: Study patients were diagnosed as having DHF by clinicians; patients studied were in shock or felt likely to develop shock, but some non-shock patients were included for comparative purposes. A standard chart for pertinent signs, symptoms, clinical laboratory findings, and treatment was instituted and maintained at each of the 4 hospitals. At the conclusion of the study, the clinical charts were reviewed and the following criteria were used to grade patients studied into 4 groups based on severity of illness.

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 ($\leq$20 mm Hg) or hypotension (systolic pressure <90 mm Hg).

Grade IV: Moribund patients with undetectable blood pressure or pulse.

Techniques for viral isolation, identification of isolates, and serologic tests have been described in previous annual reports. Viral isolation attempts were made on initial serum samples obtained from all study patients. In hemagglutination-inhibition (HI) tests, antigen prepared from dengue-1 (Hawaii), dengue-2 (New Guinea C), dengue-3 (H-87), dengue-4 (H-241), Japanese encephalitis (Nakayama), and Chikungunya (Ross) viruses was used. Acetone extracted sera were tested against 8 units of antigen. All sera from individual patients were tested simultaneously.

For immunochemical quantitation of complement proteins, the single radial immunodiffusion method was used. Monospecific antiserum to human complement proteins (C1q, C1s, C3, C4, C5, C6, C8, C9, and C3 Proactivator) was incorporated at a previously determined, optional concentration into 1.5% agarose gel, containing isotonic buffer, pH 8.0 and 0.01 M EDTA. Seven µl of 3 different test serum dilutions were applied into 3 mm wells and the diameter of the precipitin rings was measured after 24 hr. at room temperature. Each immunoplate received 5 different dilutions of a standard serum. The absolute amount of a complement protein in a given test serum was determined graphically using an r^2 vs concentration of complement proteins in the standard serum had previously been quantitated in La Jolla using highly purified proteins as reference substances. Transferrin was assayed with reagents made available by Dr. Ursula Muller-Eberhard. All assay reagents and the 10 different types of immunoplates were prepared in La Jolla. Complement determinations were performed in Bangkok and at La Jolla by Dr. Muller-Eberhard and associates.

Quantitation of the levels of clottable fibrinogen and split products of fibrinogen plus fibrin was done by a radial diffusion method utilizing antiserum to fibrinogen.

The values in µg/ml of the various proteins in the normal serum pool used in this study are as follows: C3 1500, C4 400, C5 75, C6 60, C3 PA 230, transferrin 2500, and fibrinogen 1720. These values were quite similar to mean serum protein levels determined in 30 Thai children, and were used as normal levels in the following analysis.

Results: The original study comprised 94 patients. Of these, 55 individuals who were adequately studied and had isolation or serologic evidence of recent dengue virus were selected to form the group of patients upon which this report is based. All patients with grade III and IV disease initially studied are included in this analysis. Five of these patients were grade I, 14 grade II, 23 grade III, and 13 grade IV. Pertinent signs, symptoms, and clinical laboratory findings of these patients are summarized

in Table I. The clinical symptomatology of patients studied were similar to those described in previous outbreaks. Of the 4 major manifestations of DHF, fever and hepatomegaly were found consistently in patients of all 4 grades of disease. Hemorrhagic manifestations were found in all grades of disease but Grade I (by definition), but severe bleeding-melena and or hematemesis-was observed only in shock cases. The last manifestation of DHF shock was limited (by definition) to Grade III and IV. Hemoconcentration (a high hematocrit falling by at least 20% on recovery) was observed in all grades of disease, but most frequently in Grade III patients. The lower incidence of hemoconcentration in Grade IV patients probably reflects blood loss from gastrointestinal hemorrhage.

Patients were considered to have had dengue infections if $\geq$4-fold rises in antibody titer to at least 2 of the group B antigens were found between acute and convalescent serum or if convalescent antibody titers to at least 2 antigens equalled or exceeded 1:640. Forty-five of the 55 study patients had diagnostic rises in antibody titer consistent with dengue infection and an additional 8 had high, fixed titers suggestive of recent dengue infection. Convalescent serum was not obtainable on 2 patients who died with symptoms suggestive of DSS.

An attempt was made to determine what proportion of the children studied had primary or secondary dengue infections. Since most patients were studied at least 4 days after the onset of illness, determination of type of infection could not be assessed by presence or lack of antibody in acute serum, but was assessed in this study by the magnitude of HI antibody titers in convalescent serum. Patients with HI antibody titers of $\geq$1:640 to at least 2 dengue antigens were considered to have secondary infections, while those with convalescent antibody of $<$1:640 were considered to have primary dengue infections. By these criteria, 51 of the 53 patients from whom convalescent serum were tested had secondary infections, as shown in Table 2. The 2 patients with primary infections were both infants (6 months of age or less) whose mothers possessed dengue HI antibody.

A dengue virus was isolated from the acute serum specimen of 9 of the 55 study patients; 8 isolates were dengue 2 and one isolate dengue 1. In addition an isolate of dengue 3 and isolates of chikungunya virus were obtained in 1970 from Bangkok children not included in the study. An isolation of dengue virus from study patients correlated with time after onset of disease the patient was initially studied (7 of the 9 patients with dengue isolates were studied on or before the fourth day of disease) and with the dengue 2 HI antibody titer in the serum used for isolation (Table 3). Five of the 9 children with dengue isolates had virus present with dengue antibody in early serum.

One hypothesis that could be tested in the study was whether there was a difference in HI antibody response between children with shock and children without shock. Shown in Figure 1 are $\log_{10}$ geometric mean dengue 2 HI antibody titers and 95% confidence limits by day after onset of illness for both categories of patients. There is no significant difference in HI antibody titer between shock and non-shock patients at any time after onset of illness.

Since no differences in antibody titers were found between the 2 groups, it is permissible to use HI titers of patients with or without shock in order to describe antibody patterns of either group. Shown in Figure 2 are geometric mean dengue 2 HI antibody titers for patients with serum tested on day 3 or 4 and day 5 or 6 and day 7 or 8. Similary shown are titers for patients with serum tested on day 4 or 5 and day 6 or 7, and day 8 or 9. Between 4 to 7 days after onset dengue antibody titers rose geometrically. It is precisely during this time after infection when shock developed in the majority of severely ill children in this study.

Sequential levels of complement components and transferrin on 4 representative patients are shown in Figure 3, supplied through the courtesy of Dr. Muller Eberhard. Patient #26 (Grade IV) was a 6 month old Thai female admitted on the 4th disease day in shock and passing melena. On examination she was unconscious, cyanotic, and unresponsible to pain with a pulse exceeding 180/min, unobtainable blood pressure, scattered skin petechiae, and a liver palpable 2 cm below the RCM. Initial laboratory findings included a Hct of 24%, WBC of 19,300, and a platelet count of 27,000/mm^3. She responded to I.V. infusions of fluids and blood within 6 hours of admission. The platelet count remained low until the 7th disease day when it rose to 221,000/mm^3. Dengue 2 virus was isolated from serum obtained on admission. Dengue 2 HI antibody titer was $<$ 1:20 on admission and 1:160 7 days later. This is the only child with a primary dengue infection who developed shock; the infant's mother had serum HI antibody to all 4 dengue serotypes. This child exhibited very low C3 and C4 levels and less depressed C3 PA and C5 levels until the 10th day of disease. Between day 10 and day 21, a recovery of all complement proteins to normal or supranormal levels was noted. Transferrin levels were relatively stable throughout.

Patient #33 (Grade IV) developed shock while hospitalized on the 6th disease day. Dengue 2 virus was isolated from serum on admission (DD4) in which no dengue HI antibody was detectable at 1:20 dilution. Dengue antibody at a level of 1:320-1:640 was present on the day of shock and rose to between 1:5120-$\geq$1:20480 12 days after the onset of illness. The child recovered from shock on DD9. On day 5, before shock was manifest, C3 and C3PA levels were 70-75% normal, C5 was 50% normal, and C4 supranormal (150%). With onset of shock C3 and C4 fell, whereas C3PA was little effected. Transferrin remained between

80-90% normal throughout the preshock and shock period falling 70% normal after shock. By day 12, values of all complement components were approaching normal levels.

Patient #13 (Grade III) was admitted on the 6th DD with a blood pressure of 50/0, cold and clammy skin with occasional petechiae, a Hct of 45%, WBC of 20,200, and decreased platelets on smear. Dengue virus was not isolated from her initial serum which contained HI antibody to the 4 dengue serotypes at levels of 1:320-1:1280. A greater than 4-fold rise in antibody to all dengue serotypes was demonstrated between acute and convalescent serum. Shock responded to infusions of plasma within 4 hours of admission. This child shows very low C3, C4, and C5 levels following the onset of shock with C3PA less severely affected. Transferrin remains between 80-90% normal with a late rise. C3, C3PA, and C5 levels rose to about 70% or normal by day 12 when C4 was still markedly low.

Patient #41 (Grade II) is a 10 year old male admitted on the 4th day of illness with fever, anorexia, and abdominal pain. On admission, he had scattered petechiae, a liver palpable 3 cm below the RCM, and a Hct of 50%. Three days later his Hct was 40%, WBC 4,150, and platelet count 14,000/mm^3. No evidence of shock or further bleeding manifestations developed during his hospitalization. Dengue virus was not isolated from serum obtained on the 6th disease day which had HI antibody to all dengue serotypes at levels greater than 1:20,480. The pattern of complement protein changes was mildly abnormal with C3, C4, C5, C3PA, and transferrin initially being 50-90% of normal on admission; by day 10 all values fell within 84-153% of normal.

Table 4 shows the proportion of shock patients with greater than 50% reduction in the complement proteins C3, C4, C5 and C3PA and transferrin on days after onset of shock. Between 60-82% of shock patients had significant C3 reduction on the day of or 2 days after onset of shock. On the other hand, 50% reduction in the non-complement protein transferrin were uncommon in shock patients, suggesting that reductions in C3 were due to consumption rather than inhibition of synthesis or extravasation from the vascular compartment. Significant depression of C5 and C4 levels were evident in 30-50% of shock patients and of C3PA levels in 15-30% of shock patients during the shock or immediate post shock period. In more convalescent samples obtained 6 days after the onset of shock, no patients had greater than 50% reductions of C3, C5, or C3PA, but about 25% of patients still had C4 reduction. Thus significant depression of serum complement protein levels in DSS patients was temporarily related to the shock or immediate post shock phase of illness.

Table 5 shows the proportion of patients with greater than 33% reductions in various blood proteins and platelets on the day of onset of shock and

2 subsequent days in patients developing or not developing shock. Of shock patients, 100% showed depression of C3, 89% of C5, 77% of fibrinogen, 72% of C4, and 67% of C3PA. However, 56% of shock patients showed similar reductions of transferrin levels. Consequently, depression of complement proteins or fibrinogen at this level may be caused by mechanisms other than specific consumption - i.e. by failure of synthesis or by extravasation of these proteins from the vascular space across endothelium of increased permeability.

Table 6 shows the proportion of patients with greater than 50% reductions of the same blood components. At this level, relatively few shock patients (11%) had depressed transferrin concentrations; thus greater than 50% depression of complement proteins were likely to reflect specific consumption. Of shock patients, 89% had depressed C3, 51% depressed C5, 58% depressed C4, and 33% depressed C3PA concentrations in serum obtained on the day of or 2 days subsequent to shock. Although 44% of children not developing shock had depressed C3 levels, significant depression of C5, C4, and C3PA were unusual in these patients with clinically milder illness.

Discussion: The primary question posed in this study was whether the shock syndrome accompanying dengue was due to underlying immunopathological processes. All but 2 of the study patients had secondary dengue infections which are characterized by an early (4-5 days after onset of illness) and geometric rise in dengue antibody. The 2 exceptions were infants who may well have had circulating maternally acquired dengue antibody at the time of infection. Thus antibody was present at infection or mobilized early in infection in these patients at a time when dengue virus or non-infectious viral antigens may be present. The presence of dengue virus in serum containing detectable quantities of reactive antibody was shown in 5 of the 9 viremic patients and fulfills in vivo conditions for immunocomplex formation in these patients. Attempts to demonstrate circulating immunocomplexes by precipitation with C 1q and monoclonal Rheumatoid factor were made in this study; the specificity of precipitins found as immune complexes remains questionable and must be sought by more specific and sensitive techniques in future studies. The virologic data however provide reasonable circumstantial evidence for the formation of immune complexes in this disease. It seems likely that these immune complexes could fix complement in vivo.

Significant depression of complement components during or shortly after the shock phase were evident in all shock patients studied. Greater than 1/3 reductions in C3 were found in all patients and similar reductions in C5, C4, and C3 PA were found in 67-89% of children with DSS. However since similar depression of transferrin, a non-complement protein not consumed in immunologic reactions, was found in over 50% of such patients, a 1/3 depression in complement protein concentration may

not necessarily reflect immunologic consumption in these patients. Only a small proportion of shock patients (11%) had greater than 50% depression of transferrin levels during shock; thus a 50% reduction of complement proteins would suggest specific immunologic activation of the complement system. Since 89% of shock cases had 50% depression in C3 levels and over 50% had similar depression in C4 and C5 levels, specific activation nad consumption of these complement proteins is likely in DSS patients. Significant depression of complement proteins was found generally only in the immediate shock phase of disease and was uncommon 3 or more days after the onset of shock. A much smaller proportion of non-shock patients had 50% depression of individual complement proteins than shock patients, suggesting a correlation between shock and depressed complement concentrations.

Several other points accrue from the complement data. First, most shock patients showed depressed C4 and C3PA levels, suggesting that the complement system was activated in vivo through both of the 2 known mechanisms - classical (involving the C1 proteins and C4 and C2) and through the recently described C3PA system. Secondly, almost all shock patients had evidence of specific consumption of C3 and C5. Specific actibation of C3 and C5 implies the enzymatic liberation in vivo of 2 biologically active anaphylatoxins, C3a and C5a, which are the most potent mediators of increased vascular permeability known. Data from this study suggests that the increased vascular permeability responsible for shock in these DSS patients could be mediated by these anaphylatoxins released as a result of in vivo immunological activation of the complement system.

Further studies will be required to test this hypothesis. Indeed studies to determine the decay of radioactively labelled C3 in DSS patients, to determine if C3a and C5a can be detected in shock phase serum, to determine the role of serum inhibitors of activated complement components (such as C3a and C5a) are planned in the coming year.

Table 1. Summary of clinical signs and laboratory findings in 55 study patients

Findings	Severity of Illness			
	Grade I(5) %	Grade II (14) %	Grade III (23) %	Grade IV (13) %
Fever	100	100	100	100
Hepatomegaly(2-5cm)	100 (4/4)	91 (10/11)	100 (20/20)	100(13/13)
Positive tourniquet test	50 (1/2)	92 (11/12)	84 (16/19)	62(5/8)
Platelet count				
$<50,000/mm^3$	60 (3/5)	54 (7/13)	85 (17/20)	92 (12/13)
Petechiae	0	100 (12/12)	52 (12/23)	69 (9/13)
Epistaxis	0	0	17 (4/23)	8 (1/13)
Hematemesis/melena	0	0	13 (3/23)	69 (9/13)
Hemoconcentration (≥20% increased hematocrit)	60 (3/5)	71 (10/14)	91 (21/23)	69 (9/23)

Table 2. Proportion of DHF Patients Studied with Primary or Secondary Dengue Infections

Grade Disease	Primary Dengue Infection (conv. titer <640) (No.)	Secondary Dengue Infection (conv. titer ≥640) (No.)	Unclassifiable (No.)
I and II	1	18	0
III	0	23	0
IV	1	10	2
Totals	2*	51	2**

* Both infants <6 months of age

** Both fatalities, convalescent serum not available

Table 3. Dengue Isolation in Relationship to Initial Dengue 2 HI Titer in DHF Patients

Dengue 2 HI Titer	No. of Patients	No. with Isolates
≤ 80	16	9 (56%)
≥ 160	39	0

Table 4. Proportion of Shock Patients with >50% Reduction in Indicated Serum Protein on Day after Onset of Shock

Day after onset of shock	No. Studied	% with 50% reduction in indicated serum protein				
		C_3	C_5	C_4	C_3PA	Transferrin
0	27	17(63%)	11(41%)	10(37%)	6(22%)	2 (7%)
1	34	28 (82%)	10(29%)	17(50%)	10(29%)	3 (9%)
2	27	16 (59%)	8(30%)	11(41%)	4(15%)	0 (0%)
3	24	6 (25%)	1(4%)	6(25%)	4(17%)	0 (0%)
4	21	1 (5%)	0(0%)	4(19%)	2(10%)	0 (%)
≥ 6	17	0 (0%)	0(0%)	4(24%)	0(0%)	0 (%)

Normal levels of serum proteins used for analysis

C_3	1500 ug/ml.	C_3PA	230 ug/ml.
C_5	75 ug/ml.	Transferrin	2500 ug/ml.
C_4	400 ug/ml.		

Table 5. Proportion of Patients with >33% reduction in indicated blood constituent on day of onset of shock or the 2 subsequent days

Serum Protein (or platelets)	Patients with shock		Patients without shock*	
	No. Studied	No. with 33% reduction (%)	No. Studied	No. with 33% reduction (%)
C_3	36	36(100%)	18	14(78%)
C_5	35	31(89%)	18	9(50%)
C_4	36	26(72%)	18	9(50%)
C_3PA	36	24(67%)	18	2(11%)
C_6	35	21(60%)	18	1(6%)
Transferrin	36	20(56%)	18	3(17%)
Fibrinogen	35	27(77%)	16	3(19%)
Platelets	31	28(90%)	12	9(75%)

Normal levels of blood constituents used for analysis

C_3	1500 ug/ml	C_6	75 ug/ml
C_5	75 ug/ml	Transferrin	2500 ug/ml
C_4	400 ug/ml	Fibrinogen	1720 ug/ml
C_3 PA	230 ug/ml	Platelets	100,000/mm^3

* Disease day 5, 6, or 7 or disease day 6, 7, and 8 in patients admitted after disease day 5.

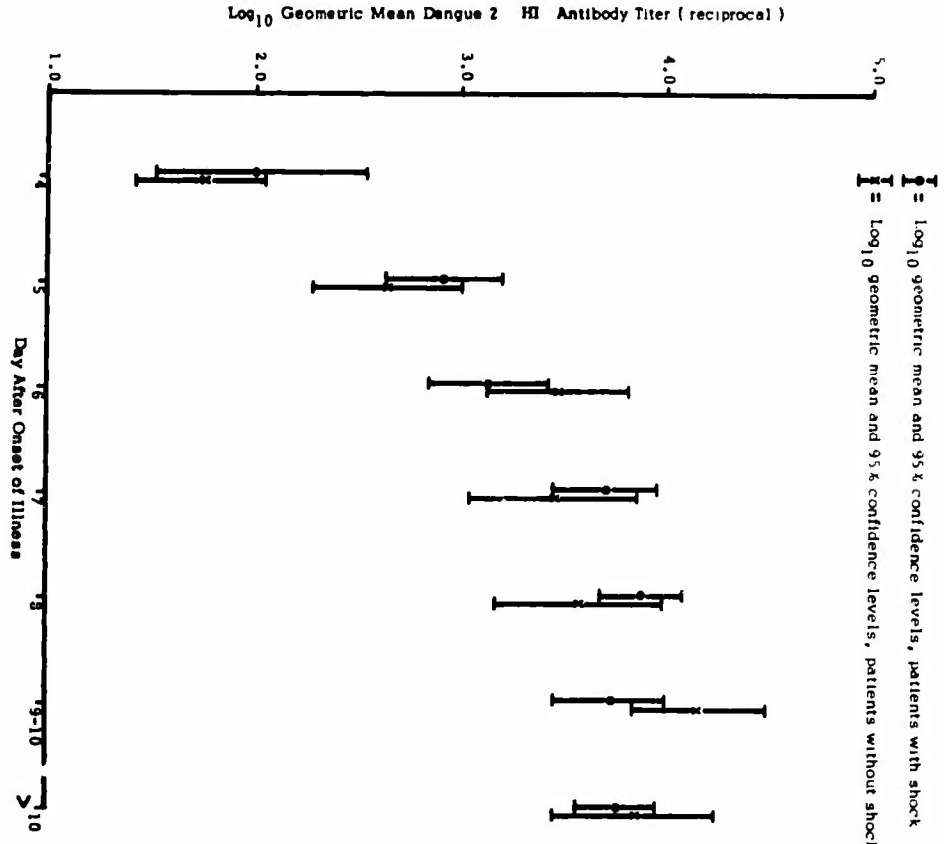
Table 6. Proportion of Patients with >50% reduction in indicated blood constituent on day of onset of shock or the 2 subsequent days

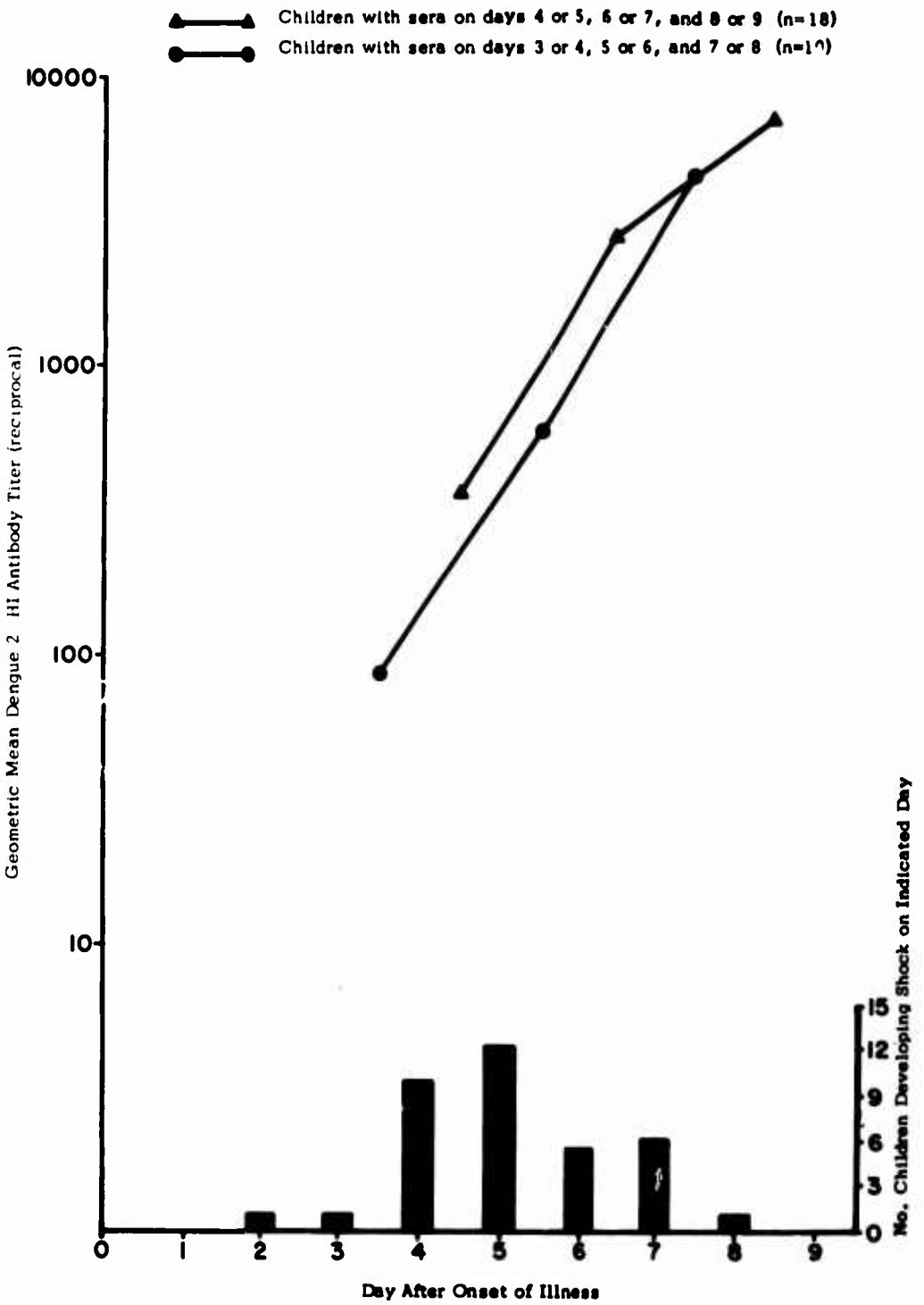
Serum Protein (or platelets)	Patients with Shock		Patients without Shock*	
	No. Studied	No. with 50% Reduction (%)	No. Studied	No. with 50% Reduction (%)
C_3	36	32 (89%)	18	8 (44%)
C_5	35	18 (51%)	18	1 (6%)
C_4	36	21 (58%)	18	1 (6%)
C_3 PA	36	12 (33%)	18	0 (0%)
C_6	35	7 (20%)	18	0 (0%)
Transferrin	36	4 (11%)	18	0 (0%)
Fibrinogen	35	10 (29%)	16	0 (0%)
Platelets	31	28 (90%)	12	7 (58%)

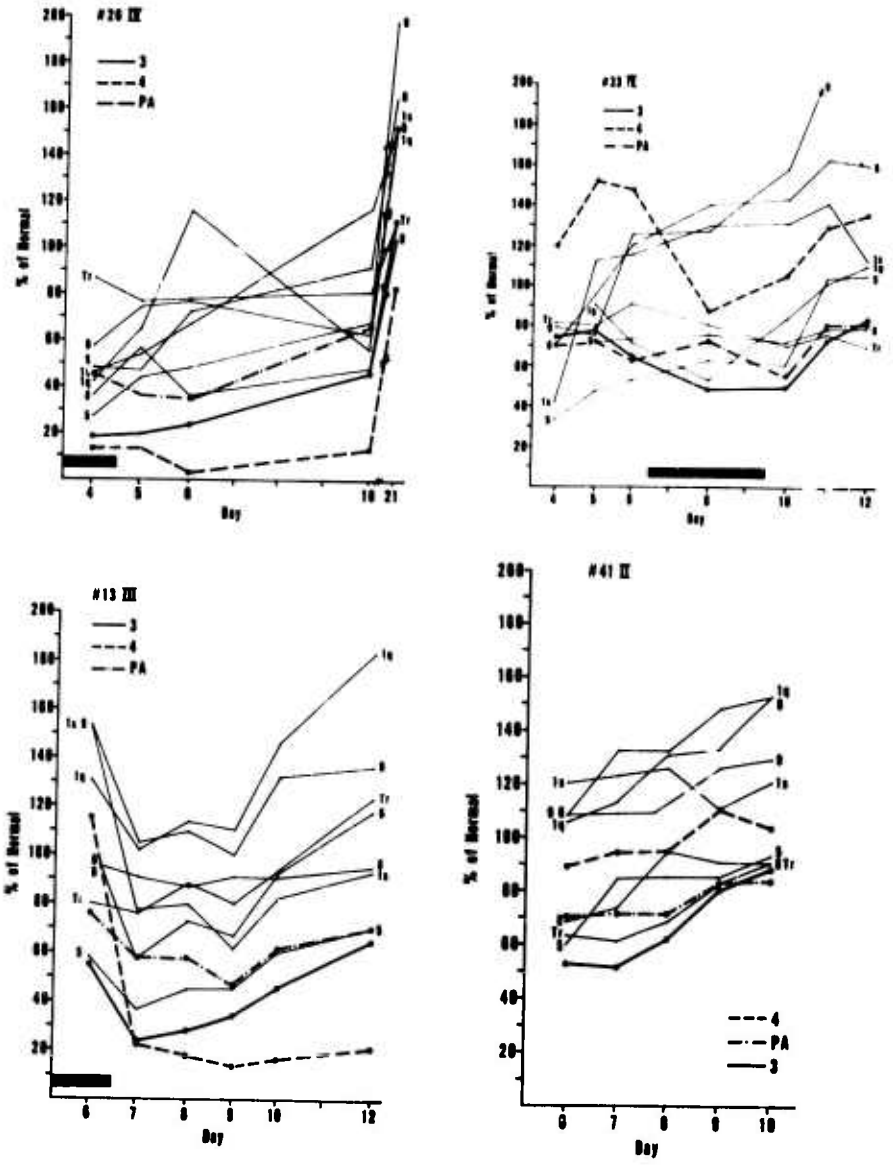
Normal levels of blood constituents used for analysis

C_3	1500 ug/ml.	C_6	75 ug/ml
C_5	75 ug/ml.	Transferrin	2500 ug/ml
C_4	400 ug/ml.	Fibrinogen	1720 ug/ml
C_3PA	230 ug/ml.	Platelets	100,000/mm^2

* Disease day 5, 6, and 7 or Disease day 6, 7, and 8 in patients admitted after disease day 5.


Log10 Geometric Mean Dengue 2 HI Antibody Titer (reciprocal)
1.0
2.0
3.0
4.0
5.0
Day After Onset of Illness
4
5
6
7
8
9-10
<10
= Log10 geometric mean and 95% confidence levels, patients with shock
= Log10 geometric mean and 95% confidence levels, patients without shock


Children with sera on days 4 or 5, 6 or 7, and 8 or 9 (n=18)
Children with sera on days 3 or 4, 5 or 6, and 7 or 8 (n=1^)
10000
1000
100
10
Geometric Mean Dengue 2 HI Antibody Titer (reciprocal)
15
12
9
6
3
0
No. Children Developing Shock on Indicated Day
0
1
2
3
4
5
6
7
8
9
Day After Onset of Illness


#26 III
3
4
PA
% of Normal
Day
#33 VI
3
4
PA
% of Normal
Day
#13 III
3
4
PA
% of Normal
Day
#41 II
4
PA
3
% of Normal
Day

Isolation of dengue virus from dengue hemorrhagic fever patients

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Purpose: To increase the efficiency of isolation of dengue viruses from patients with dengue hemorrhagic fever (DHF)

Background: This Laboratory's standard method of isolating dengue viruses from patients uses serum inoculated into MK-2 cells in the direct and delayed plaque system. Although this procedure permits isolation of dengue viruses from 40-50% of patients with primary dengue infections and from a similar proportion of patients with secondary infections sampled before the fifth day of disease, isolation rates from children with DHF approximate 15% (see previous section). Future studies of the role of specific dengue antigens and antibodies in the pathogenesis of DHF and DSS are likely to be critically dependent upon identification of the dengue serotype currently infecting the patient. Such identification is not currently possible by serologic means, but can only be accomplished by isolation of the agent. Thus we elected to study patients with DHF to see if a number of different isolation techniques might increase isolation efficiency in these patients who are often admitted late in the course of their infection with high dengue antibody titers.

I. Attempts to isolate dengue and chikungunya viruses from throat swabs of children with dengue fever.

Children with DHF have early and rapid rises in serum IgG antibodies to dengue which may result in <u>in vivo</u> neutralization of circulating virus. Neutralization of viruses in respiratory tract secretions appears dependent upon locally formed IgA rather than IgG antibody. Although the kinetics of local IgA dengue antibody rises in secondary dengue infections is unknown, we hypothesized that it likely was slower than serum IgG antibody rises and that dengue virus, if present in the nasopharynx, might be still unneutralized when it could no longer be detected in serum.

Ten patients presenting to the outpatient department of Children's Hospital with fevers of unknown origin and shown retrospectively to have serologic evidence of dengue or chikungunya infection were studied.

Throat swab and serum were obtained from each patient and were inoculated onto MK-2 monolayers in the direct and delayed plaque system. Viruses were isolated from serum of 5 patients (3-dengue 2, 1-dengue 1 and chikungunya) but were not isolated from throat washings of any patient.

II. Dengue isolation attempts from serum or plasma of DHF patients.

Studied were 57 patients admitted to Children's Hospital with a diagnosis of DHF. Serum was drawn on admission and on discharge for dengue HI antibody determinations. Serum (0.3 ml) was also obtained on admission and on discharge for dengue HI antibody determinations. Serum (0.3 ml) obtained on admission was inoculated freshly onto MK-2 monolayer for isolation by the direct and delayed plaque method. Plasma was prepared from heparinized blood (100 µg/ml). Adult female Aedes aegypti mosquitoes, obtained from the Department of Medical Entomology, were inoculated intrathoracically with needles dipped in freshly obtained heparinized blood. Additionally A. aegypti mosquitoes were allowed to feed on fresh heparinized blood from patients through a Baudrache membrane and engorged mosquitoes were collected. Fifteen mosquitoes of each group were kept in the insectary for 14 days when they were triturated and tested for virus by the direct and delayed plaque method using MK-2 cells. An additional volume of plasma was allowed to settle for 3 hours in the refrigerator or centrifuged at 600 RPM in an International Centrifuge for 3 minutes. The top layer containing WBC and platelets was collected. The remaining specimen was centrifuged at 5,000 or 10,000 RPM for 3 minutes and the remaining plasma collected. WBC and platelet counts were performed on the 2 plasma samples and then 0.3 ml volumes were inoculated onto monolayers of MK-2 and Aedes albopictus cells (Fluid Phase Cultures). Maintenance medium from these cultures were harvested at 7,12, and 14 days after inoculation and titered for virus in direct MK-2 plaques.

At the conclusion of this study, serum stored at -70°C from patients with an isolate by any technique was inoculated onto fluid cultures of MK-2 cells as above, and centrifuged plasma samples from these patients were inoculated onto MK-2 cells in the direct and delayed plaque system.

Progress. Of the 57 patients studied, 33 had serologic evidence of recent dengue infection and are included in the following data. No isolates were obtained from any of the 15 patients without serologic evidence of dengue infection, and one isolate was obtained from the 9 patients without convalescent serum samples on whom serologic confirmation of infection was not possible.

Dengue 2 virus was isolated from 6 of the 33 patients with confirmed dengue (18%). A summary of virus isolations by the various techniques from these 6 patients is shown in Table 1. Dengue 2 virus was isolated

from plasma samples of all 6 patients inoculated into MK-2 fluid phase cultures. Virus was isolated from plasma from 3 of 5 of these patients in direct and delayed plaques. It should be noted that centrifuged plasma stored at -70° C for 3 months was used in this latter test. (Fresh centrifuged plasma inoculated into fluid phase cultures in these 5 patients yielded 4 isolates in fluid phase cultures). In contrast, fresh serum from the 6 patients yielded but 2 dengue strains in the direct and delayed MK-2 plaque system, while but one isolate was obtained from the 5 patients whose frozen serum was inoculated into MK-2 fluid phase cultures after storage for 3 months. No dengue isolations were obtained from fresh plasma inoculated into fluid phase cultures of A. albopictus cells. One of the 6 heparinized bloods yielded dengue 2 virus after intrathoracic inoculation and membrane feeding using A. aegypti mosquitoes.

Attempts were made to ascertain whether isolations from fresh plasma in fluid phase MK-2 cultures correlated with the presence of platelets or WBC in plasma. These results are shown in Table 2. The centrifugation technique used did not completely separate WBC or platelets from most plasma samples. Virus was recovered from both uncentrifuged and centrifuged specimens from 5 patients and only from uncentrifuged plasma in one patient. A correlation of virus recovery and platelet or WBC counts in either plasma specimen is not apparent.

We were initially disappointed that the most efficient isolation system in this study (plasma into MK-2 cell cultures) yielded an isolation rate of 18%, similar to the 15% obtained in the DSS study in which serum-MK2 plaques system was used. However, populations sampled in these 2 studies differed in at least one important aspect. A much higher proportion of patients in the DSS study (29%) had dengue 2 antibody titers of ≥1:80 when studied than patients in this study (6%). In the DSS study, dengue was only isolated from patients' serum with dengue 2 HI antibody titers of ≥1:80 (see Table 3 - DSS study, this report). In the present study, dengue virus was isolated from the 2 patients with titers ≥1:80, but also from 4 patients with HI antibody titers ranging from 1:160 or 1:1280 (Table 3). Only one of these latter 4 patients had an isolate from serum. This finding of dengue isolations from heparinized plasma containing relatively large quantities of dengue antibody suggests that heparinized plasma may be a more efficient sample for isolation of dengue virus from DHF patients. Reasons for this finding in the small number of patients with isolations studied is unclear and will require further investigation. A. aegypti mosquitoes or A. albopictus cell cultures were not effective isolation systems in this study.

Table 1. Dengue isolates obtained from DHF patients employing different isolation techniques.

Specimen #	MK-2 cells				A. albopictus cells	A. aegypti mosquitoes		Identity of Isolate
	Fluid phase cultures		Delayed or direct plaque		Fluid phase culture	Intrathoracic Inoculation	Membrane feeding	
	(Plasma)	(Serum)	(Plasma)	(Serum)	(Plasma)	(Heparinized Blood)		
49236	+*	not done	not done	+	0**	0	0	dengue 2
49649	+	0	0	0	0	0	0	dengue 2
49678	+	0	0	0	0	0	0	dengue 2
49693	+	+	+	+	0	+	+	dengue 2
49734	+	0	+	0	0	0	0	dengue 2
49786	+	0	+	0	0	0	0	dengue 2

* positive isolation

** no isolation

Table 2. Relation of dengue virus recovery to presence of cellular elements in plasma

Specimen Number	Centrifugation Speed	Platelet Count (per mm^3)	WBC Count (per mm^3)	Virus Recovery in MK-2 Fluid Phase Cultures
49236	none or 600	53,000	0	+*
	5,000	3,000	0	+
49649	none or 600	63,000	700	+
	10,000	53,000	0	0**
49678	none or 600	141,000	4,400	+
	10,000	28,000	100	+
49734	none or 600	63,000	1,700	+
	10,000	18,000	0	+
49786	none or 600	50,000	4,800	+
	10,000	9,000	0	+

* + = isolate

** 0 = no isolate

Table 3. Dengue isolations from plasma -- MK-2 fluid phase cultures in relationship to dengue 2 HI titer in plasma used for isolation.

Reciprocal dengue 2 HI titer	Number of patients studied	Number of patients with isolates
≤20	1	1*
40	0	0
80	1	1
160	2	1
320	5	2*
640	4	0
1280	6	1
2560	9	0
5120	2	0
10240	1	0
≥20480	2	0
Total	33	6

* includes 1 patient with isolation from serum.

Title: Cellular Immunity in Dengue Infections

Principal Investigators: Varanya Sangpethsong, M.S.
Robert Edelman, LTC, MC

Associate Investigators: Ananda Nisalak, M.D.
Thomas J. Smith (Deceased) COL, MC

Objective: 1) To develop an in vitro system to measure cell-mediated immunity (CMI)

2) To determine whether or not CMI to dengue virus appears in primates and humans following dengue infection.

Background: Little is known of the processes which confer lasting and effective immunity to arthropod-borne viruses. Measurement of any single parameter of the immunological response, such as circulating antibody, may not correlate with resistance to disease under all circumstances. There is increasing evidence that lymphocyte-mediated cellular-immunity (CMI) confers protection against virus diseases caused by paramyxo, herpes, pox and certain oncogenic viruses. Conversely, in some virus infections, it is likely that CMI may actually participate in the pathogenesis of illness. There have been no published studies of the development and function of lymphocyte-mediated CMI in arbovirus infections of man or primates. This project was designed to test for the presence of CMI in arbovirus infections of these hosts using newly-developed in vitro techniques.

Progress: Work on this project started several years ago by COL Thomas J. Smith and Dr. Ananda Nisalak, who introduced the macrophage inhibition assay for macrophage inhibition factor (MIF) into this laboratory. The current investigators have attempted to refine this highly-specific test for cell-mediated immunity and adapt it to the requirements of the project.

Briefly the technique is based on the observation that lymphocytes obtained from immunized donors, incubated in vitro with the immunogen (antigen) excrete a factor (MIF) which inhibits migration of macrophages out of capillary tubes. In the method employed, peritoneal exudate cells from adult guinea pigs are allowed to migrate from capillary tubes onto cover slips in small culture (Mackaness) chambers. MIF is considered present in the culture medium when the measured area of macrophage migration in "MIF" medium is less than 80% of the area measured in the presence of Control Media not containing MIF. Table 1 describes the 4 media used in each MIF assay.

First a number of different culture media were tested for suitability for sustaining migration of guinea pig exudate cells (macrophages) from capillary tubes. The most suitable medium consisted of medium 199, 0.1% glutamine, 7% $NaHCO_3$, 10% inactivated guinea pig serum, 5% fetal bovine serum, antibiotics, and 5% CO_2.

The migration of macrophages obtained from female and male guinea pigs was next compared. The area of macrophage outgrowth from the cut and of capillary tubes was photographed at 24 & 48 hrs, the photograph traced, and the area under the tracing measured with a planimeter. The average area of cell migration for 7 male guinea pigs was 1.71 cm^2 and for 7 females, 1.40 cm^2. Males were therefore selected as macrophage donors.

Next studied was the inherent error and reproducibility of the MIF technique. With the collaboration of LTC R.A. Grossman, Dept. of Epidemiology, a statistical evaluation was made of macrophage outgrowth, looking for possible sources of error. The design of this experiment involved testing macrophages taken from 3 guinea pigs (4 tubes per animal), with area measurements made at 24 & 48 hours. The data was analyzed by the factorial statistical analysis. Our conclusions were 1) repeated measurements of the same tracing are fairly precise (±5%). 2) differences existed between the 3 animals used, although in this particular test they were reflected only in differences in the variability of the observations on capillary tubes and not of their means. Variability found was principally due to the inclusion of outgrowth patterns which were difficult to trace and it was concluded that such tracings should be eliminated. Since occasionally 1 or even 2 of the capillary tubes show unexplained irregularly contoured or incomplete outgrowth patterns at least 4 tubes per test were required. 3) Tracings at 24 hours were as reliable as tracings at 48 hrs.

We next attempted to demonstrate release of MIF by lymphocytes taken from PPD skin test positive donors. Lymphocyte purified to >90% on glass bead columns, and mixed leukocytes containing 50% lymphocytes were incubated with PPD (40 ug/ml) for 3 days. Two "MIF" media were examined; one was not concentrated, and the second was concentrated to 1/5 the original volume on an Amicon ultrafiltration unit and then incubated with macrophages in capillary tubes. Macrophages were similarly incubated with concentrated or unconcentrated control media (see Table 1 for basic experimental design). No inhibition of macrophage migration was observed on several attempts. Unsuccessful experimental variations were attempted: 1) use of killed Mycobacterium tuberculosis instead of PPD, 2) mixing human buffy coat cells with guinea pig macro-

phages in the capillary tube and then adding PPD antigen to this culture medium, 3) adding lymphocytes and antigen directly to the culture medium bathing the capillary tubes. In addition we incubated streptokinase-streptodornase (SK-SD) antigen (50 units) with lymphocytes ($3-5x10^6$ cells/ml) taken from a SK-SD skin test positive donor. One-half of the medium was withdrawn everyday, and fresh antigen containing medium added. The medium was pooled, concentrated 5 to 10 fold, and incubated together with suitable control media within capillary tubes in Mackaness chambers. On 2 occasions inhibition of migration (25% and 50%) was noted in the immune lymphocyte and antigen preparations but in both experiments one of the 3 control preparations also produced inhibition of migration. A third attempt showed no inhibition of migration by the immune or control preparations. Further attempts to demonstrate release of MIF have been indefinitely postponed. Attention will now focus on the less-specific, but technically easier lymphocyte transformation technique. Whatever in vivo CMI technique is finally selected, it must work with no more than 10-15 ml whole blood, which is the maximum volume of blood that can be drawn from children or primates at any one time.

Table 1. Media used for MIF assay

Medium	Culture medium incubated 3 days with: Lymphocytes	Antigen
1. "MIF"	+*	+
2. Control	+	0**
3. Control	0	+
4. Control	0	0

*+ = present in culture medium
** 0 = not present in culture medium

Infection and Malnutrition: Immune Function in Children with Protein-Calorie and Vitamin A Malnutrition.

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**Robert M. Suskind, M.D.

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** Field Director, St. Louis Anemia and Malnutrition Research Center (Malan) Faculty of Medicine, Chiangmai University, Chiangmai, Thailand.

Associate Investigator: Anong Pariyanonda, M.S.

Introduction: This is a collaborative project between SMRL, the Faculty of Science, Mahidol University, and the St. Louis Anemia & Malnutrition Research Center, Chiangmai, Thailand.

Background: The broad purpose of this project is to clarify why malnourished individuals are more susceptible to microbial infections than well nourished persons. In order to elucidate some of the possible mechanisms leading to increased susceptibility, we are evaluating the cellular and humoral immune status of children with protein-calorie malnutrition (PCM). Specifically the humoral parameters being investigated are serum and naso-pharyngeal immunoglobulin levels, serum levels of complement components, including $C1_q$, $C1_s$, C3, C4, C5, C6, C8, C9, C3-proactivator, and total serum hemolytic complement activity ($C'H_{50}$). The cell-mediated immune (CMI) status of the children is being evaluated by measuring the cutaneous response to dinitrofluorobenzene (DNFB) sensitization and to Monilia and Streptokinase-streptodornase (SK-SD) skin test antigens.

We anticipate that better understanding of host defense immune mechanisms against microbial infection in healthy, in addition to malnourished individuals will result from a study of malnourished children.

Study design: This project is designed to conform to the diagnostic and treatment schedule already in effect at the Anemia and Malnutrition Research Center of St. Louis University, Nakorn Chiangmai Hospital (MALAN), Chiangmai Thailand. The clinical phase of the study outlined below is the responsibility of Dr. Robert M. Suskind, and LTC Robert

Edelman, SMRL. The laboratory phase, which consist principally of measuring the levels of immune components in serum, is the responsibility of Dr. Satit Sirising and LTC Edelman.

All patients admitted to the 14 bed research ward of MALAN are treated, studied for 3 months (84-92 days) and then discharged. On admission the patients are clinically evaluated and scored for the presence of maramus, marasmus-kwashiorkor, kwashiorkor according to the modified criterias of McLaren and Gomez (see Table 1). Children are admitted to the study if they have primary malnutrition and weigh more than 3.0 kg. and less than 12 kg.

In addition to this protocol each patient is being extensively studied in another and larger research protocol directed by Dr. Suskind for blood coagulation factors, red cell survival, serum proteins (albumin, globulins, ceruloplasmin and copper, TIBC and iron, lipoproteins, glycoproteins, retinol binding protein, haptoglobin, hemopexin, blood & urine amino acid patterns, serum lipids, erythrocyte & leukocyte enzymes, Australian antigen, and electron and light microscopy of liver biopsies.

At the time of admission each patient is placed in one of 4 dietary groups so that approximately equal numbers of marasmic, marasmic-kwashiorkor, and kwashiorkor patients comprise each group. The 4 groups and their diets are listed in Table 2. Details of the diet fed during the day 1-7 stabilization period are given in Table 3. The stabilization period may be extended if the patient is not able to tolerate the diet given, but this rarely has been necessary. In addition to the diets listed in Tables 2 & 3, large doses of supplemental vitamins and minerals are given starting on day 2. On admission, all patients are treated vigorously for infection and for fluid and electrolyte imbalance. The large majority of patients have infections on admission.

Progress A) Cell-mediated immunity

Contact sensitization to 1-nitro, 2, 4-difluorebenzene (DNFB) is a standard method used to test cell-mediated immunity in vivo. Pre-existing sensitization is rare, approximately 95% of normal subjects can be sensitized to DNFB, and circulating antibodies do not develop from contact sensitization.

The children are sensitized with 2 mg of DNFB in acetone applied to the forearm which is then allowed to dry. The site of application was protected for 24 hours with an acclusive dressing. The children are tested for sensitization to DNFB by applying 100 ug DNFB in acetone

to the opposite forearm 12-14 days after the sensitizing dose was applied. Two days after the skin test dose, the inflammatory skin response at the test dose site is graded. If positive the children are considered immunized.

A total of 19 patients were exposed to 2 mg DNFB on admission; Most were first skin tested with 100 ug DNFB on day 15 while a few patients were 1st tested on day 29 & 44. Skin tests were repeated at 2 week intervals until positive. The results of the attempt to induce DNFB sensitivity in these patients are given in Table 4. Assuming all children were skin test negative on admission. Only 21% of children had converted and were skin test positive when first tested on day 15. An increasing percentage of patients converted to positive after day 15, with 100% of patients skin test positive by day 56. The low percentage of positive patients on day 15 could be explained by a defective immune response, a faulty inflammatory response, or by defects in both of these mechanisms. The increasing percentage of reactors to the skin test dose after day 15 could be due to improved nutritional status leading in turn to repair of a defective effector (antigen recognition & response) limb of CMI or to repair of a deficient inflammatory response. Furthermore repeated skin test doses of 100 ug DNFB may have contributed to the gradually increasing conversion rate to subsequent skin testing by stimulating the affector (lymphocyte sensitizing) limb of CMI. We therefore designed additional experiments to clarify the mechanism of the defective response to DNFB. These experiments, most of which are in progress, are described below.

DNFB is a potent non-specific skin irritant in addition to being a contact allergen. Application of a 2 mg dose to the skin of a non-immune results in marked inflammation (erythema and edema) within 12-72 hours which subsides over 3-5 days. We attempted to measure the skin inflammatory response to DNFB. Three groups of non-immune patients were first sensitized with 2 mg DNFB on admission, on days 15, or on day 56. The degree of skin inflammation was graded 2 days after DNFB challenge, and the results obtained to date are shown in Table 5. Only 9 of 25 (36%) of those sensitized on admission had an inflammatory response consisting of induration and/or vesicle or bleb formation, whereas 3 of 3 children sensitized on day 56 had an inflammatory response. Greater than 75% of normal adults can be expected to show an inflammatory response according to a previous report. It therefore appears that in PCM, the inflammatory response may

be deficient on admission but improves coincident with nutritional repair. Further studies are planned to study their inflammatory skin responses using the Rebuck skin window technique.

We attempted to determine when the CMI response can be induced by testing 5 patients from each of the 3 treatment groups shown in Table 5. These patients, sensitized for the first time on days 1, 15, or 56, were all skin tested with 100 ug DNFB on day 70. The results shown in Table 6 show that none of 4 pts could be sensitized on day 1, but 3 of 3 patients could be sensitized on day 56. The failure to sensitize on day 1 strongly suggests that a defect in the CMI response exists on day 1. Because the effector portion of the CMI response (Table 7) and the inflammatory response (Table 5) is intact on day 56, the failure to respond implies that a defect existed on day 1 in the ability to be immunologically sensitized (affector limb). Results pending (Table 6) will determine whether this defect is repaired by day 15.

As previously discussed, the 100 ugm skin test doses of DNFB repeated while nutritional repair was occurring may have stimulated the affector limb of a recovering CMI response and may have thereby contributed to the rising conversion rates noted in Table 4. In order to test this possibility the 4 children in Table 6 who were skin test negative on day 70 were challenged again; Two were skin tested with 100 ug DNFB on day 84 and two on day 92. One child on each of these 2 retest days converted to skin test positive, indicating that the skin test dose of 100 ugm may indeed immunize some nutritionally repaired children.

Between 50 and 90% of healthy individuals can be expected to have been naturally sensitized to monilia or streptococcal antigens by the age of 6-12 months, and therefore show positive delayed hypersensitivity to intradermal antigenic challenge. Accordingly in order to test immunological recall in PCM, patients were inoculated intradermally on day 1 with 0.1 ml of monilia (1:100) and streptokinase-streptodornase (SK-SD) (50 units) skin test antigens. The patients served as their own controls in that they were retested when their nutritional status was partially (day 29) and completely (day 70) normal. The results shown in Table 7 indicate that on admission only 7% and 14% of children were skin test positive to monilia and SK-SD, respectively. By day 70, 70% of the children were positive to monilia and 50% were positive to SK-SD. The poor response to antigenic challenge on day 1 can be ascribed either to a deficient inflammatory response (Table 5) or to a defective effector (recall) portion of the CMI response. It is apparent that by day 70, both mechanisms were largely intact. A study is in progress to test the CMI effector limb independently of inflammatory response by measuring lymphocyte

transformation in vitro in the presence of monilia and SK-SD antigens. In addition, 20 pts are being repeatedly skin tested starting on day 70 in order to determine whether these test antigens can immunize and induce a positive test on rechallenge.

Total peripheral lymphocyte counts were performed on admission. The results, grouped according to clinical diagnosis, show no decrease in lymphocyte counts compared to normal and no differences between 7 marasimic ($\bar{x}$ = 6,770 cumm), 9 marasmic-kwashiorkor ($\bar{x}$ = 6,178 cumm) and 10 kwashiorkor ($\bar{x}$ = 5,812 cumm) patients. Thus the immune defects noted in PCM are the results of factors other than a quantitative deficiency of circulating lymphocytes.

Further correlations were made between clinical diagnosis (m, m-k, k) and the immune and inflammatory response on admission. No clear relationship emerged between the clinical state of the patient and their inflammatory and immune responses on day 1; the 3 groups appear to be equally defective. The levels of serum complement components (C'1q, C'1s, C'3 proactivator, C'3, & C'5) on admission did not correlate with the degree of impairment of inflammation or of CMI. Finally the effect of the 4 protein-calorie diets (table 2) on DNFB skin test responses on day 15 and 29 was studied; results show no clear differences between the 4 dietary groups; all diet groups showed nearly equal and gradual improvement. It thus appears that the low calorie-low protein diet with vitamin and mineral supplements supplies adequate nutrients for physiological repair of the DNFB skin test. Physiological repair with 1 gm protein/Kg diet was not invariable; the low serum complement levels for example, shown in the next section, rose only with a 4 gram protein/Kg diet.

B. Serum Complement and Immunoglobulin.

Background: Serum complement plays a major role in mediating inflammation, chemotaxis, immune cytolysis and phagocytosis of bacteria. Complement is involved in endotoxin metabolism and perhaps in endotoxin shock. Infection, particularly bacterial with endotoxin shock, is a common event in PCM; the previous section on CMI function provides evidence for defective inflammatory response. Thus a study of complement in PCM may provide additional clues as to the mechanism mediating infections and the many physiological malfunctions noted in malnourished individuals. Our initial studies have dealt with the "profiles" of serum C' component levels and the change in these levels following nutritional repair. Dr. H. J. Muller-Eberhardt generously provided the immunodiffusion plates for determining the levels of 9 complement components. Duplicate data for C'3 was also obtained using commercial immunoplates (Hyland Lab).

Serum or heparinized plasma was obtained on days 1, 8, 29, and 84. The serum was stored for weeks or months at -20°C and then shipped to Bangkok. All specimens were run with complement standards supplied by Dr. Muller-Eberhardt or Hyland Labs.

Serum immunoglobulin levels were also determined in these serum specimens by radial immunodiffusion using commercial IgA, IgG, IgM & IgD plates.

Progress: The statistical analysis of data from 20 well-nourished control patients and 10 marasmic and 10 Kwashiorkor patients is not completely finished. A narrative summary of the data obtained to date follows. All complement components were depressed on day 1 below the mean control levels; some components, such as C'3 proactivator and C'9, measured more than 2 standard deviations below the control mean, while others, such as C'4 & C'5 were within 1 standard deviation of the mean. The C' levels began to rise by day 8, with recovery of all components by day 29. The levels for several components on day 29 were in fact significantly higher than the mean, but fell to normal levels by day 84, producing a "rebound" effect. In general the levels of C' on day 1 were lower in Kwashiorkor than in marasmic patients. Evidence is accumulating which indicates that the depressed levels of C' on admission are the result of depressed synthesis rather than of increased consumption or loss. For example, comparisons were made of C' levels on days 8 & 29 in children fed 1 gm protein - 175 cal/kg diets and those fed 4 gm protein - 175 cal/kg diets. The C' levels did not rise between days 8 & 29 in children fed the low protein diet, whereas all C' components rose markedly on the high protein diet. Complement turnover studies are being planned.

The low titers of C' components measured immunochemically does not necessarily reflect imparied complement biological activity. Therefore an attempt was made to elucidate the biological activity of serum C' by titering total hemolytic complement activity ($C'H_{50}$). In a series of preliminary experiments we found that $C'H_{50}$ titers are not lowered by repeated freeze-thawing, storage at room temperature (25°C) for 6 hours, or addition of heparin or CO_2 atmosphere to the storage tubes. However storage prolonged beyond 2-3 weeks, even at -90°C, seems to result in a gradual loss of $C'H_{50}$ titer over time. Thus the very low $C'H_{50}$ titers we measured in children with PCM may have resulted from prolonged storage of their serum (1-5 months) before testing. We are now measuring $C'H_{50}$ titers in serum stored less than 2-3 weeks in order to eliminate this possible laboratory artifact.

Measurement of serum immunoglobulin (Ig) levels has shown that on admission IgG & IgM are slightly increased in those children clinically infected, while IgD and IgA are markedly increased. In no patient were admission Ig levels below those considered normal for Thai children. The IgA & IgD levels tended to fall to normal levels over several weeks. Plans are underway to test the quality rather than the quantity of serum Ig in PCM by measuring the immune response to specific antigenic challenge.

Summary: This data emphasizes the disordered CMI function, inflammatory response, and complement metabolism in PCM. The data thus provides a sound basis for further studies of PCM and infection.

Table 1. Criteria used to score the clinical nutritional status of each PCM patient on admission.

I. Clinical impression

II. McLaren's criteria

Sign		Point Score
1. Edema		3
2. Dermatosis		2
3. Edema + Dermatosis		6
4. Hair change		1
5. Hepatomegaly		1
6. Serum albumin (gm/100 ml	Total serum protein (gm/100 ml)	
-1.00	-3.25	7
1.00-1.49	3.25-3.99	6
1.50-1.99	4.00-4.74	5
2.00-2.49	4.75-5.49	4
2.50-2.99	5.50-6.24	3
3.00-3.49	6.25-6.99	2
3.50-3.99	7.00-7.74	1
≥4.00	≥7.75	

McLaren's score

Clinical diagnosis	Total point score
marasmus	0-3
marasmus-kwashiorkor	4-8
kwashiorkor	9-15

III Gomez criteria

Clinical criteria	Measured weight/ expected weight for age (Thailand)	Edema
Marasmus	<60%	No
Marasmus-Kwashiorkor	<60%	yes
Kwashiorkor	>60%	yes
underweight child	>60%	No

Table 2. Dietary groups

Group	Days following admission 1-7	8-29	30-70	70-84
I	Stabilization	1 gm protein/kg 100 calories/kg	4 gm protein/kg 175 calories/kg	solid food ad lib (4gm.175 cal.)
II	"	4 gm protein/kg 100 calories/kg	"	"
III	"	1 gm protein/kg 175 calories/kg	"	"
IV	"	4 gm protein/kg 175 calories/kg	"	"

Table 3. Stabilization diet (days 1-7)

Day	Component
1	IV therapy
2 & 3	1 gm protein and 25 cal/kg - if tolerated
4 & 5	1 gm protein and 50 cal/kg - if tolerated
6 & 7	1 gm protein and 100 cal/kg - if tolerated

Table 4. Contact Sensitization to DNFB[1/] in Protein-Calorie Malnourished Children

Day skin tested[2/]	Total pts	Skin response[3/] Positive	Negative	% Converted
15	19	4	15	21
29	14	7	7	50
44	11	8	3	73
56	8	8	0	100

1/ Sensitizing dose of 2 mgm DNFB applied to skin on day 1.

2/ Day 100 ugm skin test dose DNFB applied to skin; skin response graded 2 days later.

3/ Positive = induration and/or vesicle or bleb
Negative = no reaction or erythema only.

Table 5. Skin Inflammatory Response to 2 mgm DNFB

Day tested*	Total pts.	pts completed	Skin response[xx] Positive	negative	% Positive
1	25	25	9	16	36
15	5	0			
56	5	3	3	0	100

* Skin response read on day 3, 17, and 58 respectively

xx Negative = no reaction or erythema; positive = induration and/or vesicle or bleb formation.

Table 6. Attempt to induce DNFB sensitivity on different days after admission

Day Sensitized*	Total pts.	Pts completed	Skin response[xx] Positive	Negative
1	5	4	0	4
15	5	0		
56	5	3	3	0

* Day sensitizing dose of 2 mgm DNFB applied to skin

xx Skin test dose of 100 ug DNFB applied on day 70 and read on day 72; positive = induration and/or vesicle or bleb; negative = no reaction or erythema

Table 7. Skin Test Response to Monilia and Streptoccocal Antigens

Antigen	Day* tested	Total no. patients	Skin Response Positive	Negative	% Positive
Monilia[xx]	1	13	1	12	7
(1:100)	29	12	8	4	66
	70	10	7	3	70
SK-SD***	1	29	4	25	14
(50 units)	29	27	9	18	33
	70	25	13	12	52

* Skin test read two days after 0.1 ml antigen injected intradermally.

xx Monilia positive test = > 5mm induration and/or vesicle

xxx Streptokinase-streptodornese positive test = erythema and/or induration.

Isolation and Characterization of Influenza A Viruses from Korat

Principal Investigators: Rapin Snitbhan, M.D.
Pricha Singharaj, M.D.
Franklin H. Top, Jr., M D.

Background: In late February 1972, members of the School of Public Health noted and excess in febrile respiratory disease among Thai Nationals seen in their health clinic in Korat, Thailand. Although no increase in URI rates were reported in U.S. military personnel in Thailand in February-March 1972, attempts were made to determine whether the civilian outbreak was associated with influenza virus.

Methods: Throat washing (TWS) in Hank's balanced salt solution with 0.4% bovine plasma albumin and acute serum were obtained from 11 patients with febrile URIs. TWS were frozen and transported to Bangkok on dry ice. Two week convalescent sera were obtained on 4 of the 11 patients originally sampled. Aliquots of TWS were inoculated into the amniotic and allantoic cavities of embryonated eggs; fluids were harvested 3 days after inoculation and tested for hemagglutination. Aliquots of TWS were also inoculated into tube cultures of primary Rhesus monkey kidney cells (MKR) which were observed for cytopathic effect (CPE) every other day and tested for hemadsorption at 7 and 14 days. Isolates were characterized in hemagglutination-inhibition (HI) tests using hyperimmune antisera to reference Influenza strains. Acute and convalescent sera from patients were treated with receptor-destroying enzyme (RDE) prior to use in HI tests with 4 units of A_2/HK/68 antigen.

Progress: Influenza A strains were isolated from TWS of 10 of the 11 patients sampled; 9 strains were isolated in embryonated eggs and 8 in MKR cell cultures. Strains isolated in MKR cultures showed CPE similar to that found with the 1968 Influenza A strains. Table 1 shows results of antigenic analysis of one of the isolates, A_2/Korat 305/72, against hyperimmune antisera to reference Influenza strains. The 1972 isolate is obviously closely related by HI test to the 1968 A influenza strains isolated in Hong Kong and Korat. All 4 patients tested had $\geq$4 fold virus in HI antibody to A_2/HK/1/68 between acute and convalescent sera.

We conclude that the Influenza A strains isolated from Korat in 1972 have similar HA antigens to 1968 strains. One isolate (A_2/Korat 305/22) was shipped through WRAIR to the WHO Influenza Reference Laboratory, National Center for Disease Control for more detailed antigenic analysis including characterization of its neuraminidase antigen.

Table 1. Antigenic analysis of Korat Influenza Isolate by HI Tests

Antiserum of Reference Strain	Homologous Titer	Titer to A_2/Korat 305/72
A_2/Japan 305/57	160	10
A_2/Puerto Rico/64	160	10
A_2/Korat 1/68	320	320
A_2/Hong Kong 1/68	320	320

The Study of Coronavirus OC43 Infection

Principal Investigators: Chaninthorn Suwongse, M.D.
Franklin H. Top, Jr., LTC MC

Objective: To determine the past incidence of coronavirus infections in people living in the tropical environment of Thailand.

Background: Coronaviruses are a newly described group of RNA viruses isolated from patients with upper respiratory tract illness. At present, at least 3 distinct strains of coronaviruses have been isolated - B814, 229E, and NIH O.C. (organ culture) strains. Strain B814 was isolated and has only been grown in human embryonic tracheal organ cultures. Strain 229E was isolated in human cell cultures and can be grown in various human derived cell lines. The NIH O.C. viruses (OC38, OC43), originally isolated in human organ culture, have been successfully adapted to grow in suckling mouse brain. Although the antigenic characteristics of these 3 prototype viruses are not completely described, strains 229E and OC43 appear to be antigenically distinct.

Coronaviruses produce upper respiratory disease as shown by studies in England and the United States. Infections tend to occur in autumn and winter months in temperate climates and in some years about 20% of upper respiratory diseases are associated with coronavirus infections. Although their detailed epidemiology is not yet clear, the URI's produced by this virus have a tendency to appear as epidemic outbreaks in addition to more sporadic illness. Serological surveys of patients with lower respiratory disease in temperate and tropical environments have been negative for coronavirus infections. The incidence of coronavirus URI in the tropics is still unknown.

Materials and Methods:

Virus Seed and HA Antigen Preparations: Strain NIH OC43 was obtained from Dr. D.J. Tyrrell in the lyophilized form of a 10% suspension of infected mouse brain containing 5% calcium gluconate lactobionate. After reconstitution in 1 ml of phosphate buffered saline pH 7.2, the suspension contained approximately $10^{4.5}$ (suckling mouse lethal dose$_{50}$) (SMLD$_{50}$). A dose of 0.02 ml containing 10^{3} SMLD$_{50}$ was inoculated intracerebrally into 3-5 day old suckling mice. Infected mice showing typical encephalitic symptoms, usually within 48-60 hours after inoculation, were sacrificed and their brains pooled and made up into the following antigen preparations.

a. Virus seed. A 10% suspension of brain was made in tryptose phosphate broth containing 0.5% gelatin. Brain suspensions were distributed in 2 ml aliquots into ampules, lyophilized and then kept at -20°C. The infectivity titre of the suckling mouse brain seed prepared was $10^{4.6}$ $SMLD_{50}$.

b. Haemagglutinating antigen; Antigen was prepared by making 10% suspensions of infected brain in phosphate buffered saline pH 7.2, which were clarified by refrigerated centrifugation at 600g for 20 minutes, kept at -70°C, and used as HA antigen. As an antigen control, normal mouse brains were pooled and made up to 10% suspension in veronal buffered diluent and clarified in the same manner as the antigen. The haemagglutinating antigen prepared agglutinated chicken red blood cells at a 1:640 to 1:1280 dilution.

Haemagglutination inhibition test. The reaction was carried out by microtiter technique at room temperature, using adult chicken erythrocytes at 0.5% concentration with PBS pH 7.2 as a diluent and 4 units of HA antigen. Sera were heated at 56°C for 30 minutes prior to dilution for the test.

Neutralization test. Neutralizing antibody was measured by adding serial virus dilutions with 1:2 dilution of serum tested. The virus-serum mixture was incubated for 1 hour in room temperature before inoculation into suckling mice. A neutralization index (NI) of 1.7 $\log_{10}$ or greater was considered to reflect neutralizing antibody.

Sucrose density gradient centrifugation. A 0.25 ml volume of human serum was layered on a preformed 10-40% sucrose density gradient and centrifuged at 35,000 RPM for 18 hours in an SW-39 rotor. Twelve fractions were collected, and each fraction tested for OC43 HI antibody and IgG and IgM concentrations.

DEAE-Sephadex extraction. Selected sera were extracted by the DEAE-Sephadex method described by Altemeier, et al (Applied Microbiology 19:785, 1970) which removes all serum proteins except IgG. Purity of the extracts obtained was tested by immunoelectrophoresis of whole and treated serum using goat anti-whole human serum.

Results:

Prevalence of Coronavirus OC43 HI antibody. Two populations were studied; 832 adult made blood donors in Bangkok and 476 residents of the Chiangmai valley. This latter group involved residents of village Maerim (88), Sanpatong (86), Sankampang (81), Saraphi (104) and school children

in Chiangmai City (117). As shown in Table 1, only about 7% of Bangkok adults and 13% of Chiangmai residents lacked OC43 HI antibody. The majority of both populations had antibody at a titer between 1:10 and 1:40. The age-prevalence of OC43 antibody is shown in Table 2. Although the majority of children less than 2 years lacked antibody, 65% of the 3-4 year old tested, 85% of the 5-9 year old tested, and 95% of the population 10 years or older had OC43 HI antibody.

Persistence and acquisition of OC43 HI antibody. Sera from Chiangmai villagers were collected in November 1969, March 1970, July 1970, and November 1970. Sequential sera over a 9 to 12 month period from 278 villagers were tested for antibody persistence. As shown in Table 3, 248 villagers had HI antibody in November 1969, and 90% had no significant change in antibody titer; 21(9%) of these had $\geq$4 fold antibody rises. Of the 30 villagers lacking HI antibody to OC-43, 13(43%) had significant rises in antibody suggesting infection with this virus or an antigenically related virus during the year.

Identification of HI activity in human sera as antibody. Since sera from most people sampled had HI activity, it was important to determine whether this activity could be related to antibody. Results of fractionating 4 human sera by sucrose density gradient centrifugation are shown in Table 4. HI antibody was found only in fractions containing IgG.

In addition, 7 sera with HI titers $\geq$1:40 were extracted with DEAE-Sephadex. Serum electrophoresis of 2 extracted sera revealed 2 lines - a strong IgG line and a faintly visible line toward the anode. All 7 sera tested had HI activity after DEAE-Sephadex extraction, suggesting that serum HI activity was associated with IgG.

Neutralization tests were performed on sera from 16 individuals. Sera of the 9 persons tested who lacked HI antibody lacked neutralizing activity, while sera of 6 of the 7 persons who had HI activity had neutralizing activity (Table 5).

Summary: The association of HI activity with the IgG fraction of serum and the correlation between serum HI activity and serum neutralizing activity suggests that the assay used is detecting HI antibody rather than non-specific inhibitors. The antibody data is consistent with the possibility that viruses identical or serologically related to OC-43 commonly cause infections in Thais and indeed infect most persons by 3-4 years of age.

Table 1. Distribution of Coronavirus OC43 Antibodies in 2 Thai Populations.

HI antibody titre	Adults (Bangkok) No. with HI titre	(%)	All ages (Chiangmai) No. with HI titre	(%)
<10	61	7.2	63	13.4
10	126	15.5	98	20.1
20	299	35.9	181	38.5
40	254	30.5	102	21.3
80	180	9.6	29	5.8
160	12	1.4	3	0.6
Total	832		476	

Table 2. Prevalence of Coronavirus OC43 HI Antibody by Age, Chiangmai Population.

Age	No. studied	No. with antibody	% with antibody
0-2	15	2	12.5
3-4	32	20	64.5
5-9	183	155	84.7
10-14	84	80	95.2
15-19	37	35	94.6
20-29	32	30	93.7
30-39	75	74	98.6
40	18	18	100.0
Total	476	409	86.8

Table 3. Persistence of Coronavirus OC43 HI Antibody, Chiangmai Villages.

Initial Antibody status	No.	No. without rise or fall	No. with ≥4-fold rise	No. with ≥4-fold fall
≥10	248	221	21	6
<10	30	17	13	-

Table 4. Immunoglobulin Concentration and HI Antibody Activity of Fractions from Sucrose Gradient Centrifugation.

Serum No.	HI Whole serum	Fraction No.	Immunoglobulin concentration IgG mg%	IgM mg%	HI titre
1849	<10	3	<10	23	<4
		4	<10	24.5	<4
		5	<10	30.5	<4
		6	-	-	<4
		7	<10	0	<4
		8	-	-	<4
		9	94	0	<4
		10	20	0	<4
2418	<10	3	<10	0	<4
		4	<10	16	<4
		5	<10	13.8	<4
		6	-	-	<4
		7	<10	0	<4
		8	138	0	<4
		9	-	-	<4
		10	<10	0	<4
1856	40	3	<10	0	<4
		4	<10	16	<4
		5	-	-	<4
		6	10	0	<4
		7	10	0	4
		8	74	0	16
		9	82	0	32
		10	-	-	8
2410	40	3	<10	20	<4
		4	<10	24.5	<4
		5	-	-	<4
		6	<10	0	<4
		7	<10	0	4
		8	58	0	16
		9	74	0	32
		10	-	-	4

Table 5. Correlation between OC43 HI and Neutralizing Antibody

HI Antibody	Log Neutralization Index <1.7 (No.)	Log Neutralization Index ≥1.7 (No.)
<10	9	0
≥10	1	6

Reservoirs of Rabies in Thailand

Principal Investigator: Robert L. Hickman, MAJ, VC

Associate Investigators: Kwanyuen Lawhaswasdi, DVM
Dennis O. Johnsen, MAJ, VC

Part I. Rabies Diagnostic Service

OBJECTIVE: The objective was to maintain a competent facility capable of providing a rapid and accurate rabies diagnostic service in order to determine the risk of human exposure to rabiesvirus infection from animal bites. In addition to providing an important service, this activity was a reliable source of information regarding rabiesvirus infections in the canine population of Thailand.

DESCRIPTION: Suspect rabies specimens are accepted from U.S. military and approved Thai sources. The fluorescent rabies antibody (FRA) test and intracranial inoculation of weanling mice (MI) are the methods used routinely for diagnosis. The indirect FRA test is used to evaluate the presence of rabiesvirus antibody in human sera when requested.

PROGRESS: A total of 780 specimens were submitted for examination during the past 12 months. The results are presented in Table 1. A comparison of the FRA and MI results is presented in Table 2. Based on the MI results, the accuracy of the FRA results were 99.1% when negative and 98.2% when positive. During this report period, rabies antibody titers in 151 human sera were determined by the FRA technique.

SUMMARY: Of a total of 780 specimens submitted for rabies diagnosis, 331 (42.4%) were infected with rabies virus. These results are similar to those reported earlier. Although dogs continue to be the major species involved and constitute the greatest hazard to man, other species, particularly cats, should not be ignored.

Table 1. Rabies Diagnostic Results

Species	Number Examined	Number Positive	Percent Positive
Canine	625	320	51.2
Feline	72	7	9.7
Others	83	4 *	4.8
Total	780	331	42.4

* 2 human, 1 monkey, 1 bat.

Table 2. Comparison of FRA and MI Rabies Diagnostic Results

		MI		
		POS	NEG	
FRA	POS	325	4	329
	NEG	6	445	451
		331	449	

Part II. Survey of Domestic Animals for Rabiesvirus Infection.

OBJECTIVE: The purpose was to determine the prevalence of rabiesvirus infections in asymptomatic stray dogs captured by municipal and federal authorities in Thailand.

DESCRIPTION: Canine specimens were obtained from two sources. Stray dog control programs were conducted by the Division of Communicable Disease Control, Thai Ministry of Health, and cooperating U.S. Air Force Installations in Thailand. From 10 to 30 of the dogs collected each day during the operation of a program were submitted for rabiesvirus examination. The total number of dogs captured in the communities involved is not known. The second source of specimens was the Bangkok Municipal Health Department which operates a continuous stray dog pickup program. Each week, 10 percent of the dogs picked up on a single day were randomly selected for examination. The total figure, therefore, approximates 1.6 percent of all the stray dogs destroyed during the 18 month period of

the survey (estimated 32 thousand). From both groups, only dogs without clinical signs of rabies were examined. All specimens were examined by the FRA technique. Rabiesvirus was isolated from FRA positive specimens by mouse inoculation and confirmed by serum neutralization test.

PROGRESS: A total of 906 dogs was examined during the 18 month study period. The examination results are presented in Table 1. Since these animals were asymptomatic at the time of euthanasia and salivary gland examinations were not done, it is not possible to estimate how many of the FRA positive animals were capable of transmitting the disease. It is assumed that all were in some stage of virus inoculation and that all would have eventually died of rabies. Certainly many would have been responsible for disease transmission to other animals and perhaps to man at some time before death. During the same period, 881 suspect canine specimens were submitted for routine rabies diagnosis and 449 or 51.0 percent were found to be positive.

SUMMARY: The number of isolations obtained from the two dog populations emphasizes the extent of canine rabies in Thailand and the continuing need for more adequate control programs if the problem is ever to be eliminated.

Table 1. Isolation of Rabiesvirus from Asymptomatic Stray Dogs in Thailand.

Source	Number Examined	Number Positive	Percent Positive
Ministry of Health:			
Udorn	177	6	3.4
Korat	140	3	2.1
NKP	47	1	2.1
Ubon	30	1	3.3
Bangkok Health Department			
Bangkok	512	25	4.9

Part III. Survey of Selected Rabiesvirus Isolates for the Presence of "Rabies-Like Viruses".

OBJECTIVE: The purpose was to study 50 isolates of rabiesvirus obtained from animals having no clinical history of rabies in order to confirm their identification or, in failing to do so, provide material for further investigation into the occurrence of "rabies-like viruses" in Thailand.

DESCRIPTION: Selected isolates were to be inoculated into weanling mice to provide aliquots of infected mouse brain suspension. The virus titer of each isolate pool would be determined. A neutralization-screening test using 100 MLD_{50} of virus and antisera calculated to neutralize 5 MLD_{50} of CVS rabiesvirus would be conducted. In addition, FRA examination of each isolate would be performed at 2, 4, 24 and 48 hours after slide preparation. Isolates not neutralized and/or demonstrating a weakening FRA reaction with time would be studied further by neutralization tests.

PROGRESS: A total of 60 isolates were selected for study on the basis of available clinical history. All but 3 were recovered from weanling mice. Five of the latter were recovered after inoculation into suckling mice. All recovered isolates were FRA positive after 4 hours fixation in acetone. An infected brain suspension pool of each of the 56 isolates was produced from weanling mice and divided into 5 aliquots. Virus titrations in suckling mice were completed with 26 of the isolates. Mouse brain suspensions of CVS and a virulent street virus were produced and titrated. Normal and hyper-immune horse serum were prepared and aliquoted for use in the neutralization screening test. Virus titration is continuing and initial neutralization screening tests and FRA studies are in progress. Final results are pending completion of the latter procedures.

SUMMARY: Fifty-six of 60 selected rabiesvirus isolates were recovered from storage and aliquots prepared from weanling mouse suspensions. Virus titers were determined for 26 and others are in progress. Initial mouse neutralization screening tests and FRA studies are underway.

Identification of Vertebrate Hosts of Ectoparasites in Thailand

Principal Investigator: Joe T. Marshall, Jr., Ph.D.

Associate Investigators: Guy G. Musser, Ph.D.*
Sawart Ratanaworabhan, Ph.D.**
K. C. Emerson, Ph.D.***
Alfred Gropp, M.D.****
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Vandee Nongngork

Objective: To provide proper scientific names for the wild vertebrates from which ectoparasites are collected or which are suspected of being reservoir hosts of disease.

Description: Vertebrates, especially those voucher hosts of ectoparasites, are prepared as study specimens and identified by comparisons with other specimens in museums. For the rodents and some of the birds this entails a taxonomic revision with examination of type specimens, further collecting, and study of their ecology, cytogenetics, and karyology.

Progress: The checklist of rats and mice of Thailand, Table 1, has been changed because of finding four species of the niviventer-fulvescens group on Doi Inthanon and discovering that the lesser bandicoot of Thailand is not Bandicota bengalensis but a distinct species, B. savilei. Table 1 includes identifications of lice found on rats and mice. It will be seen that each subgenus of rats is characterized by two unique species of lice, one from Hoplopleura, the other from Polyplax. Additional collections of lice by Vandee and Marshall, now being studied, are all accompanied by voucher museum specimens of their hosts. They will fill most gaps on the table. Karyotypes are similarly diagnostic of the subgeneric groupings of rats and mice; the karyology study is now ready for publication. Taxonomic revision of the genus Mus exclusive of Africa is also ready for publication, with results shown in the accompanying key to Eurasian species. The six native species of Thai mice have now been colonized for cytogenetic study in 10 laboratories around the world.

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** Rice Protection Research Center, Bangkok
*** Smithsonian Institution, Washington, D.C.
**** Pathologisches Institut der Universitat Bonn
***** Kasetsart University, Bangkok
****** Applied Scientific Research Corporation of Thailand, Bangkok

Summary: The number of species of rats and mice occurring in Thailand is now understood to be 36 of which 11 are commensal with man and one is non-native (R. norvegicus). Progress in the taxonomic revision of these rodents, in order to provide scientific names, has involved karyology, cytogenetics, host-specific ectoparasites, ecology, and anatomical study of the skull.

Table 1. Check-list of Rats and Mice of Thailand, Status of Louse Identifications (from Dr. K. C. Emerson) and Back-up Specimens of Hosts Actually Preserved in Museums

underline = museum skin and skull identified by Marshall
"....." = museum skin and skull, from the literature

	Hoplopleura	Polyplax
Bandicoots		
Bandicota indica	malabarica "skins in USNM"	asiatica
B. savilei curtata	malabarica "skins in USNM", V184	asiatica "Elbel F875"
Subgenus Berylmys		
Rattus berdmorei	kitti "skins in USNM from Aranya Pradet"	spinulosa
R. bowersi	diaphora ITM 6780	
R. mackenziei		
Subgenus Stenomys		
R. mulleri	dissicula	
Subgenus Rattus		
R. remotus	pacifica	
R. rattus thai (north and central Thailand)	pacifica "skins in USNM"	spinulosa "skins in USNM are Elbel F935, F949, RE 1333, RE1723, RE1725, RE1781, RE464, RE465"
R. rattus robinsoni (Koh Samui)	pacifica	
R. r.? jalorensis	pacifica	spinulosa
R. r.? diardii		
R. sladeni		

	Hoplopleura	Polyplax
R. nitidus		
R. exulans	pacifica	spinulosa
R. losea exiguus		
R. argentiventer	pacifica	
R. norvegicus	pacifica	spinulosa "skin in USNM, Elbel Y206"
Subgenus Leopoldamys		
Rattus edwardsi		close to insulsa ITM 6746, 6753
R. sabanus	malaysiana	insulsa (spinulosa)
Subgenus of Rajah Rats		
R. surifer	pectinata USNM 86750	
R. rajah pellax		
R. whiteheadi	(pectinata)	
Subgenus of Niviventer Group		
Rattus rapit orbus		
R. fulvescens	sicata ASRCT 54-654, 54-727	
R. bukit	(pectinata) sicata USNM 355181 ASRCT 54-707, 54-722	
R. niviventer		pricei ASRCT 54-738
R. cremoriventer	(pectinata) sicata ASRCT 54-661	
R. langbianus		

	Hoplopleura	Polyplax
Subgenus Leggadilla		
Mus shortridgei	nov. sp. ITM 6686, ITM 6741	
Subgenus Coelomys		
Mus pahari	nov. sp. ITM 6779	
Subgenus Mus		
Mus cookii		
Mus caroli	johnsonae ITM 6754, 6755b	
Mus cervicolor	johnsonae USNM 294944, 294946, ITM 6756 captiosa USNM 294944, 294946, 294947; "RE 460" (Mus sp?)	
Mus musculus castaneus	captiosa	serrata
Genera of Tree-rats		
Chiromyscus chiropus		
Vandeleuria oleracea		
Chiropodomys gliroides		
Hapalomys longicaudatus		

KEY TO EURASIAN SPECIES OF THE GENUS MUS

GENUS MUS Length of first upper molar more than half the toothrow; postero-internal cusp of first molar absent; head and body about 100 mm; plantar pad round rather than long and pointed; prelambdoidal fenestra exposes a slender paraoccipital process at least in the young; anterolateral corner of parietal projects forward in a point.

KEY TO EURASIAN SUBGENERA OF THE GENUS MUS

1 Supraorbital ridge present, eye and ear large (rat-like adaptation) ..... Subgenus Leggadilla

1' No supraorbital ridge 2

2 Interorbitum broader than 4 mm, incisive foramina broad and short, fur velvety or spiny, eye small (shrew-like adaptation). Subgenus Coelomys

2' Interorbitum narrower than 4 mm, incisive foramina slender and long, fur not spiny, eye large (commensal adaptation) ..... Subgenus Mus

KEY TO SPECIES OF THE SUBGENUS LEGGADILLA

1 The only southeast Asian species, fur spiny, dorsal color light gray-brown, ventral fur white with conspicuously gray bases, anterior border of zygomatic plate swept back in covex arc, incisive foramina penetrating deep between the first molars, molars broad lacking accessory cusp, upper incisors notched, mammae 3 + 2, grass beneath deciduous forest in Burma, Thailand, Cambodia - ..... Mus shortridgei

1' Indian species, ventral fur white including the bases 2

2 Mammae 4+2, anterior border of zygomatic plate arched forward in a convex semicircle, incisive foramina long, functional anterior accessory cusp on first upper molar, medium size, skull averaging 25 mm, notch shallow to none ..... Mus saxicola (There are two subspecies: M. s. gurkha with soft fur, dorsal surface light sandy brown, Simla, Nahan, Kumaon, Nepal; and M. s. saxicola with dorsal fur spiny and grayish brown, Sind, Kangra, Cutch, Poona, Madras).

2' Mammae 3 + 2, anterior border of zygomatic plate approximately vertical or curved back, incisive foramina reaching only to level of anterior cusp of first molar (exception: see no. 4, below) no accessory cusp, fur spiny above and below 3

3 Large size, skull 26-30 mm averaging 27, no notch on upper incisors, black spot on hind foot, Punjab? (type of spinulosus not seen), Kumaon, Bihar, Poona, Deccan, Kanara, Coorg. Mus platythrix (There is subspecific variation in color. Sooty brown in the north, purplish brown with ochraceous flanks in Coorg).

3' Smaller size, skull 21-25 mm averaging 22.6, notch of incisors variable, purplish brown with ochraceous flanks/white, Central Provinces, Bellary, Madras, Ceylon.. Mus phillipsi (The distinctive subspecies, M. p. fernandoni, with foreshor tened skull, occupies Ceylon).

4 Same as 3' but with long incisive foramina and no notch, Rajputana, Gujerat, Central Provinces..... Mus sp?

KEY TO SPECIES OF THE SUBGENUS COELOMYS

1 Pelage spiny or stiff, not wooly, ear small.....2

1' Pelage dense, wooly, velvety, ear large.....3

2 Size large, skull length greater than 27 mm, Ceylon.....Mus mayori

2' Size medium, skull length under 27 mm, Sikkim to Vietnam..Mus pahari (with three subspecies: jacksoniae, Assam and N. Burma to Yunnan and Szechuan; pahari, Sikkim, Darjeeling; gairdneri, mountains of northern Thailand, Laos, and Vietnam).

3 Skull long and slender as in mayori and pahari, supraorbital area smoothly rounded.....4

3' Skull short and broad with flattened top of rostrum like Mus musculus and sharp supraorbital angle like Mus cookii, mammae 1 + 2, coloration dark brown/ochraceous buff, Nilgiri Hills of southern India.....Mus famulus

4 Coloration similar to that of M. famulus with dark feet and tail and pelage chocolate brown/bronzy buff, mammae 1+2, tail shorter than 105 mm, incisive foramina 5 mm or longer, mountains of Java..... ..... Mus vulcani

4' Coloration steely gray/silvery, feet white, tail longer than 110 mm, incisive foramina shorter than 5 mm, mammary formula unknown, mountains of Sumatra, Mus crociduroides.

KEY TO SPECIES OF THE SUBGENUS MUS

1 Upper incisors curve forward and downward perpendicular to palate (pro-odont), narrow interpterygoid space, ventral color whitish, tail bicolored.....2

1' Upper incisors markedly recurved (opisthodont).....3

2 Nasals very short, exposing to dorsal view the upper incisors, whose anterior surface is brown; incisive foramina intruding only slightly between anterior tips of first upper molars, posterior palatine foramina situated at rear of palatal bridge; tail blackish on top and longer than head and body; ricefields and grassy areas in Ryukiu Islands, Taiwan, southeastern China to Thailand, reappearing in Sumatra, Java, Madura and Flores.....Mus caroli (Well marked subspecies of Mus caroli include a long-furred, richly colored form on mountains of Vietnam, and lighter brownish

-gray subspecies in Thailand of which that of the north has silvery gray underparts, that of central and southeast Thailand is almost pure white ventrally).

2' Nasals longer, overhanging the upper incisors, which are buff colored on anterior surface; bicolored tail paler gray on top than in caroli and shorter than head and body; feet always pure white; incisive foramen extending between anterior molars; posterior palatine foramina in middle of palatal bridge (as in all species except caroli); Nepal to Vietnam, reappearing in Java.....Mus cervicolor (There are three subspecies: M. c. cervicolor, small, weight averaging 15 g., brownish gray/white with pale gray bases, ricefields in Nepal, Burma, Vietnam, Thailand, Java. This commensal population surrounds the following two subspecies of larger size, averaging 22 g., and darker coloration, that live in natural forest. M. c. popaeus, brown/white with gray bases; grass beneath deciduous forest in Burma and Thailand. M. c. annamensis, deep brown/white with slate bases, ochraceous along flanks; mountains of Laos and Vietnam).

3. Length of upper molar row 4 mm or more, fur stiff and dense, ventral color whitish, incisive foramina terminating opposite anterior cusp of first molar as in M. caroli, broad interpterygoid space; grass in mountain forests of Assam, Burma, Yunnan, Laos, northern Thailand, and Vietnam.....Mus cookii

3' Length of molar row less than 4 mm, fur soft.....4

4 Rostrum shallow, its least depth only one-half of rostral length (measured from gnathion to inferior anterior corner of zygomatic plate), ventral fur whitish.....5

4' Rostrum flat on top, deep (in side view), its least depth two thirds of rostrum length; rostrum short, cranium broad and flat on top; Eurasia.....Mus musculus, with two groups of subspecies...7

5 Larger (a miniature of cookii), skull longer than 21 mm, tail averaging 77 mm at least as long as head plus body; vertical anterior border of broad, approximately square zygomatic plate; incisive foramina terminating opposite anterior cusp of first molar as in cookii; India (Poona), Nepal, Sikkim, Assam, Burma, Yunnan.....Mus kakhyenensis (A dark, rich brown/buffy subspecies, M. k. palnica occurs in the mountains of southern India).

5' Smaller, skull less than 21 mm, tail less than 72 mm.....6

6 An exact miniature of kakhyenensis with narrow, gracefully tapered and rounded skull; narrow delicate molars lacking bold division of cusps; tail averaging 63 mm, slightly less than head and body; skull length averaging 20 mm, incisive foramen rarely reaching level of anterointernal cusp of first molar; Poona, Madras, Ceylon.....
.....).....Mus fulvidiventris (with two subspecies: M. f. dunni, brownish-gray, Punjab and peninsular India. M. f. fulvidiventris of Ceylon, with richer rufous-brown dorsal coloration).

6' A miniature of molossinus (except for relatively shallower rostrum and relatively large teeth); skull, averaging 19 mm long, is broad-beamed, angular, robust; teeth large, squarish, broad, deeply sculptured with all cusps equally distinct, interpterygoid space very narrow; incisive foramina penetrating deeply between anterior molars; zygomatic plate broad, set out laterally from rostrum from which it is marked off beneath by a groove; its anterior border markedly arched forward in a semicircule, with robust masseteric knob at its base; tail averaging 51 mm, much shorter than head plus body; ricefields of Nepal, India, Burma ..... Mus booduga

7 Zygomatic plate slender, hugging close to rostrum, usually with straight vertical anterior border and slight masseteric knob; size medium to smallest; Eurasia except northeast and southeast Asia.....BACTRIANUS Group of Subspecies:
(M. m. bactrianus and other desert forms; size medium; tail bicolored, shorter than head and body; fur long and silky, sandy buff-brown dorsally, with underparts and feet pure white; deserts of northern Africa, Mediterranian Region and Mid-East. M. m. homourus, size medium, tail as long as head and body, below white with gray bases and a peculiar singed brown color along the flanks; wild habitats in Ladak, Kashmir, Nepal, Sikkim.
M. m. tantillus, like homourus except very small and tips of ventral fur either white or a salmon tint (like muralis); Szechuan, Shensi, northern Vietnam.
M. m. musculus, size medium, brownish gray/slate, tail longer than head and body; inside buildings of Europe, introduced to Americas, some Pacific islands, and Australia. Progenitor of large European laboratory strains.
M. poschiavinus. Dr. A. Gropp is accumulating evidence that this Swiss offshoot of M. m. musculus may be a distinct species. We have seen no specimens of it).

7' Zygomatic plate exactly as described for booduga (6'), size small, eastern Asia.....
.....MOLOSSINUS Group of Subspecies:
(M. m. wagneri and allies of northern Asian deserts, desert type silky pelage, coloration, and short tail as described above for bactrianus.)
M. m. molossinus, Japan (and allies such as yamashinai in Korea), dark gray/white with slate bases, short bicolored tail. Inhabits buildings and outdoors in Japan, where it is the only member of the genus. Progenitor of small Asian laboratory mouse.
M. m. castaneus, underparts concolorous with the back, which is dark ochraceous brown; tail longer than head and body; lives in buildings and warehouses in Ceylon, Calcutta, Kathmandu? (the

type locality, but not collected there since Hodgson's specimens of 1845), Sikkim, Burma, Yunnan, Kweichow, Fukien, Taiwan, Pescadores, Vietnam, Thailand, Malaya, Singapore, Indonesia, Philippines. Introduced into some Pacific islands where overlapping with musculus).

The Prevalence of Hepatitis-Associated Antigen (HAA) among Thais Hospitalized with Acute Hepatitis

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Associate Investigators:	Bunterng Dechjun Nathada Plavooth Pranom Vangnai
Assistant Investigators:	Chariya Hussen Ovath Tonglee Yupadee Vanichakorntanes

OBJECTIVE:

1. To gain experience in the laboratory detection of HAA.
2. To build a bank of HAA positive sera for subsequent experiments.
3. To determine the prevalence of serum hepatitis among hospitalized patients with acute hepatitis.

DESCRIPTION: According to Krugman[1], "the specific association of HAA with serum hepatitis has been amply confirmed." Using this assertion as a basic premise, Thai inpatients with acute hepatitis were surveyed to determine what proportion had serum* hepatitis.

This project was conducted during the 16 month period ending in August, 1971. The Medicine and Pediatric Wards at the Royal Thai Army Hospital and Women's Hospital served as the study site. Nurses from SEATO Laboratory visited these wards several times a week to obtain blood samples from patients with hepatitis. At the same time, blood

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* Alternatively, one may discard previously used nomenclature and classify acute hepatitis into 2 types: HAA positive and HAA negative hepatitis. In so doing, however, many of the traditional distinctions between serum and infectious hepatitis, originally described using the tools of epidemiology and now confirmed as a consequence of the discovery of HAA, would be neglected. Thus I prefer only to modify the traditional distinctions, first by admitting that serum hepatitis may be transmitted by other than parenteral means, and then by adding HAA to the list of attributes of serum hepatitis (RBC).

was obtained from hospitalized patients without liver disease to serve as age and sex matched controls. The blood specimens, collected weekly throughout hospitalization, were tested for HAA and liver function indices. A diagnosis of hepatitis was accepted if the SGOT or SGPT level exceeded 100 Sigma Units and if the history and clinical findings were compatible with the diagnosis.

PROGRESS: One hundred nineteen patients ranging in age from 2 months to 70 years were accepted as cases of hepatitis. Fifty-one (43%) of these cases had at least one serum specimen positive for HAA.

The antigen was less frequently detected when the initial serum specimen was obtained late in the course of the disease. For example, the initial serum specimen for study was obtained 3 weeks or more after the onset of jaundice in 21 cases. Only 4 (18%) of these had detectable HAA.

One or two age, sex, and time matched controls were studied for each of 68 hospitalized Thai adults with acute hepatitis. Thirty-one (46%) of the hepatitis cases had sera positive for HAA compared with 15 (12%) of the controls. Thus, antigenemia was associated with hepatitis cases rather than being a non-specific function of hospitalization.

The bimonthly distribution of cases shows that HAA positive hepatitis (that is, serum hepatitis) occurs year round in Bangkok (Fig. 1).

HAA was detectable in the serum from hepatitis cases of all ages (Fig. 2). As seen in this age distribution of hospitalized hepatitis cases, serum hepatitis was most frequently encountered, however, in the young adults. (The shaded area represents cases of serum hepatitis).

Serial serum specimens were obtained weekly for 4 or more weeks in 28 cases that were initially positive for HAA. Twenty-six of these became negative for HAA or had an appreciable CF titer fall. The study period for the remaining 2 cases lasted only 4 and 5 weeks. This evidence indicates that the antigenemia was related to the disease in these cases. In other words, these patients do not appear to have been chronic carriers of HAA who incidentally developed acute hepatitis.

Literature Cited

1. Krugman, S and Giles, JP: Viral hepatitis: New light on an old disease. JAMA 212: 1019, May 11, 1970.

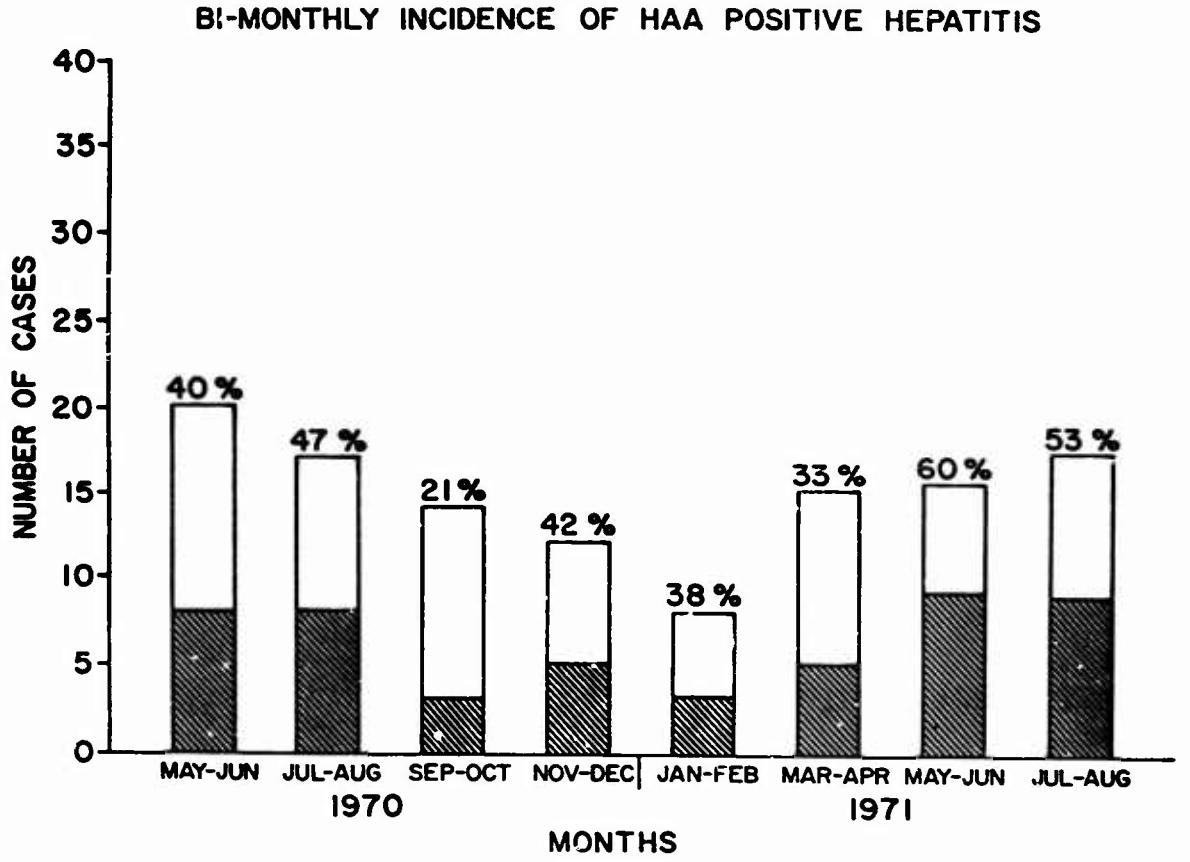
BI-MONTHLY INCIDENCE OF HAA POSITIVE HEPATITIS
NUMBER OF CASES
0
5
10
15
20
25
30
35
40
40 %
47 %
21%
42 %
38 %
33 %
60 %
53 %
MAY-JUN
JUL-AUG
SEP-OCT
NOV-DEC
JAN-FEB
MAR-APR
MAY-JUN
JUL-AUG
1970
1971
MONTHS

FIGURE 1.

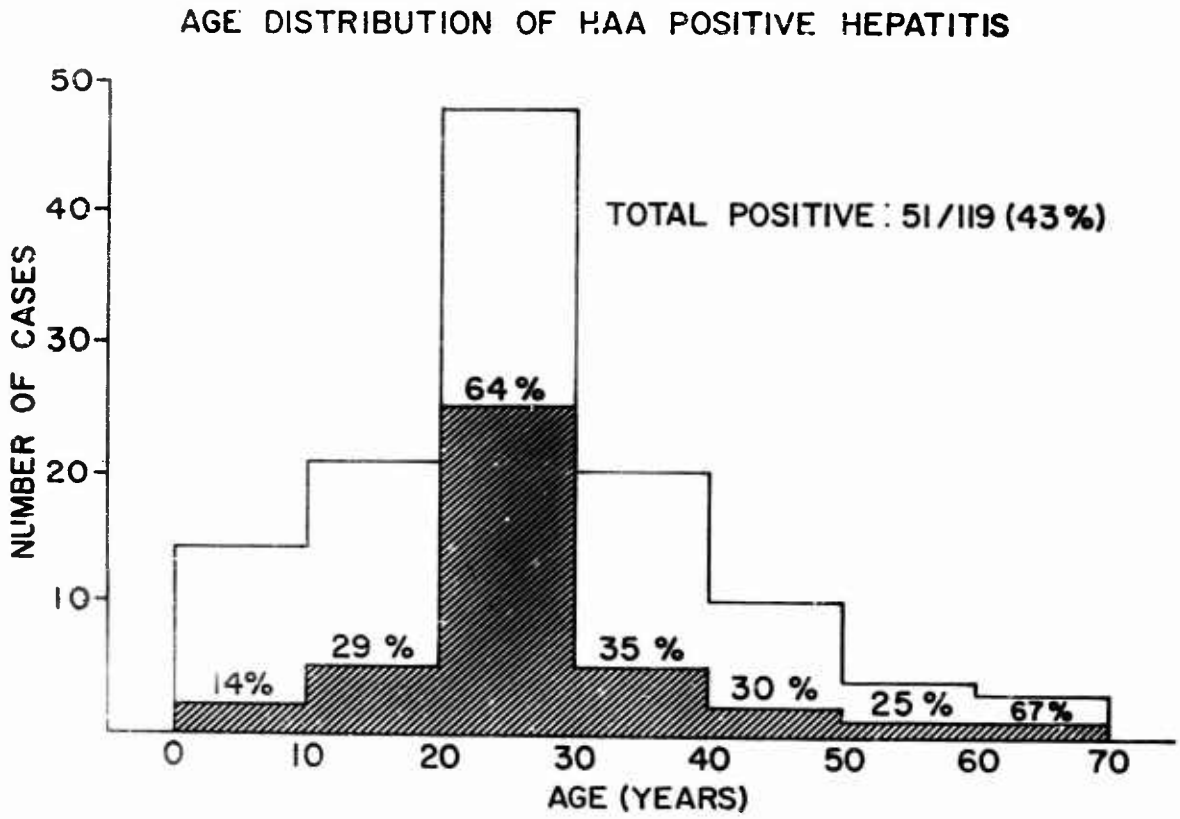
AGE DISTRIBUTION OF HAA POSITIVE HEPATITIS
NUMBER OF CASES
50
40
30
20
10
TOTAL POSITIVE: 51/119 (43%)
64%
14%
29 %
35 %
30 %
25 %
67%
0
10
20
30
40
50
60
70
AGE (YEARS)

FIGURE 2.

Study of Hepatitis in a Pediatric Outpatient Department

Investigators: Richard A. Grossman, LTC, MC
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Milton Willhight, SFC
Dumrong Chiewsilp, MD
Songsri Buranakarl
Pethai Mansuwan, MD*

OBJECTIVE: To determine the proportion of acute hepatitis cases seen in a pediatric outpatient department which are associated with HAA and to determine the duration of antigenemia.

DESCRIPTION: HAA has been found much less frequently in inpatient pediatric than adult hepatitis cases in Bangkok. Since available outpatient data suggested that most of the pediatric hepatitis cases are not hospitalized, a study of this population was indicated. By selecting age-and sex-matched case controls and by obtaining sera from family members of both cases and controls, background HAA prevalence could be controlled for and risk factors could be better assessed. The study was performed at the Bangkok Children's Hospital Outpatient Department throughout 1971. Detailed historical information was obtained from all cases, controls and their family members. Controls were of the same sex, age (usually within ± one year of the cases), and lived in the same general area of Bangkok-Thonburi (whenever possible) as the cases. Controls were usually selected on the same or following day at the outpatient clinic. Control diagnoses were almost all uncomplicated upper respiratory infections or pertussis. CF testing for HAA and anti-HAA was run on all sera; where CF results (HAA) were questionable, the IEOP test was also performed. An attempt was made to obtain serial sera from the families of all HAA positive persons (whether they were cases, controls or family members).

PROGRESS: From January to December, 1971, 71 patients were given a diagnosis of hepatitis. Of these, 62 had a clinical and laboratory picture compatible with the diagnosis. Two pairs of male-female siblings were included, one pair in April, one in November, and each time the sibs became symptomatic within 3 days of each other. None of these 4 cases were positive for HAA. Eleven of the 62 cases were residents of provinces outside the greater Bangkok area - 3 from Ayutthaya, 3 from Pathumthani, 2 from Nonthaburi and one each from

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* Director, Children's Hospital

Chachoengsao, Chainat and Chiangmai. Of the remaining 51 Bangkok area cases, controls were matched for 42 cases (82%) - 21 males and 21 females. The Bangkok cases occurred in every month except December. Although no sharp peak occurred, there were noticeably fewer cases in the final 3 months of 1971 (Jan-Mar: 18; Apr-Jun: 19; Jul-Sept: 10; Oct-Dec: 4). The age and sex distribution of these 51 cases is shown in Table 1. Males accounted for 53% of the cases and the males were 1-2 years older, on the average, than females.

Background information on the 42 cases and their controls is summarized in Table 2. Age and sex were, of course, comparable, but so too were household size and other characteristics except for contact with a jaundiced person. The typical symptoms of acute hepatitis are apparent in the case sample while fever was not often present in either group at the clinic visit. The cases were seen 2-15 days after the onset of symptoms (median 5 days). Comparability of non-hepatitis-associated variables is also shown by similar distributions of hemoglobin in cases and controls (Table 3). Liver function test results are clearly different. The other 9 cases without controls did not differ from the rest of the cases for the variables considered. The liver function test results were very similar between the family members of the 51 Bangkok area cases and the 42 controls (Table 4). Although only a small number of both family groups had slight elevations in these tests, such elevations were more frequent in case family members.

Antibody to HAA was not detected in any of sera tested during the study. HAA was detected in 9.5% of the 42 cases and 4.8% of their matched controls (Table 5). Although the difference is not statistically significant, there are two features which point to this being a real difference. First, the rate in the family members of both cases and controls is comparable to that in the controls, which is also approximately the prevalence found in the general Bangkok population (See Huay Khwang Study). Second, and more pertinent to the association of HAA with pediatric hepatitis cases, is the question of persistence of HAA positivity. Our results to date have indicated that HAA is usually a transient phenomenon in symptomatic hepatitis cases while asymptomatic Thais who have been found to have HAA in their sera have usually remained positive for up to 2 years of observation. Table 6 summizes the serologic history of the 20 HAA positive persons found in the study. Three of the 4 positive cases who were followed past the initial serum collection had titers less than 1:64 and rapidly reverted to negative. The fifth positive case was not followed, but was positive only by IEOP on the initial serum. All but one of the other 14 positive persons

(controls and family members) who were followed from 4 to 36 weeks had high, persistent CF titers (almost always ≥1:64). Liver function tests were normal in every HAA-positive control and family member. Without having the necessary pre-infection HAA status on the cases and a larger series of cases, it can only be suggested from these data that HAA is associated with a definite, but small proportion of symptomatic pediatric hepatitis cases in Bangkok and that the risk of developing overt hepatitis may be greater in a child who lacks the antigen than in one who is a persistent carrier.

The family of case 42, while interesting of itself, may also provide support for the latter hypothesis of risk. Six of the 8 family members of the case were HAA positive, the 5 positives that were subsequently followed remaining so; they are presumed to be HAA carriers (Table 6). The positives were 2 brothers (ages 13 and 16), 2 sisters (ages 12 and 27), the aunt (age 45) and the uncle (age 47) of the case (a 12 year-old male). Unfortunately, the 27 year-old sister, who had a low titer of 1:8 on the initial serum (study No. 42h), was lost to followup. Negative were a 14 year-old sister and an 18 year-old brother. The case's initial serum was anticomplementary and all subsequent sera were negative. There was no history of transfusions, drug addiction, jaundice or prior exposure to jaundice for any of the 9 family members.

Other examples of family clusters of asymptomatic HAA positives in Thais are presented elsewhere in the Report. Further prospective study is needed to determine whether (as Blumberg hypothesizes) symptomatic hepatitis is more likely to occur in HAA negative than in HAA-positive persons in a population like the Thais where the relatively large numbers of HAA positives may be related to a genetic predisposition to the carrier state.

Table 1

Hepatitis Cases at Children's Hospital Outpatient Department in Bangkok-Thonburi Residents, 1971

Age	Male	Female	Total
0-2	3	2	5
3-5	3	6	9
6-8	8	10	18
9-11	7	4	11
12-14	6	2	8
TOTAL	27	24	51
Median	8.8	7.2	7.9

Table 2

Comparison of Hepatitis Cases with Controls for Various Background Characteristics

Background Characteristic	Cases N=42	Controls N=42
Males	21	21
Median Age	7.9	7.5
Household Size - Range	3-12	3-12
Household Size - Median	7	7
Dental work previous 6 mos.	4	5
Medical injections previous 6 mos.	23	28
History of Jaundice	3	2
Contact with Jaundiced person previous 6 mos.	9	2
History of Blood Transfusion	1	0
Present Illness		
Vomiting	24	10
Dark Urine	38	5
Light Stool	10	1
Fever (Temp. > 37.5)	3	5
Jaundice	40	0
Enlarged or Tender Liver	30	0

Table 3

Comparison of Hepatitis Cases with Controls
for Various Laboratory Test Results

Laboratory Test	No. Pairs Tested	Cases	Controls
Hemoglobin - Range	40	5.6-14.7	7.5-14.5
Hemoglobin - Median	40	11.9	12.1
SGOT - Range	42	72*-1850	11-67
SGOT - Median	42	367	30
Total Bilirubin - Range	42	0.4**-34.4	0.0-0.6
Total Bilirubin - Median	42	6.8	0.3
Alkaline Phosphatase - Range	41	1.6-23.2	2.7-10.0
Alkaline Phosphatase - Median	41	10.1	5.1
HAA Positive	42	4(9.5%)	2(4.8%)

* All but one value was > 90.
** All but 2 values were > 1.8.

Table 4

Comparison of Family Members of Hepatitis Cases and Controls for Various Laboratory Test Results

Laboratory Test	Family Members of 51 Cases* N=186	Family Members of 42 Controls N=111
SGOT - Range	10-104	10-57
SGOT - Median	27.5	27.2
SGOT - No. ≥90	2	0
Total Bilirubin - Range	0.0-1.5	0.1-2.3
Total Bilirubin - No. (%) ≥ 1.0	12(6.4)	3(2.7)
Alkaline Phosphatase - Range	1.0-16.4	1.0-9.6
Alkaline Phosphatase - No. (%) ≥ 8.0	7(3.8)	2(1.8)
HAA Positive - No. (%)	8(4.3)	5(4.5)

* Total of 49 families since 4 cases occurred in 2 families.

Table 5

Summary of HAA Positives in Bangkok Area Cases, Controls and Family Members

	No. Tested	HAA Positives					
		No.	%	No. Male	Age		Mean SGOT
					Range	Median	
Cases							
Total	51	5	9.8	3	8-9	9	1045
With Controls	42	4	9.5	2	8-9	8.5	1181
Controls	42	2	4.8	1	10-12	11	34
Family Members							
Of the 51 Cases	186	8	4.3	3	11-47	21.5	27
Of the 42 Controls	111	5	4.5	4	12-37	24	27

Table 6

Persistence of HAA Positivity in Cases, Controls and Family Members

	Study Number	Initial CF Titer	No. Weeks Followed	Followup Sera		CF Titer on Last Serum
				No. Tested	No. (+)	
Cases (5)	16	1:16	2	1	0	Neg
	18	≥1:64	53	13	13	≥1:64
	40	1:32	4	3	2	Neg
	43	Neg*	0	0	- -	- -
	61	PF+*	22	8	1	Neg
Controls (2)	C-47	≥1:64	33	11	11	≥1:64
	C-65	≥1:64	16	7	7	≥1:64
Case Family Members (8)	27c	≥1:64	4	2	2	≥1:64
	42a	≥1:64	10	4	4	≥1:64
	42b	≥1:64	36	10	10	≥1:64
	42d	≥1:64	29	7	7	≥1:64
	42f	≥1:64	32	9	9	≥1:64
	42g	≥1:64	32	9	9	≥1:64
	42h	1:8	0	0	- -	- -
	53b	≥1:64	10	3	0	Neg
Control Family Members (5)	C-32c	≥1:64	5	2	2	≥1:64
	C-39d	≥1:64	4	2	2	≥1:64
	C-52a	1:16	24	7	7	≥1:64
	C-65b	≥1:64	20	7	7	≥1:64
	C-65c	≥1:64	20	6	6	≥1:64

* IEOP (+). + PF = Partial Fixation.

Hepatitis-Associated Antigen (HAA) in American Personnel with Acute Hepatitis in the Republic of Vietnam*

Principal Investigators: David A. Neumann, CPT, MC
Michael W. Benenson, CPT, MC

Associate Investigators: Robert B. Cotton, MAJ, MC
Dumrong Chiewsilp, MD

OBJECTIVE: To determine the prevalence of HAA positive hepatitis among American Servicemen with acute hepatitis in the Republic of Vietnam.

DESCRIPTION: Patients admitted to the 3rd Field Hospital in Saigon, the 24th and 93rd Evacuation Hospitals at Long Binh Post and the 6th Convalescent Center Cam Ranh Bay, with a diagnosis of hepatitis supported by clinical and laboratory evidence were entered in the study.

Laboratory tests were performed at the admitting hospital. These included Serum Glutamic Oxaloacetic Transaminase (SGOT), direct and indirect bilirubin and Alkaline phosphatase. Interviews were performed and sera collected by members of the U. S. Army Medical Research Team (USA MRT Vietnam).

HAA testing was done in Saigon using the agar-gel duffusion technique and aliquots of the sera were also brought to Bangkok for testing by CF, and by IEOP in some cases.

PROGRESS: The study was conducted from August to December 1970. One hundred seventy five patients met the criteria for inclusion in the study.

Thirty seven (?1%) were positive for the hepatitis-associated antigen using the agar gel diffusion technique. Another thirty-four were positive using the more sensitive CF and IEOP techniques. Thus a total of 71 (41%) patients were positive for HAA.

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* This study was done jointly by the Walter Reed Army Institute of Research Medical Research Team in Saigon and the SEATO Laboratory in Bangkok, Thailand.

The HAA antigen was detected most frequently in specimens taken during the first week after clinical onset of disease. The percentage of positives decreased progressively in specimens collected after that time.

Comparing questionnaire responses for the two groups, those that had HAA detected in their sera and those that did not, there was no difference in the following variables: race, age, time-in-country, location-in-country, eating on the economy, and drug use. Although time-in-country was not related to HAA detection, the number of cases of hepatitis did increase with increasing time-in-country.

Fifteen patients admitted to the intravenous use of habituating drugs. Of these fifteen only six (40%) were positive for HAA. This rate is very similar to that of the total group.

Convalescent sera, taken from 4-18 days after the initial bleed, were available for 21 patients. Eighteen showed decreasing titers of HAA. In the other 3, the titer increased, but in these cases the second serum specimen was taken only 4 days after the initial specimen.

The Epidemiology of Hepatitis-Associated Antigen (HAA) in a Defined Urban Population. I. Prevalence Survey

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Franklin H. Top, Jr., LTC MC
Robert B. Cotton, MAJ MC
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Rapin Snitphan, MD
Walter W. Noll, MAJ MC
Nelson Davis, Jr., SFC
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OBJECTIVE: To determine the prevalence of HAA in a random sample of urban Bangkok residents and to test the genetic hypothesis of the HAA carrier state.

DESCRIPTION: Our case-control studies of both Bangkok area hepatitis patients and recipients of HAA(+) blood have clearly demonstrated the important association with HAA in these populations. A sizeable (> 5%) prevalence of HAA has also been found in presumably healthy male blood donors. Although necessary and valuable information is derived from the study of these groups, important questions about host and environmental risk factors involved in the acquisition and persistence of HAA (and anti-HAA) and their relation to subclinical as well as clinical hepatitis can best and most validly be obtained by adequate sampling of the population at risk-in this case, the total Thai population. Since the overall risk of developing hepatitis is greater in urban than in rural populations, we decided to establish a Bangkok study site, perform a cross-sectional, family-directed survey, and, based upon these results, determine the feasibility and need for continuing prospective surveillance.

The study area selected was the Huay Khwang low-income housing development. One of the 3 major sections was mapped and found to have exactly 800 houses. The rows of houses (on both sides of 8 blocks) are composed of groups of 5 adjoining houses with several yards separating each of the 5-house clusters. The 160 clusters were taken as the sampling units and 15 such clusters (75 houses) were randomly selected. Thus the sampling process was basically similar to that used in Chiangmai and Mae Sariang. Confidence limits of the various statistics

presented were derived from the method for analyzing cluster random samples. In June, 1971, comprehensive interviews were performed with the random sample residents, using standardized questionnaire forms. Special attention was paid to the family relationships in each household so that accurate pedigree analyses could be performed in the testing of whether susceptibility to being an HAA carrier is inherited as an autosomal recessive trait (as Blumberg hypothesizes). In July, 1971, blood was collected from volunteering sample members (over age one year) within a 2-week period; heights and weights were also measured. The following tests were promptly run on the blood specimens: hemoglobin, hematocrit, SGOT, HAA (CF and IEOP tests) and blood typing (ABO, MNS, CDE, Duffy, Kell and Kidd). For very young children and a few who refused venipuncture, peripheral blood was sometimes obtained instead; only a limited number of tests could be performed on these specimens. After the HAA results became known, extra effort was expended to try to get complete (as far as possible) participation of all family members where at least one member had been found to be HAA-positive. Thus numerous extra specimens were obtained from relatives living in the Bangkok-Thonburi area. These non-random sample persons are not included in the prevalence analyses (Tables 1-7), but they are included in the family analyses (Table 8 and 9).

RESULTS: The random sample included a total of 814 people in the 75 houses, or an average of 10.8 people per house. There were 147 separate families residing in these houses (average of 2.0 families per house). The average family size, therefore, was 5.5 persons. The age and sex distributions of the Huay Khwang sample are presented in Table 1. Precision of the sampling process was both good (standard errors approximately 0.01) and comparable over the entire age span. Compared to a typical Thai village, this urban sample has proportionally more young people (median age 1-3 years younger) and fewer males (46% versus the country-wide value of 49%). Nearly 90% of the sample population (over age 1 year) volunteered a blood sample (Table 2). Participation was very good for females and children but only 70% of the adult males submitted specimens.

A total of 695 sera were processed for HAA - 55 (7.9%) were positive for the antigen (Table 3); none were positive for anti-HAA. Although it appears that the prevalence may be higher in males (9.5%) than in females (6.8%), this difference is not statistically significant, either in children or in adults (Table 4). An additional 11 persons (1.8%) had anticomplementary CF reactions, but the IEOP test was negative for each specimen. The IEOP procedure proved to be slightly more

sensitive than the CF test. Only one of the 55 positives was IEOP-negative, CF-positive, while IEOP was positive in 5 sera which were CF-negative plus 3 more sera which had partial fixation or ≤1:2 CF reaction. Overall, 80% of the CF positives had high (≥ 1:32) titers which are what we have usually found in persistent HAA carriers. (Indeed, followup sera 6 months later on these persons found all such persons tested still positive, at similar high titers).

In general, past histories on HAA-positive persons differed little from those in the whole sample (Table 5). The historical variables considered, therefore, provided no clue as to possible means of HAA acquisition. Only a history of jaundice was elicited in greater frequency in HAA-positives, but only 4 of the 55 positives (7.3%) gave such a history. The HAA-positive group was also similar to the entire sample in distributions of height, weight, hemoglobin and hematocrit (Table 6). (Age-adjustment of the positives was not required since the HAA-positives were similarly distributed to the entire sample). Although the mean SGOT for both sexes was higher in the positive group (and worthy of further evaluation), 48 of the 55 HAA-positives had SGOT values below 70 and only 3 were over 90 (highest 104). Differences in the 2 blood typing groups are probably reflecting the clustering of HAA-positives in family members plus the incompleteness of the typing data; these and other important genetically-related data are presently being further evaluated.

Tables 7 and 8 present attempts to describe the distribution of HAA positives in families. The 55 positives (and presumed persistent HAA carriers) were present in 11 of the 15 five-house clusters and in 23 of the 75 houses. Although these figures do not suggest a marked clustering effect, analysis by blood relatives (i.e., families) in these houses definitely suggests a degree of clustering within families which is not necessarily shared between families or between houses. Whereas almost 1/3 of the houses had at least one HAA(+) resident, only 18.4% of the families showed this. As expected, the % of positive families increased with increasing family size, but so too did the proportion of positive people in positive families (Table 7, c/b). There were several examples of houses with 2 large (5-7) families residing together where only one family had HAA-positives. Although one family could still conceivably be sharing an environmental HAA ascertainment factor not shared by the other family, contact between both families' members is extremely close at all social levels and results such as these appear to better support a theory of familial predisposition to HAA-carriage. Table 8 shows that at least one offspring was positive in each of the 6 families in which the mother was positive

compared to only 8 out of 75 families in which the mother was negative and the father was either negative or not tested. The method used by Blumberg to test the genetic hypothesis was applied to these family data (Table 9). The segregation of HAA-positive offspring in matings where one parent is HAA-positive (upper table) shows a very close fit to the expected distribution and provides further support for the hypothesis that predisposition to the HAA-carrier state is inherited as an autosomal recessive condition. The fit is poor, for matings where both parents are negative (lower table). However, the numbers are quite small and in 7 of the 12 families HAA results are known for only one parent (the other parent was assumed to be negative). Further data may be forthcoming from a repeat serum collection from the total random sample which is being obtained in April, 1972.

Available sera from the random sample cohorts from Mae Sariang and Chiangmai Valley (see 1969-70 and 1970-71 Annual Reports) provided the opportunity to determine (by IEOP test) the prevalence of HAA in these Northern Thai populations. In Mae Sariang and in 3 of the 4 Valley villages, prevalence was lower than in Huay Khwang. The schoolchildren in urban Chiangmai (age 6-8) had a similar prevalence to Huay Khwang and one village (C) had 9 of 46 males (20%) HAA-positive. The 9 were clustered in 4 families. Each of the 22 HAA-positives in Chiangmai had persistent IEOP-positive reactions over the 2 years (Nov 69-Nov 71) of surveillance and had normal SGOT values.

Table 1

Sex and Age Distributions of Huay Khwang Random Sample

Age	Male		Female		Total				
	Frequency	Relative Freq. (%)	Frequency	Relative Freq. (%)	Frequency	Relative Freq. (%)	Cumulative Relative Frequency (%)	$\hat{Sp}$*	95% CL+
0-4	58	15.4	46	10.5	104	12.8	12.8	.0100	10.6-14.9
5-9	55	14.6	58	13.2	113	13.9	26.7	.0155	23.3-30.0
10-14	63	16.8	67	15.3	130	15.9	42.6	.0167	39.1-46.2
15-19	45	12.0	59	13.5	104	12.8	55.4	.0126	52.7-58.1
20-29	51	13.6	78	17.8	129	15.8	71.2	.0134	68.4-74.1
30-39	40	10.6	56	12.8	96	11.8	83.0	.0110	80.7-85.4
40-49	42	11.2	38	8.7	80	9.9	92.9	.0055	91.7-94.0
50-	22	5.8	36	8.2	58	7.1	100.0	- -	- -
Total	376	100.0	438	100.0	814	100.0	100.0	- -	- -
Median Age	16.3		19.1		17.9				

* Standard error of the sample proportion.
\+ 95% Confidence limits of the sample proportion.

Table 2

Participation of Huay Khwang Random Sample in Giving Blood Specimens

Age	Male		Female		Total	
	No. Sera	%*	No. Sera	%	No. Sera	%
1-9	89	89.0	87	94.6	176	91.7
10-19	100	92.6	120	95.2	220	94.0
20-29	38	74.5	71	91.0	109	84.5
30-39	27	67.5	53	94.6	80	83.3
40-49	27	64.3	37	97.4	64	80.0
50-	16	72.7	32	88.9	48	82.8
Total	297	81.8	400	93.9	697	88.3
Median Age	15.0		19.3		17.4	

* % Giving blood specimen.

Table 3

Prevalence of HAA by Age and Sex

Age	Male			Female			Total		
	No. Tested	HAA (+)		No. Tested	HAA (+)		No. Tested	HAA (+)	
		No.	%		No.	%		No.	%
1-4	34	1	2.9	31	2	6.4	65	3	4.6
5-9	54	5	9.2	56	7	12.5	110	12	10.9
10-14	61	8	13.1	66	2	3.0	127	10	7.9
15-19	39	6	15.4	54	3	5.5	93	9	9.7
20-29	38	5	13.2	70	4	5.7	108	9	8.3
30-39	27	0	0.0	53	4	7.5	80	4	5.0
40-	43	3	7.0	69	5	7.2	112	8	7.2
Total	296	28	9.5	399	27	6.8	695	55	7.9*
Median	15.0	15.0		19.3	19.2		17.4	16.4	

* 95% Confidence limits 4.5-11.3%.

Table 4

Comparison of HAA Prevalence Between Males and Females

Age	HAA Prevalence (%)		χ^2
	Male	Female	(1 DF)
<15	9.4	7.2	0.48 NS*
≥15	9.5	6.5	1.19 NS
Total	9.5	6.8	1.19 NS

* NS= Not Significant.

Table 5

Comparison of Historical Characteristics
between HAA-positive Persons and Total Sample

History of	HAA(+) N=55 (%)	Total Sample* N=697 (%)
Hospitalizations	16.4	16.8
Medical injections previous 6 mos.	23.6	20.2
Dental work previous 6 mos.	5.4	4.4
Tattoo	3.6	4.7
Blood donations	1.8	2.7
Blood transfusions	3.6	3.2
Jaundice	7.3	3.6

* Total sample submitting serum specimen.

Table 6

Comparison of HAA-positive Pers' with Total Sample for Various Measurement and Laboratory Test Results

Statistic		HAA (+) Group N=55*	Total Sample N=697*
Mean Height (cm)	Male	142.8	140.8
	Female	138.6	140.6
Mean Weight (Kg)	Male	37.9	38.6
	Female	38.4	41.7
Mean Hemoglobin (g%)	Male	11.9	12.6
	Female	11.2	11.6
Mean Hematocrit (%)	Male	38.0	38.7
	Female	35.2	36.2
Mean SGOT (sf units)	Male	39.0	24.7
	Female	37.0	26.8
Blood Type %	O	29.6	33.9
	A	25.9	26.0
	B	27.8	31.1
	AB	16.7	8.9
%	M	50.9	43.2
	N	14.5	13.2
	MN	34.5	43.6

* No. results slightly less for some tests.

Table 7

Distribution of HAA-positive Families and People by Family Size

Family Size*	a No. Families	b No. Families HAA (+)+	b/a (%)	c No. People HAA(+)	c/b
1-2	47	2	4.3	2	1.0
3-5	47	10	21.3	15	1.3
6-8	40	8	20.0	16	2.0
9-11	8	4	50.0	5	1.2
12-14	4	3	75.0	17	5.7
19	1	0	0.0	0	-
Total	147	27	18.4	55	2.0
Median	4.7	6.4	-	7.5	-

* No. in family submitting sera.
\+ One or more HAA (+) person in family.

Table 8

HAA Results in Families

Offspring*	Mother		Total
	LAA(+)	HAA(-)	
≥1 HAA(+)	6	12+	18
All HAA(-)	0	67	67
Total	6	79	85

* At least 2 offspring per family (Median=4).

\+ Father was HAA(+) in 4, HAA(-) in 4 and unknown in 4 families.

Table 9

Segregation of HAA-positives in Families with at Least One HAA-positive Offspring

No. Offspring in Family	No. of Families	No. HAA(+)		Variance
		Observed	Expected	
MATING TYPE: HAA(+) x HAA(-)				
1	2	2	2.000	0.000
2	4	5	5.332	0.888
3	2	4	3.428	0.980
4	1	1	2.133	0.782
6	1	1	3.048	1.379
7	1	6	3.528	1.667
8	1	4	4.016	1.945
Total	12	23	23.485	7.641
		χ^2(1 DF)=0.033 p~0.85		
MATING TYPE: HAA(-) x HAA(-)				
1	2	2	2.000	0.000
2	1	1	1.143	0.122
3	1	1	1.297	0.263
4	3	3	4.389	1.260
5	2	3	3.278	1.184
6	1	2	1.825	0.776
8	2	2	4.446	2.344
Total	12	14	18.378	5.949
		χ^2(1 DF)=3.222 p~.075		

Table 10

Prevalence of HAA in Northern Thailand Study Groups

Study Area*	Age Range Tested	No. Tested	No. HAA(+)	HAA Prevalence (%)		
				Male	Female	Total
Chiangmai Valley						
Village A	1-69	84	0	0.0	0.0	0.0
Village B	1-39	84	1	2.0	0.0	1.2
Village C	1-39	80	9	19.6	0.0	11.2
Village D	1-39	91	1	2.3	0.0	1.1
Urban School E	6-8	161	11	7.5	6.2	6.8
Mae Sariang Town	1-79	177	5	4.9	1.0	2.8

* All samples were randomly selected except for school E. Blood collected June, 1969 in Mae Sariang; others in July, 1971.

Observations of HAA in A Blood Donor/Recipient System in Thailand

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DESCRIPTION:

Background Studies in the United States have left little question that patients receiving blood containing HAA run a greater risk of post-transfusion hepatitis than patients receiving blood that does not contain HAA. The attack rate, however, appears to be a function of the population of recipients studied. While Gocke (1) has shown that over 50% of recipients of HAA positive blood develop evidence of hepatitis, Cherubin (2) reported a much lower attack rate, and speculated that his study population, a low socioeconomic group, many of whom were parenteral drug users, was largely immune to serum hepatitis.

* Thai Component

In view of this discrepancy in results between different populations in the United States, it would be hazardous to extrapolate these findings to Thailand and other areas of Southeast Asia, which have a number of pertinent differences from the United States. Whereas the prevalence of blood donors positive for HAA is 0.1-0.5% in the United States, 5-10% or more of the presumably healthy people in parts of Southeast Asia and other tropical areas are carriers of HAA. Furthermore, some investigators have presented evidence that the distribution of the carriers in these populations is genetically determined.

Assuming that the prevalence of HAA in Thailand would be high, as in other areas in Southeast Asia, the Hepatitis Study Group at this Laboratory felt it worthwhile to undertake a survey of Thai blood recipients in order to learn what happens to the recipient of blood containing HAA. The primary goal of this survey is to assess the risk of post-transfusion hepatitis in a patient who receives blood containing HAA. In addition, the survey allows us to describe responses by the recipient other than the development of hepatitis.

Study Site The Royal Thai Army Hospital (Pramongkutklao Hospital) was selected to provide the study population of blood recipients. This is a large, multispecialty general hospital administered by the Royal Thai Army.

The blood requirement of the Royal Thai Army Hospital is provided by the Blood Bank of the Royal Thai Army Institute of Pathology, located adjacent to the Hospital. Virtually all the blood processed by the Blood Bank is used to fill requests submitted by the Royal Thai Army Hospital. Almost 90% of the blood comes from a group of paid donors who donate blood at the Blood Bank. The remainder is supplied by the Thai Red Cross.

In February, 1971, permission was given SEATO Laboratory to obtain a blood specimen and questionnaire information from all donors at the Blood Bank. If donors were not present, as in the case of Red Cross blood, extra serum from the "side tubes" accompanying the blood unit would be made available to our laboratory. In addition, the Blood Bank agreed to let SEATO Laboratory establish a screening station in series with the regular donor processing system. This permitted us to create a complete donor registry of our own, enabling donor identity to be double-checked.

In March, 1971, permission was obtained from the hospital commander to follow all blood recipients. The project was explained to the chiefs-of-services, who agreed to cooperate actively in helping this laboratory study their patients.

In order to test the HAA status of recipients before they receive blood, the Blood Bank sets aside part of the "type and crossmatch" specimen for our use. To ensure that an adequate volume of serum would be available for these additional tests, the Blood Bank persuaded the Hospital, whenever possible, to submit 10 ml of blood along with each "type and crossmatch" request. As a result of this cooperative measure we are missing pre-transfusion HAA results in less than 5 percent of the recipients.

Each week, an average of 122 units of blood are "deposited" in the Blood Bank. Of these approximately 110 are withdrawn and transfused at the Royal Thai Army Hospital. The remaining units either are used in clinics not a part of Royal Thai Army Hospital, or are lost through contamination or expiration. Blood components and derivatives are only rarely used by the Hospital.

We offered to provide the Blood Bank with our HAA test results as soon as possible, so that units of blood containing HAA could be removed before transfusion. Since the CF test was to be our method of HAA testing, results would not be available for at least 24 hours after donation. Thus, the recipients of blood positive for HAA would come from that group of patients who received blood before the HAA test results were known. Once the study began, however, the Blood Bank Director elected not to remove units of blood containing HAA.

Survey Population A "potential" recipient is assigned a study number the first time the Blood Bank receives a "type and crossmatch" request. When that "potential" recipient receives blood, he becomes an "actual" recipient (see Fig.1). "Actual" recipients may be inpatients (IN) or outpatients (OUT). They may reside in the Bangkok-Thonburi area (BT) or outside this area ($\overline{BT}$). If outpatients, they may be alive (ALIVE) or dead (DEAD). They may become lost (LOST) to follow-up. If a patient receives blood within 6 months before entry into the survey, or receives blood during the survey that has not been tested for HAA, he is a recipient of uncharacterized blood units (UBU). Otherwise, he is a recipient of only characterized blood units (CBU). Recipients are further charcterized according to the HAA status of the blood received. They may be recipients of blood positive (RPH), indeterminate (RIH), or negative (RNH) for HAA (see below).

Because of the numerous difficulties in following a large number of recipients for six months, we have imposed criteria to restrict the number of patients that we follow actively. This active category is made up of recipients from boxes a through g in Fig.1. In order to assess the risk of post-transfusion hepatitis following the receipt of blood containing HAA, comparisons will be made between the RPH recipients (boxes a, b, and e) and the RNH recipients (boxes d and g).

For the purposes of this survey, recipients represented by the numbered boxes in Fig.1 are considered "inactive" and are not followed actively.

"Active" recipients are followed weekly or biweekly while inpatients. After discharge form the hospital, we attempt to follow the recipient every two weeks. In some cases, it has only been possible to contact the recipient monthly.

At each follow-up visit, blood is obtained from the recipient for HAA and liver function tests. A medical history emphasizing the signs and symptoms of hepatitis is obtained from each recipient intially and medical progress notes are recorded at subsequent contacts. We attempt to follow each active recipient for 6 months.

Interpretation of HAA Test Results Initially, only the CF test was used for the detection of HAA and anti-HAA. The advantages of the IEOP test for the detection of HAA, and more recently, anti-HAA, soon became apparent, and we began routinely testing all sera obtained in this survey by both techniques.

Experience with the CF test convinced us that modification of the classic scheme of reporting results would be necessary. According to this modification, there are 5 possible CF test results:

1. "$<$ 1:2" This report indicates that the serum is negative for HAA by complement fixation.

2. "$\leq$ 1:2" This report indicates that some ($<$ 4 U) complement is fixed at a serum dilution of 1:2, and that fixation disappears over subsequent dilutions. Although this result would be reported as $<$1:2 according to the classic rules of CF testing, we have encountered such a result frequently as the titer of HAA falls during convalescent period of HAA positive hepatitis, indicating that HAA is probably present, but in an amount too small to fix at least 4 of the 5 units of complement.

3. " 1:D" This report indicates that the serum is positive for HAA up to dilution D ($2 \geq D \geq 64$).

4. "PF" This report indicates that there is a partial fixation of complement, the degree of which either rises and falls, or remains constant, over several dilutions. This result does not reflect very low titer HAA (as does the result " $\leq$ 1:2", but indicates that the HAA present does not react with the antiserum for optimal complement fixation. Most of the sera that yield this result are positive by IEOP.

5. "AC" This report indicates that the serum is anti-complementary.

All sera collected in this survey are now tested for HAA and anti-HAA by IEOP, as well as by CF. IEOP results are recorded as "positive" or "negative". When the precipitin line is very faint, the serum is retested until the examiner is satisfied with his interpretation of the reaction.

Classification of a Serum Specimen: After a serum specimen is tested for HAA (and anti-HAA) by CF and IEOP, it is classified as "positive", "indeterminate", or "negative" according to the scheme shown in Fig. 2. This scheme also is used when the serum specimen is tested by only one method. If only CF were used, the IEOP result is called "negative" in this scheme. Similarly, if only IEOP were used, the CF result is called " < 1:2" and the serum is then classified using the same scheme.

The use of this scheme permits us to minimize false negatives and to acknowledge and identify indeterminate results that could complicate the later interpretation of survey findings.

Classification of A Recipient: As described above, a recipient is classified as RPH, RIH, or RNH according to the HAA status of the transfused blood. The HAA status of a bottle of blood is determined by the CF and IEOP tests for HAA performed using serum obtained from the donor at the time the bottle of blood was donated. Thus, the status of a unit of blood may be positive, indeterminate, or negative for HAA (and anti-HAA). Consequently, each "active" recipient (see Fig.1) can be assigned to one of three categories:

1. Recipient of blood positive for HAA (RPH): One who has received at least one unit of blood positive for HAA.

2. Recipient of blood indeterminate for HAA (RIH): One who has received at least one unit of blood indeterminate for HAA and no units

of blood positive for HAA.

3. Recipient of blood negative for HAA (RNH): One who has received only units of blood negative for HAA.

Personnel Nurses from the Department of Medicine work in the Blood Bank processing donors Monday through Friday. A nurse-assistant is kept on duty to log in all "type and crossmatch" requests and to separate an aliquot of serum from the "type and crossmatch" blood sample.

Public Health nurses from the Department of Epidemiology and nurses from the Department of Medicine follow the active recipients. After discharge from the hospital, these nurses visit the recipients at home or, in some cases, arrange for the recipient to return to SEATO Laboratory for follow-up.

A nurse and nurse-assistant log in all blood samples generated by the survey. These two workers also separate the serum into aliquots for HAA and liver function tests.

Six technicians working under the supervision of Dr. Dumrong Chiewsilp of the Thai Component perform the CF and IEOP tests for HAA and anti-HAA. Two technicians working under the supervision of Dr. Rapin Snithbhan in the Department of Virology perform the IEOP test for HAA.

Biochemical liver function tests (SGOT, SGPT, total and direct bilirubin, alkaline phosphatase, and thymol turbidity) are performed by the staff of the Biochemistry Laboratory of the Department of Experimental Pathology.

There is one full time data processing specialist who is responsible for the transfer of primary data from manually-entered data sheets to Hollerith cards for later automatic processing.

Data Processing The large number of data transactions and the complexity of the information flow associated with this survey created a need for automatic data processing assistance. This aspect of the survey is described in a separate report.

PROGRESS:

Donors Since 1 March 1971, 7349 units of blood have been deposited into the Blood Bank. Three thousand three hundred nineteen paid donors

gave 6367 of these units. The remaining 982 units came from Red Cross sources.

Comparison of CF and IEOP test results: Figure 3 shows the distribution of CF and IEOP results obtained from testing 2571 serum specimens from blood donors. (In some cases, there were 2 or more sera from one donor.) The " $\leq$ 1:2" and "PF" CF result categories have been combined. Based on the scheme of HAA test result interpretation described earlier, 10.7% of these sera were positive for HAA, 3.6% were indeterminate, and 85.7% were negative.

Prevalence of HAA in donors: Table 1 shows the results of testing sera from 1322 donors. Each serum was tested by both CF and IEOP.

Prevalence of HAA by blood group: There was no apparent difference in the distribution of ABO blood groups between HAA positive and HAA negative donors (Table 2). The HAA indeterminate group was not included in this analysis.

HAA status and SGOT level: The distribution of SGOT levels in 1080 HAA negative donors and 128 HAA positive donors is displayed in Fig.4. The mean SGOT level of the HAA negative donors was 26.7 Sigma Units (standard deviation = 7.9) and the median level was in the 25-29 interval. Twelve (1.1%) HAA negative donors had an SGOT level of 70 Sigma Units or more. The mean SGOT level of the HAA positive donors was 32.8 Sigma Units (standard deviation = 10.6) and the median level was in the 30-34 interval. Seven (5.5%) HAA positive donors had an SGOT level of 70 Sigma Units or more. There is a tendency for HAA positive donors to have a slightly higher SGOT level than the HAA negative donors. In individual cases, however, the SGOT level does not discriminate between the HAA positive and HAA negative donor.

Recipients From March,1971, through March,1972, there were 1929 requests for transfusion, of which 594 did not receive blood. Thus 1335 (69.2%) of the "potential" recipients became "actual" recipients. Of these 1335 patients, 369 (27.6%) received one or more HAA positive units. There were 525 positive units transfused; 91 patients received more than one HAA positive unit. The maximum number of positive units given to a recipient was 14.

Pre-transfusion sera were not available for testing from 86 patients. Of the remaining 1843 patients, 133 (7.2%) were positive for HAA before receiving any blood; 25 (1.3%) were positive for anti-HAA by CF testing*. This prevalence of HAA in the serum of ill patients requiring a transfusion is similar to the prevalence in an urban population of Bangkok (see Huay Khwang report) and in the donor population, both presumably healthy groups of people.

At the time of this report (April 1972) 123 patients have been followed for at least ten weeks (Table 3). Seventy-nine patients received at least one unit of HAA positive blood (RPH group) and 44 patients received only negative units of blood (RNH group).

Recipients of HAA positive blood (RPH): For purposes of analysis, three patients with a pre-transfusion sera positive for HAA and two patients positive for anti-HAA were excluded, leaving 74 patients for consideration. There were 42 (57%) males and 32 (43%) females in this group. The mean age overall is 35.4 years and median age, 34.0 years.

The most frequent diagnoses in the group receiving positive units were trauma (30%), followed by obstetric and gynecologic disorders (16%), gastrointestinal disorders (13%), carcinoma (12%), hematological problems (8%), and liver disease (3%). There were 6 deaths in the RPH group.

Patients in this group received a mean of 8.8 units of blood, females averaging 6.0 units and males 10.9 units.

Thirteen of the 74 recipients in the RPH group developed detectable HAA in their sera following transfusion. Of these 13, 6 were antigenemic without convincing biochemical or clinical evidence of hepatitis (Fig.5), 6 had anicteric hepatitis (Fig.6), and 1 had icteric hepatitis (Fig.7). HAA became detectable from 1-98 (median 21) days after receipt of the first blood containing HAA. In 6 recipients, HAA has continued to be detectable 41 to 231 days after receipt of the initial positive unit. Antigenemia has continued longer than 15 weeks in 4 of these patients. There are no obvious differences in sex, age,

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* Anti-HAA data in this report result from the CF test only. IEOP results for anti-HAA are not used.

number of units received, titers of units received, blood group, or SGOT-levels between those recipients persistently antigenemic and those antigenemic for only a short time.

Twenty-two recipients developed anti-HAA between 1 and 54 days (median 7 days) after receipt of the first blood containing HAA. Anti-HAA was detectable for a short time in 19 recipients. In the other 3 recipients, antibody has remained detectable throughout the follow-up period, 49, 64, and 68 days after it was initially detected. The remaining 40 members of the RPH group developed neither HAA nor anti-HAA. Although none of these 62 recipients (the 40 that developed neither HAA nor anti-HAA and the 22 that did develop anti-HAA) developed detectable HAA, 5 had SGOT levels greater than 90 Sigma Units sometime during follow-up. In 4 of these, the SGOT elevation was associated with a CF antibody response, raising the possibility that an immune response may play a role in mediating hepatic cellular necrosis. The recipient record in Fig.8 represents such a case.

Recipients of only HAA negative blood (RNH): Forty-four recipients in this category have been followed for at least 10 weeks, 26 (59%) females and 18 (41%) males. The mean age over-all is 35.6 years with a median age of 34.5 years. The predominance of females and their younger age is likely due to the input from the obstetric and gynecologic service. On this service the patients tend to be young and usually do not require multiple transfusions.

Diagnoses in this category (RNH) of recipients, in contrast to the RPH group, included a larger proportion of obstetric and gynecologic disorders (48%) and a smaller proportion of traumatic injuries (11%). The percentages for the other diagnoses are essentially the same as in the RPH group.

Patients in the RNH group received a mean of 2.7 units of blood (males, 3.1; females, 2.4).

None of the recipients in the RNH group developed detectable HAA during follow-up, and only 3 have had an SGOT level greater than 90 Sigma Units. Only one of these appeared to have acute hepatitis. Another had cirrhosis and jaundice. The elevated SGOT level (98 Sigma Units) in the third was unexplained.

Literature Cited

1. Gocke, DJ: A prospective study of posttransfusion hepatitis: the role of Australia antigen. JAMA 219:1165, Feb.28, 1972.

2. Cherubin, CE: Risk of post-transfusion hepatitis in recipients of blood containing SH antigen at Harlem Hospital. Lancet i:627, Mar.27, 1971.

Table 1.

PREVALENCE OF HAA IN THAI BLOOD DONORS

HAA RESULT	NO.	%
POSITIVE	139	10.5
INDETERMINATE	39	3.0
NEGATIVE	1,144	86.5
TOTAL	1,322	100.0

Table 2

BLOOD GROUP DISTRIBUTION IN THAI BLOOD DONORS

BLOOD GROUP	HAA POSITIVE (N = 139)	HAA NEGATIVE (N = 1144)
O	44 %	41 %
A	19 %	18 %
B	31 %	35 %
AB	6 %	6 %

TABLE 3

Active Recipients Followed Ten or More Weeks

	RPH	RNH
Number of patients	74 (100%)	44 (100%)
Males	42 (57%)	18 (41%)
Females	22 (43%)	26 (59%)
Mean age	35.4	35.6
Males	32.5	37.1
Females	39.2	34.7
Median age	34.0	34.5
Males	24.5	39.0
Females	40.5	33.0
Units per recipient	8.8	2.7
Males	10.9	3.1
Females	6.0	2.4
Developed detectable HAA	13 (18%)	0
Males	6 (14%)	0
Females	7 (32%)	0
Developed detectable anti-HAA	22 (30%)	0
Males	12 (29%)	0
Females	10 (45%)	0

Figure 1. Classification of Recipient Population

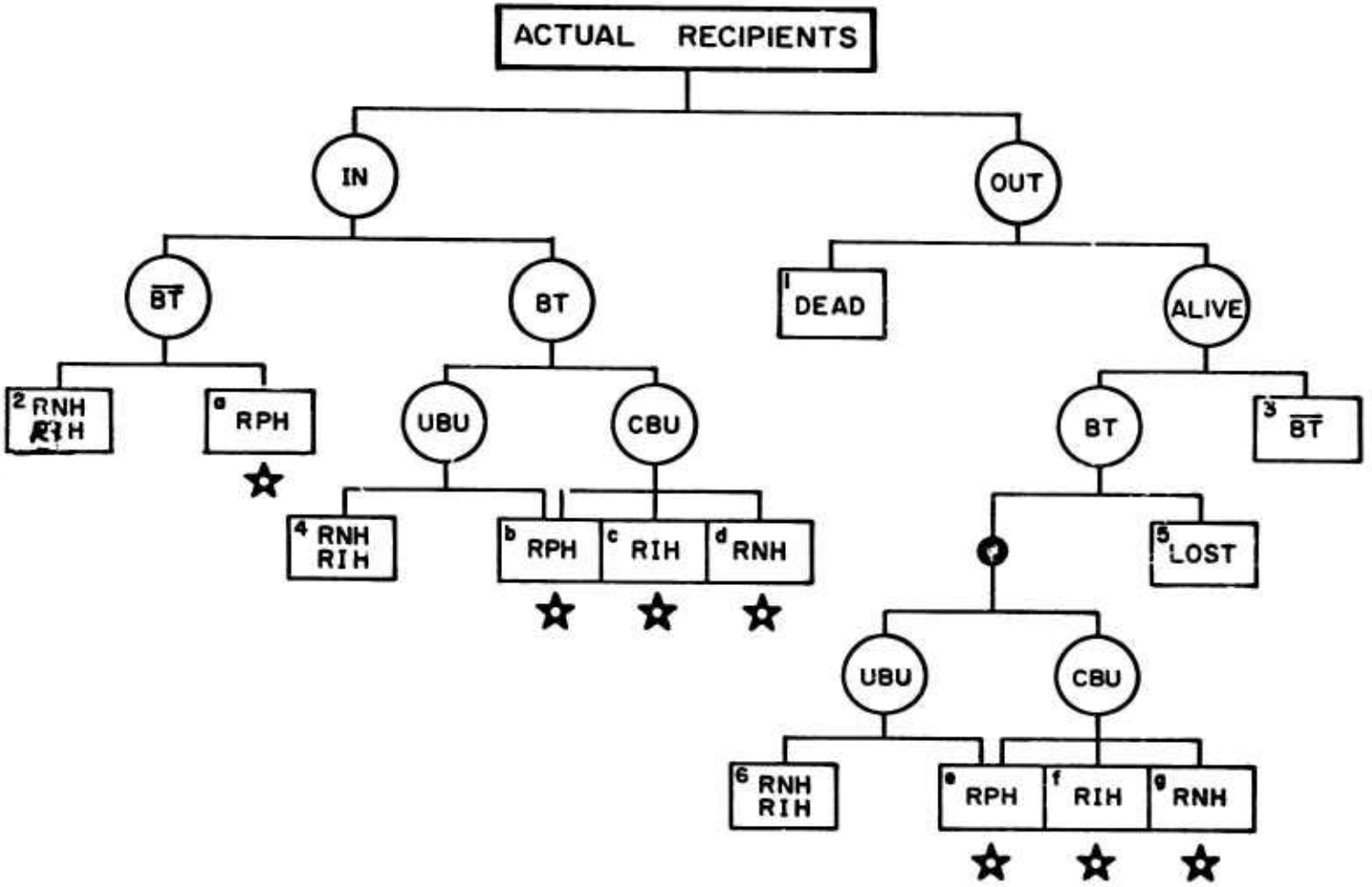


Figure 2

Scheme Used to Derive A Consensus Result
from CF and IEOP Results

CF Result:	IEOP Result: +	IEOP Result: -
< 1:2	P	N
≤ 1:2	P	I
PF	P	I
1:D	P	P
AC	P	I

P = positive

I = indeterminate

N = negative

Figure 3.

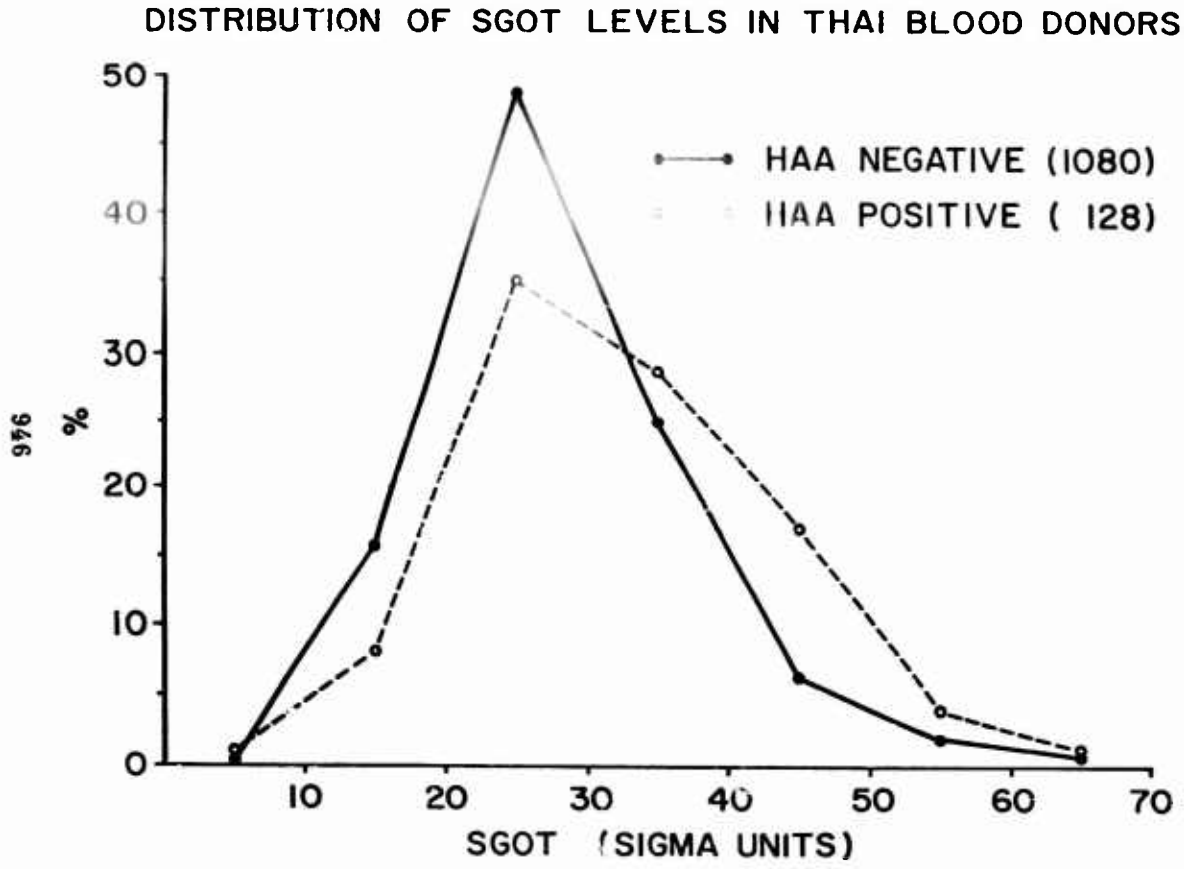
DISTRIBUTION OF CF AND IEOP TEST RESULTS

CF RESULT	AC	IEOP RESULT +		IEOP RESULT −	
< 1:2		P	0.2	I	3.2
1:2 to ≥ 1:64		P	0.5	N	85.7
≤ 1:2 AND PF		P	6.8	P	0.4
		P	2.8	I	0.4

TOTAL = 100.0 %
(N = 2571)

POSITIVE	275 (10.7%)
INDETERMINATE	93 (3.6%)
NEGATIVE	2203 (85.7%)

Figure 4


DISTRIBUTION OF SGOT LEVELS IN THAI BLOOD DONORS
HAA NEGATIVE (1080)
HAA POSITIVE (128)
%
50
40
30
20
10
0
10
20
30
40
50
60
70
SGOT (SIGMA UNITS)

R-0296 — Figure 5. Recipient record facsimile exemplifying the development of antigenemis without convincing biochemical evidence of hepatitis.

TRANSFUSIONS

TRANSF. DATE	BLOOD UNIT	DONOR NO.	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE
07MAY71	1607-0.	0997	03715	LT2			LT2			
12MAY71	1767-0.	1149	04214	64			LT2			
12MAY71	1769-0.	0236	04216	LT2			LT2			
17MAY71	1831-0.	1202	04335	32	P		AC			
10JUN71	2324-0.	1588	06067	LT2	-		LT2			
22JUN71	2500-0.	0201	06594	LT2	-		LT2			
22JUN71	2496-0.	1771	06590	LT2	-		LT2			
09JUL71	2720-0.	0894	07050	LO2	P		LT2			
15JUL71	2815-0.	2059	07224	LT2	-		LT2			

LABORATORY RESULTS

DATE	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE	SGOT	SGPT	TCT. BIL.	DIR. BIL.	ALK. PHO.	T.T.
11MAY71	04298	LT2			LT2									
17MAY71	04699	LT2	-		LT2									
25MAY71	05134	LT2	ND		LT2				37	20	00.0	00.0	03.3	06.8
01JUN71	05600	PF	P		LT2				21		00.0	00.0		
08JUN71	06094	PF	P		LT2				36	20	00.6	00.3	01.9	04.6
10JUN71	06169	PF	P		LT2									
22JUN71	06675	PF	P		LT2									
09JUL71	07358	64	P		LT2									
15JUL71	07592	LT2	-		LT2									
10AUG71	09341	64	P		LT2				34		00.6	00.2	04.8	13.2
16SEP71	10642	64	P		LT2				41	58	00.3	00.1		11.6
30SEP71	11321	64	P		LT2				55	99	00.0	00.0		10.5
02NOV71	12520	64	P		LT2	-			29	55	00.7	00.2	04.6	13.4

R-052

Figure 6. Recipient record facsimile exemplifying the development of anicteric hepatitis.

TRANSFUSIONS

TRANSF. DATE	BLOOD UNIT	DONOR NO.	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE
18JUN71	2327-0+	0329	06070	64	P		LT2			
18JUN71	2431-0+	0217	06280	LT2	-		LT2			
18JUN71	2344-0+	0681	06087	LT2	-		LT2			
19JUN71	SP15-0+	1765	00000	ND			LT2			
19JUN71	X52350-0+	1766	06580	LT2	-		LT2			
21JUN71	X01996-B+	1777	06626	LT2	-		LT2			
21JUN71	X070-0+	1778	00000	ND			LT2			

LABORATORY RESULTS

DATE	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE	SGOT	SGPT	TOT. BIL.	DIR. BIL.	ALK. PHO.	T.T.
18JUN71	06537	LT2	-		LT2									
20JUN71	06574	4	P		LT2									
21JUL71	07864	64	P		LT2				88		00.3	00.1	01,8	03.0
04AUG71									218		00.8	00.4	03,8	03.3
05AUG71	09894	64	P		LT2									
23AUG71	09755	16	P		LT2				230	255	00.7	00.2	04,9	02.9
30AUG71	09981	PF	P		LT2				164	440	00.9	00.4	04,1	02.8
14SEP71	10554	LT2	-		LT2				242	112	00.8	00.4		02.1
28SEP71	11201	LT2	-		LT2				18	55	00.6	00.3		02.5
29OCT71	12377	LT2	-		LT2	-			19	14	00.8	00.2	01,8	02.3
30NOV71	14010	LT2	-		LT2	-			11	7	00.3	00.1	01,9	02.8

R-02331

Figure 7. Recipient record facsimile of the single recipient who developed icteric hepatitis.

TRANSFUSIONS

TRANSF. DATE	BLOOD UNIT	DONOR NO.	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE
26APR71	PT10-B+	0985	03759	LT2	-		LT2			
26APR71	PT12-B+	0986	03760	LT2	-		LT2			
30APR71	1658-B+	1066	03827	64			LT2			
30APR71	1663-B+	1069	03850	LT2			LT2			
04JUN71	2179-B+	1460	05682	LT2	-		LT2			
04JUN71	2187-B+	0294	05690	PF	P		LT2			
05JUN71	2258-B+	1524	05904	LT2	-		LT2			
05JUN71	2259-B+	1525	05905	LT2	-		LT2			
08JUN71	2281-B+	1541	05990	LT2	-		LT2			
08JUN71	2282-B+	1542	05991	LT2	-		LT2			
22JUN71	X53117-B+	1821	06682	64	P		LT2			
22JUN71	X53146-B+	1822	06680	LT2	-		LT2			

LABORATORY RESULTS

DATE	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE	SGOT	SGPT	TOT. BIL.	DIR. BIL.	ALK. PHO.	T.T.
26APR71	03700	LT2			LT2									
30APR71	03925	LT2			LT2									
25MAY71	05158	32	P		LT2				34	60	01.1	00.4	05.4	04.8
01JUN71	05597	64	P		LT2				1380		01.0	00.5		
04JUN71	05882	64	P		LT2									
08JUN71	06111	16	P		LT2									
15JUN71	06362	64	P		LT2				585	870	05.2	02.8	07.8	05.8
22JUN71	06663	PF	P		LT2				635	1030	07.3	04.1	07.8	02.4
24JUN71	06684	PF	P		LT2									
29JUN71	06992	PF	P		LT2									
06JUL71	07250	LT2	-		LT2				160		22.6	15.7	06.6	03.1
13JUL71	07446	LT2	P		LT2				171		14.2	08.6	04.4	03.6
20JUL71	07747	LT2	-		LT2				360	171	05.3	03.3	03.1	05.7
									127	255	02.9	01.9	03.6	05.6

R-0139

Figure 8. Recipient record facsimile exemplifying the development of CF anti-HAA and elevated SGOT levels.

TRANSFUSIONS

TRANSF. DATE	BLOOD UNIT	DONOR NO.	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	ANT HAA TYPE
09APR71	1328-0+	0667	02919	AC	-		AC			
09APR71	1329-0+	0668	02920	LT2	-		LT2			
12APR71	1333-0+	0672	02924	AC	-		AC			
12APR71	1334-0+	0673	02925	LT2	-		LT2			
17MAY71	1917-0+	0248	04635	LT2	-		LT2			
17MAY71	1919-0+	1271	04637	LT2	-		LT2			
20MAY71	1889-0+	0102	04536	LT2	-		LT2			
20MAY71	1876-0+	1238	04523	16	P		LT2			

LABORATORY RESULTS

DATE	SERUM ACCESS	HAA CF	HAA IEP	HAA AGD	ANT HAA CF	ANT HAA IEP	ANT HAA AGD	HAA TYPE	SGOT	SGPT	TOT. BIL.	DIR. BIL.	ALK. PHO.	T.T.
09APR71	02978	LT2	ND		LT2									
17MAY71	04738	AC	-		AC									
20MAY71	04995	AC			AC									
27MAY71	05336	LT2	-		LT2				120	160	00.8	00.6	04.3	02.8
04JUN71	05744	LT2	-		16									
10JUN71	06147	AC	-		AC				67		00.9	00.4		
17JUN71	06504	LT2	-		LQ2				182	310	00.6	00.5	05.0	06.5
06JUL71	07253	LT2	-		PF				95		00.6	00.2	03.9	02.8
27JUL71	08293	LT2	-		LT2				445	695	00.9	00.4	04.0	04.6
10AUG71	09335	LT2	-		LT2				ND					
24AUG71	09780	LT2	-		LT2				39	31	00.5	00.2	02.6	04.7
28SEP71	11209	LT2	-		LT2				15	13	00.4	00.1		07.6
28OCT71	12346	LT2	-		LT2	P			37	18	00.3	00.1	02.6	10.2
30NOV71	14008	LT2	-		LT2	P			23	31	00.0	00.0	02.6	08.4
30DEC71	15213	LT2	-		LT2	P			24	20	00.6	00.2	02.1	08.6

Comparison of the Sensitivity of Complement Fixation (CF) Test and Immunoelectroosmophoresis (IEOP) for Detection of Hepatitis B

Principal Investigators: Rapin Snitbhan, M.D.
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Franklin H. Top, Jr., M.D.

BACKGROUND: Of the many tests described to detect HB Ag in human serum, three-Agar gel diffusion (AGD), CF and IEOP-have been used in this laboratory. This report compares the sensitivity of the 3 tests in detecting HB Ag in the serum of Thai blood donors and recipients.

METHODS: Sera tested were obtained from blood donors and recipients in a study earlier described in this report or from patients with hepatitis. Details of methods of AGD, CF, and IEOP tests were described in last year's Annual Report.

PROGRESS: Table 1 compares the results of CF and IEOP tests for detection of HB Ag in human serum. Of the 3445 sera tested, IEOP detected HB Ag in 369 (10.7%). By CF, 216 sera (6.2%) were positive for HB Ag (positive equals complete fixation at ≥1:2 serum dilution) or 306 sera (8.9%) if partial fixation reactions are deemed to reflect antigen. Relatively good agreement between CF and IEOP results were evident in sera with complete fixation, except for sera with low CF titer (2-4). The occasionally negatives by IEOP in sera containing antigen at > 1:32 was usually due to a prozone effect; dilution of such sera to 1:4 gave positive IEOP reactions.

The principal difference in the sensitivity of the tests was manifest in sera giving partial fixation. Approximately 3% of all sera tested gave such reactions and 90% of these were IEOP positive. Reasons for the partial-fixation reaction in these sera, many of which contain large amounts of antigen by titration in IEOP, is uncertain; the possibility that such sera contain anti-HB IgM antibodies which block complement-fixation by the immune antisera used in the CF test or contain principally immunocomplexes of HB Ag with saturated antibody or complement sites is intriguing. In any case, sera giving partial fixation account for about 25% of IEOP positive sera encountered in Bangkok.

Strong AGD reactions were generally found only in sera that were IEOP positive with CF titer ≥ 32. IEOP positive sera with undetermined CF reaction or partial fixation generally gave weak or no precipitins in AGD.

Table 1. Comparison of CF and IEOP tests in detection of HB antigen

CF Titer	Sera Tested (No.)	IEOP Positive (No.)	IEOP Negative (No.)
negative (<2)	3008	66	2942
2-4	20	11	9
8-32	72	69	3
>32	124	115	9
undetermined	131	27	104
partial fixation	90	81	9

An Automatic Data Processing System for the Blood Bank Project

Investigators: Robert B. Cotton, MAJ, MC
David Londe[1]
Ronald Campbell, SFC
Michael W. Benenson, CPT, MC
Nitaya Kanchanawan
Thavorn Sa-nguandej

DESCRIPTION: The Blood Bank Project had been underway for only several months when it became apparant that the voluminiously accumulating data was beginning to saturate the existing manual system of record keeping. In 3 months, over 2000 donors and almost 700 recipients had been enrolled in the survey. Several thousand data transactions were occurring each week in the process of keeping the donor and recipient records up to date.

Figure 1 is a schematic illustration showing the data pathways required to create and maintain the donor and recipient records. The donor master file (DMF) and recipient master file (RMF) are files of records describing each blood donor and recipient and the events that happen to them. The laboratory result file (LRF) contains results of laboratory tests performed on blood specimens from donors and recipients. The black boxes represent the laboratory. A DMF or RMF record is created when a donor or recipient is first encountered (double solid lines). As laboratory results become available, these entries are added to the DMF and RMF records (solid lines). As new events happen to the donors (additional donations) and to the recipients (transfusion, death, discharge, laboratory test request, etc), the master files are revised (double broken lines). A blood transfusion relates a donor to a recipient, leading to an exchange of information between that recipient record in the RMF and that donor record in the DMF (single broken lines). When manually maintained, the nature of this data flow limited the recorders access to the data sources and object records, creating numerous problems, for the investigators had to compete with the recorders for study subjects' records, compromising our ability to monitor the study. The successful execution of the project was being threatened by this sluggish and unwieldy system of manually kept data files. Thus, in August, work was begun on an automatic data processing system for the maintenance

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[1] Contracted by Advanced Research Projects Agency
U.S. Department of Defense, Bangkok, Thailand

of the donor and recipient master files.

PROGRESS: This automatic data processing system has been operational since March, 1972. Figures 2 through 5 show how the system works. Data generated by the study is entered manually into 5 types of data sheets suitable for direct key-punch transcription to data cards (Figure 2). The resulting 5 types of data cards are then used to revise the donor and recipient master files, which are kept on magnetic tape. In addition to the magnetic tape output containing up-to-date donor and recipient master files, there are several kinds of printed output (Figure 3). A copy of each revised donor record and each revised recipient record is printed. An inventory of the recipient population is listed*. Finally, data errors that the system encounters are listed. Listings of the DMF and RMF are kept in bound volumes. The volumes are kept up to date by replacing obsolete records with revised records (Figures 4 and 5). The master files are revised twice a week. Facsimiles of printed recipient records are shown in Figure 5-8 in the report "Observations of HAA in A Blood Donor/Recipient System in Thailand".

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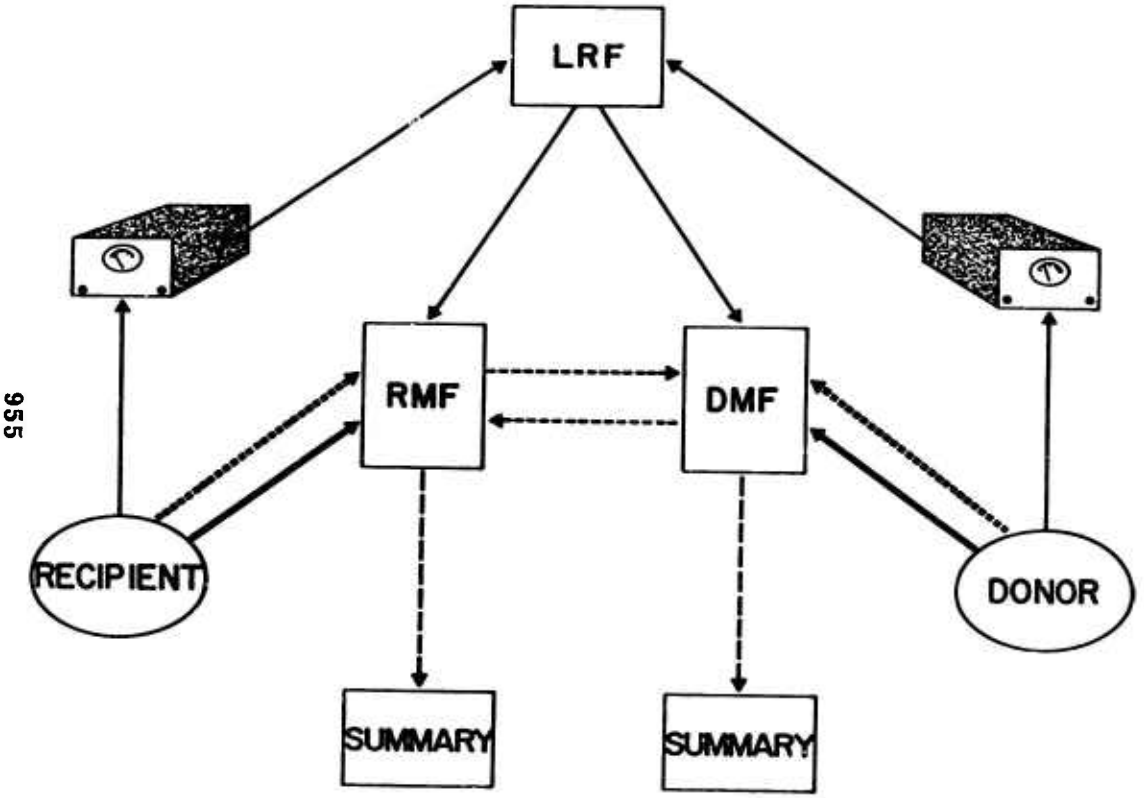
* Not yet operational

Figure 1.



INFORMATION FLOW IN BLOOD BANK PROJECT

Figure 2.

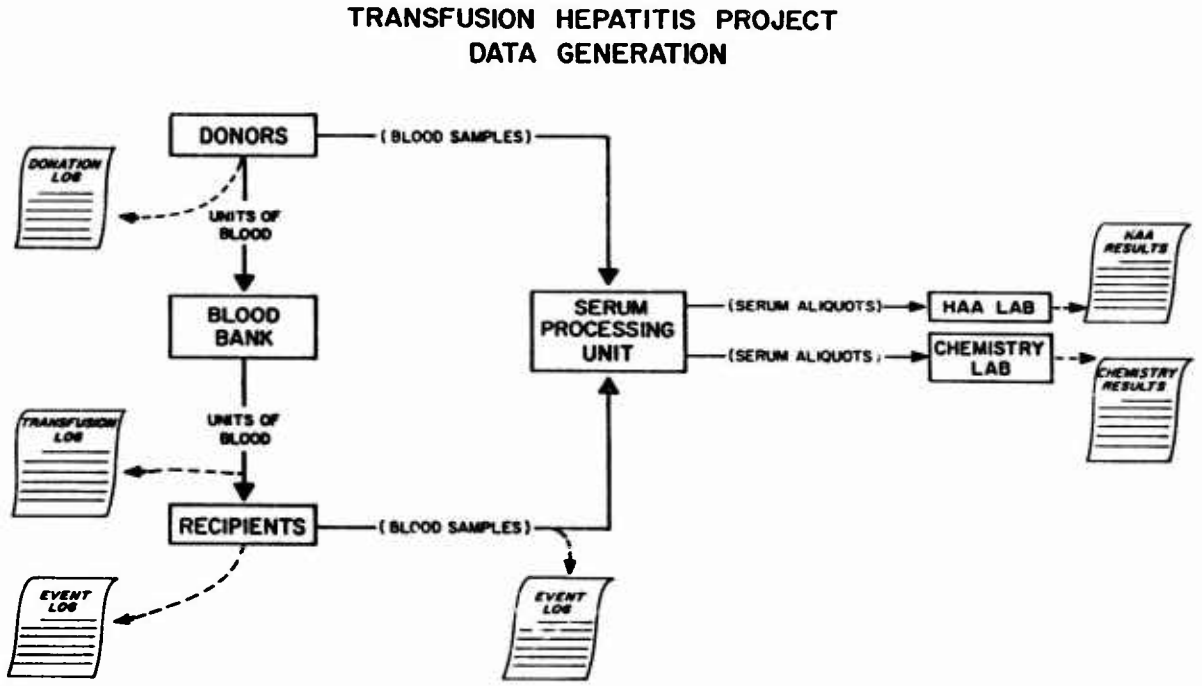

TRANSFUSION HEPATITIS PROJECT
DATA GENERATION
DONORS
(BLOOD SAMPLES)
DONATION LOG
UNITS OF BLOOD
BLOOD BANK
SERUM PROCESSING UNIT
(SERUM ALIQUOTS)
HAA LAB
HAA RESULTS
(SERUM ALIQUOTS)
CHEMISTRY LAB
CHEMISTRY RESULTS
TRANSFUSION LOG
UNITS OF BLOOD
RECIPIENTS
(BLOOD SAMPLES)
EVENT LOG
EVENT LOG

Figure 3.

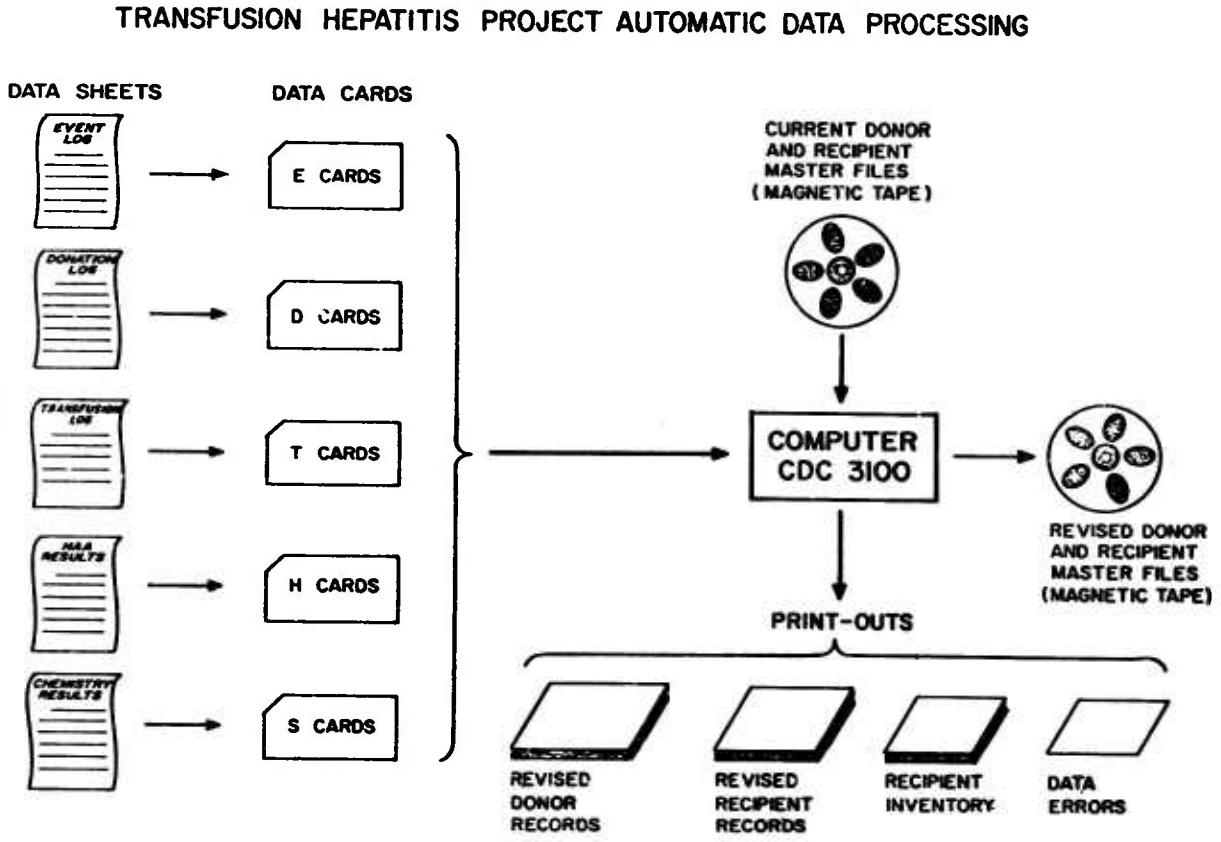

TRANSFUSION HEPATITIS PROJECT AUTOMATIC DATA PROCESSING
DATA SHEETS
DATA CARDS
EVENT LOG
E CARDS
DONATION LOG
D CARDS
TRANSFUSION LOG
T CARDS
HAA RESULTS
H CARDS
CHEMISTRY RESULTS
S CARDS
CURRENT DONOR AND RECIPIENT MASTER FILES (MAGNETIC TAPE)
COMPUTER CDC 3100
REVISED DONOR AND RECIPIENT MASTER FILES (MAGNETIC TAPE)
PRINT-OUTS
REVISED DONOR RECORDS
REVISED RECIPIENT RECORDS
RECIPIENT INVENTORY
DATA ERRORS

Figure 4.

TRANSFUSION HEPATITIS PROJECT
MANUAL DATA PROCESSING OF DONOR RECORDS

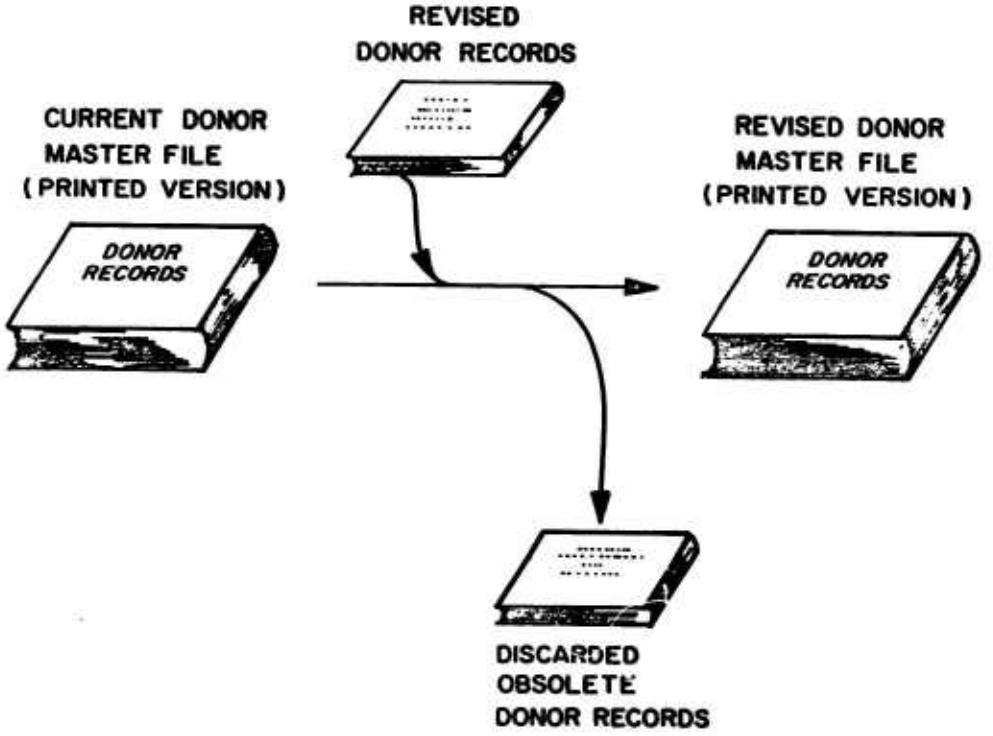
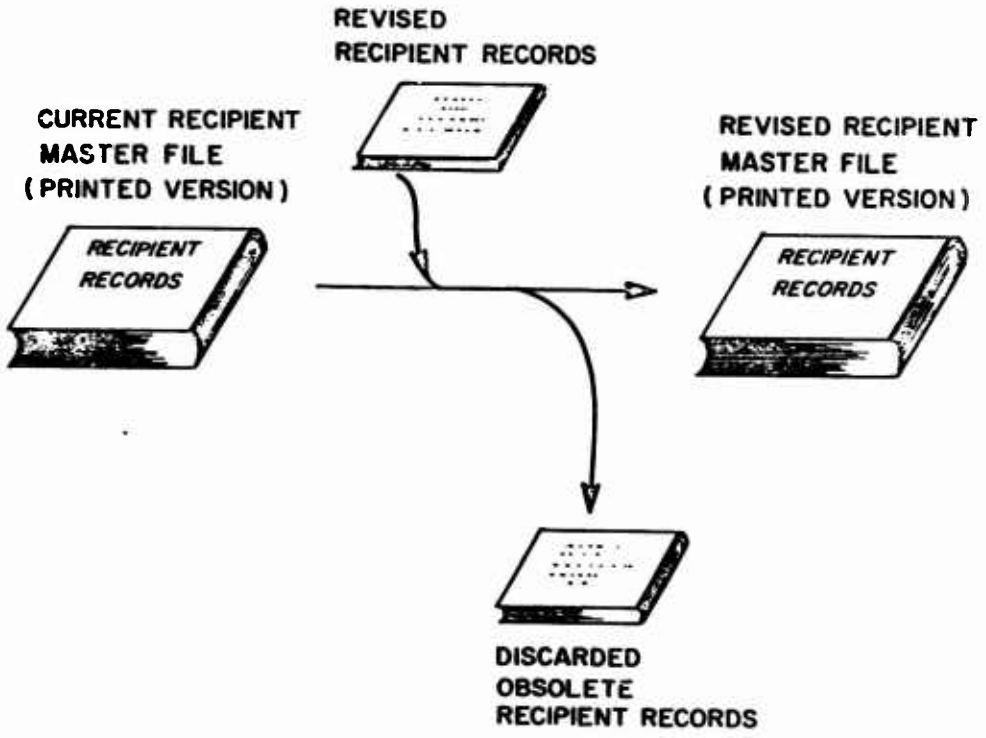


Figure 5.

TRANSFUSION HEPATITIS PROJECT
MANUAL DATA PROCESSING OF RECIPIENT RECORDS



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0B 6466	72 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. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A811	00	045
b. CONTRIBUTING				
XXXXXXXXX	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Bacterial and Mycotic Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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

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a. DATES/EFFECTIVE: NA EXPIRATION:

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18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 72	2.4	108.0
	CURRENT 73	1.0	60.0

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code) (U)Bacterial Diseases; (U)Mycotic Diseases; (U)Southeast Asia; (U)Diarrhea; (U)Pneumonia; (U)Venereal Diseases; (U)Vibrio parahaemolyticus

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

23. (U) To identify bacterial and mycotic diseases of military importance in Southeast Asia and to provide information to aid in the diagnosis, treatment, and control of the diseases.

24. (U) Diseases occurrence is certified by clinical and laboratory methods. Where relevant, long term surveillance of a population for occurrence of particular bacterial or mycotic diseases is instituted. Variables affecting transmission and virulence are studied in vivo and in vitro.

25. (U) 71 07 - 72 06 The potential enteric and systemic pathogenic bacteria, Vibrio parahaemolyticus has been demonstrated to occur throughout the year in the sea waters and sea foods of Thailand. Characteristics regarding the probable pathogenicity of V. parahaemolyticus appear to differ from isolates from temperate areas. Clandestine use of unprescribed antibiotics by promiscuous females has no effect on frequency of infection with Neisseria gonorrhea. Cultures from 148 penile lesions resembling soft chancre of Clostridium tetani will remain dormant in the leg muscle of mice for 2 months before germinating and causing clinical tetanus after subsequent inoculations of Staphylococcus aureus. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 71 - 30 Jun 72.

*Available to contractors upon originator's approval.

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Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 045, Bacterial and mycotic diseases of man and animals

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Vibrio parahaemolyticus in Thailand

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

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OBJECTIVE: To determine the characteristics and occurrence of Vibrio parahaemolyticus in Thailand.

BACKGROUND: Vibrio parahaemolyticus, a gram negative, halophilic bacillus was first reported in Thailand in 1970 by the SMRL. Since that time, much attention has been brought to bear on this bacteria's importance as a cause of gastroenteritis in Thailand. In one hospital near Bangkok, this organism has been recovered from up to 25% of the adult patients reporting to that facility with diarrhea. This figure compares with a recovery frequency at that hospital of only 15% of Salmonella spp. and Shigella spp. combined. Other reports have indicated that sea foods from Thailand served to tourists on international flights have harboured V. parahaemolyticus and caused outbreaks of diarrhea and gastroenteritis among the passengers.

The department of bacteriology and mycology has directed its attention to refining certain characteristics of the bacteria, to demonstrating the continued occurrence of V. parahaemolyticus in Thai sea waters and sea fisn throughout the year, in investigating the Kanagawa phenomenon of this bacteria, and in searching for a model of pathogenicity. Additional effort was expended in a survey of American tourists presenting with diarrhea to a dispensary in a transient billets, in determining antibiotic sensitivity profiles of various isolates of V. parahaemolyticus, and in determining the frequency of isolation from Thai pediatric patients with diarrhea. A report on a possible model of pathogeny and on the occurrence of this bacteria in pediatric diarrhea is presented elsewhere in this annual report.

PROGRESS:

CHARACTERISTICS Vibrio parahaemolyticus was identified in this laboratory by the biochemical and morphological reactions and appearances reported in the 1971 SMRL Annual Report. Extensive serology was attempted using 52 antisera from Japan, however many of the isolates from Thailand remain untypeable. Of a randomly selected 100 isolates from human diarrhea cultures, 77 were typeable. Only 50% of our isolates from natural sources are typeable. In addition to reactions previously reported, characteristics in arginine, ornithine and lysine decarboxylase media have been noted. Typical reactions in our laboratory reveal that 100% of the isolates failed to decarboxylate arginine, 79.5% exhibited a decarboxylase activity to ornithine and 98.3% were capable of decarboxylating lysine.

Our experience has also noted a need for a careful standardization of methods used to determine the halophilism character of these organisms. One procedure, that of inoculating test tubes of alkaline peptone water (APW) plus varying amounts of NaCl from KIA tubes with 3% NaCl, resulted in a very high percentage of strains growing at 10% NaCl. This characteristic (growth in APW with 10% NaCl) is important as it can often be used to determine the species of other halophilic organisms often associated with V. parahaemolyticus.

Other tests have indicated that numerous strains of V. parahaemolyticus can survive, i.e., be recovered when subsequently streaked on TCBS agar, 18-24 hours at 37C in APW with up to 20% NaCl. If halophilism is measured in this manner - recovery from the test media, then numerous strains would be erroneously classified. Our data indicate that there is seldom any growth (increase in number of organisms/ml of media) at these increased concentrations of NaCl.

Part of the literature fails to mention the use of shaking in testing for halophilism. Our findings indicate that of 60 representative strains in APW with 10% NaCl, 34 demonstrated increased turbidity after 18 hours at 37C in a shaking machine. Only 10% of the 60 strains could produce turbidity after 18 hours at 37C without shaking.

Our results are presently reported as measurements of density in 15mm screw cap test tubes containing 5 ml of APW with 0%, 3%, 7%, and 10%

NaCl. These tubes are inoculated with 1 loopful (3 mm loop) of inocula from a 24 hour culture in APW plus 3% NaCl at 37C. Tubes are then shaken in a 37C water bath for 18 hours and turbidity is visually determined on a 1+ to 4+ scale.

OCCURRENCE: Japanese investigators have reported that V. parahaemolyticus is only rarely found in sea waters and sea foods during the winter months. This fact is probably responsible for the up to 70% of summer diarrhea in that country being attributed to this organism. Reports from Viet Nam have also indicated a lower frequency of recover in the winter months.

In order to determine if there is a fluctuation or an absence of V. parahaemolyticus in Thai sea water or sea foods throughout the year, monthly surveys were conducted during the past year by personnel of SMRL. These surveys consisted of obtaining samples of sea water near the shore line, sand from the shore, and sea fish (mollusks, shrimp, crab, and fish) from local markets. Cultures of fish pastes and sauces that are often used in Thai foods, were all negative for V. parahaemolyticus. All of the study sights were near populated areas and the markets are used daily by housewives for purchasing sea foods. Figure 1 presents the data collected since October 1970. The quantity of specimens examined is represented at the left with the shaded portion of the columns depicting those specimens with V. parahaemolyticus. During the first winter that the survey was conducted (Dec 1970; Jan, Feb, 1971) only random samples were collected. The specimens for January 1971, for example, included 65 sand and sea water samples - only 23 of which were positive. In February 1971 only 12 shrimps were examined. More control was exercised in the winter of 71-72 with more uniformity of specimens obtained. These data appear to indicate that there is no decrease in the frequency of recovery of V. parahaemolyticus during those winter months.

Table 1 shows the distribution of isolates during the past 12 months from natural (isolates from sources other than human) sources. It is evident that recovery is more uniform in sea foods than from sand and sea water. Counts of V. parahaemolyticus in sea water obtained along populated shore lines have been between 10,000 and 100,000 organisms per ml. Sand specimens were especially more varied. We suspect this variation is due to water content of the sand as drier sand specimens usually resulted in fewer positive cultures of V. parahaemolyticus.

Kanagawa Phenomenon:

In 1969, Miramoto and his colleagues reported that strains of V. parahaemolyticus isolated from natural sources failed to haemolyze blood in a special media. In their laboratory, only 0.5% of isolates from sand, sea water, and sea fish haemolyzed red blood cells while 91% of their isolates from human diarrhea patients exhibited haemolysis. This special media, Wagatsuma agar, is made without heat sterilization and contains 5% washed human (group O) or rabbit blood cells. This haemolysis is termed the Kanagawa phenomenon and has been closely associated with pathogenicity because of the above findings.

Isolates of V. parahaemolyticus obtained from natural sources in Thailand do not behave on Wagatsuma agar as do Japanese isolates. Using media made with either rabbit blood cells or human group O cells (blood is washed three times with normal saline and then reconstituted to its original volume with normal saline), this laboratory found that most Thai isolates from either human or natural sources were haemolytic.

One hundred twenty-eight isolates from human diarrhea cultures were examined as were 140 from natural sources. Table 2 presents the findings of these experiments. Figure 2 compares this data to that reported from Japan.

The organisms in Japan were tested against rabbit blood cells. Later, personal communication with Dr. R. Sakazaki suggested that we use only human blood in the Wagatsuma agar. Our data reveal that the bacteria from Thailand continued to demonstrate haemolysis in human blood.

Recovery of V. parahaemolyticus from American Tourists:

A survey was conducted at a transient billets (Chao Phya Hotel) in Bangkok to determine the frequency of recovery of V. parahaemolyticus from Americans with diarrhea temporarily residing at that facility. Most of the persons presenting to the dispensary located in the hotel were visiting Bangkok for fewer than 5 days. Most had been in the city for less than 2 days. All test subjects and controls were adult and more than 85% were male. This facility was selected as a study site because it was felt that transients would be less likely to carry their own medication and treat themselves as do many long term residents of Bangkok.

Rectal swabs were obtained from the patients, placed in Cary-Blair media immediately, and inoculated onto TCBS agar plates within 6 hours.

Table 3 shows the results of this study. It is noted that 5.6% of the 71 cases studied had V. parahaemolyticus while 4.2% had Salmonella species and only 1 of the 71 had Shigella sonnei isolated from the diarrheal stool. Seventeen American nationals seen at the dispensary for cuts or abrasions were used as controls. Their histories were identical to those patients with diarrhea. None of the 17 control specimens revealed any pathogenic enteric bacteria from stool cultures.

Antibiotic Sensitivities of Various Strains of V. parahaemolyticus:

Numerous strains of V. parahaemolyticus were tested for sensitivities against several commonly used antibiotics. The organisms were grown in BHIB with 3% NaCl at 37C for 20 hours and then seeded onto tripticase soy agar plates. Antibiotic discs were placed on the seeded plates and sensitivity was indicated by zones of inhibition around the discs.

The organisms were divided into groups according to their haemolysis on Wagatsuma rabbit blood agar and the source from which the bacteria were obtained. Group I are strains that are haemolytic, group II are non-haemolytic, group III from human diarrhea, and group IV from sources other than human diarrhea (natural). The results of this study are presented in Table 4.

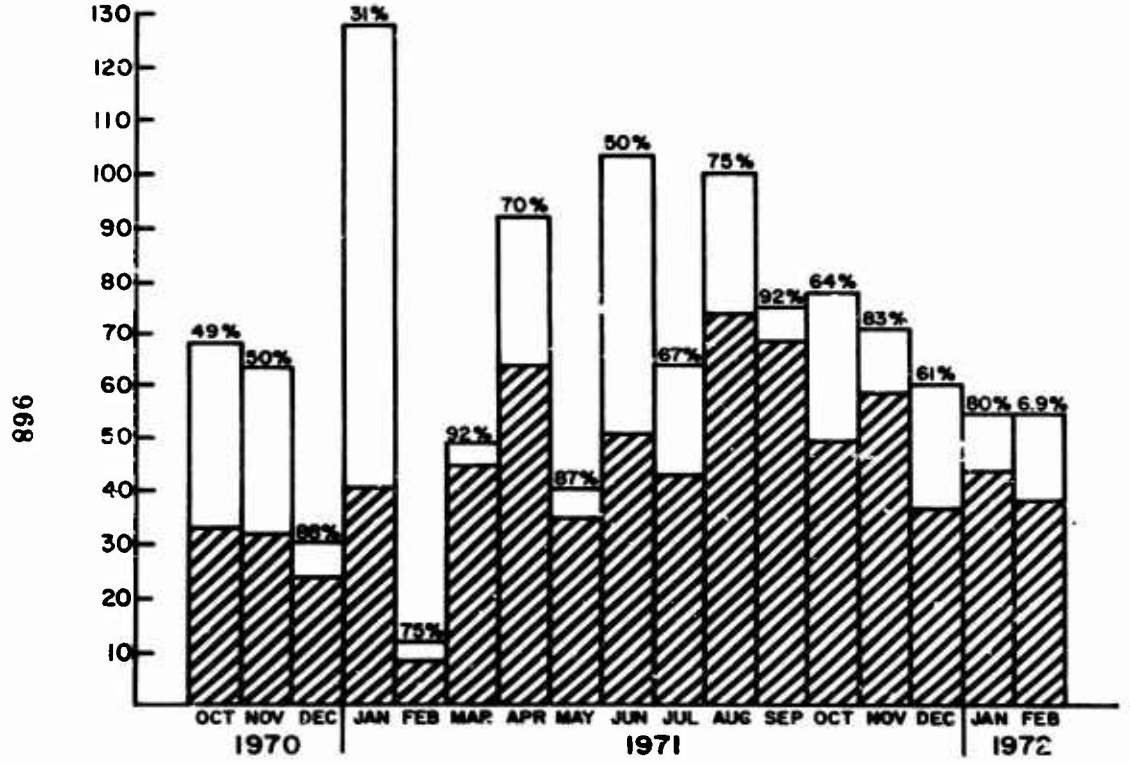

FIGURE 1. RECOVERY OF VIBRIO PARAHAEMOLYTICUS FROM NATORAL SOURCES IN THAILAND.

TABLE 1

Results of a Twelve Month Survey of Natural Sources for Vibrio parahaemolyticus in Thailand.

No. of specimens harbouring Vibrio parahaemolyticus/No. of specimens examined					
Month	Sand & Sea water	Mollusks	Fish	Crab	Shrimp
March 71	0/0	16/18	18/19	9/10	0/0
April	0/0	14/25	14/20	14/14	20/30
May	0/0	13/15	10/20	5/5	7/10
June	25/68	3/10	9/10	5/5	9/10
July	10/29	10/10	8/10	5/5	10/10
August	27/34	10/10	10/15	6/15	10/10
September	4/5	9/10	14/17	8/8	10/10
October	11/19	3/10	9/11	7/8	9/10
November	20/26	10/30	8/10	5/5	10/10
December	9/20	4/10	9/15	5/5	10/10
January 72	14/20	8/10	8/10	5/5	9/10
February	7/20	10/10	7/10	5/5	9/10
March	11/20	7/10	9/15	5/5	8/10

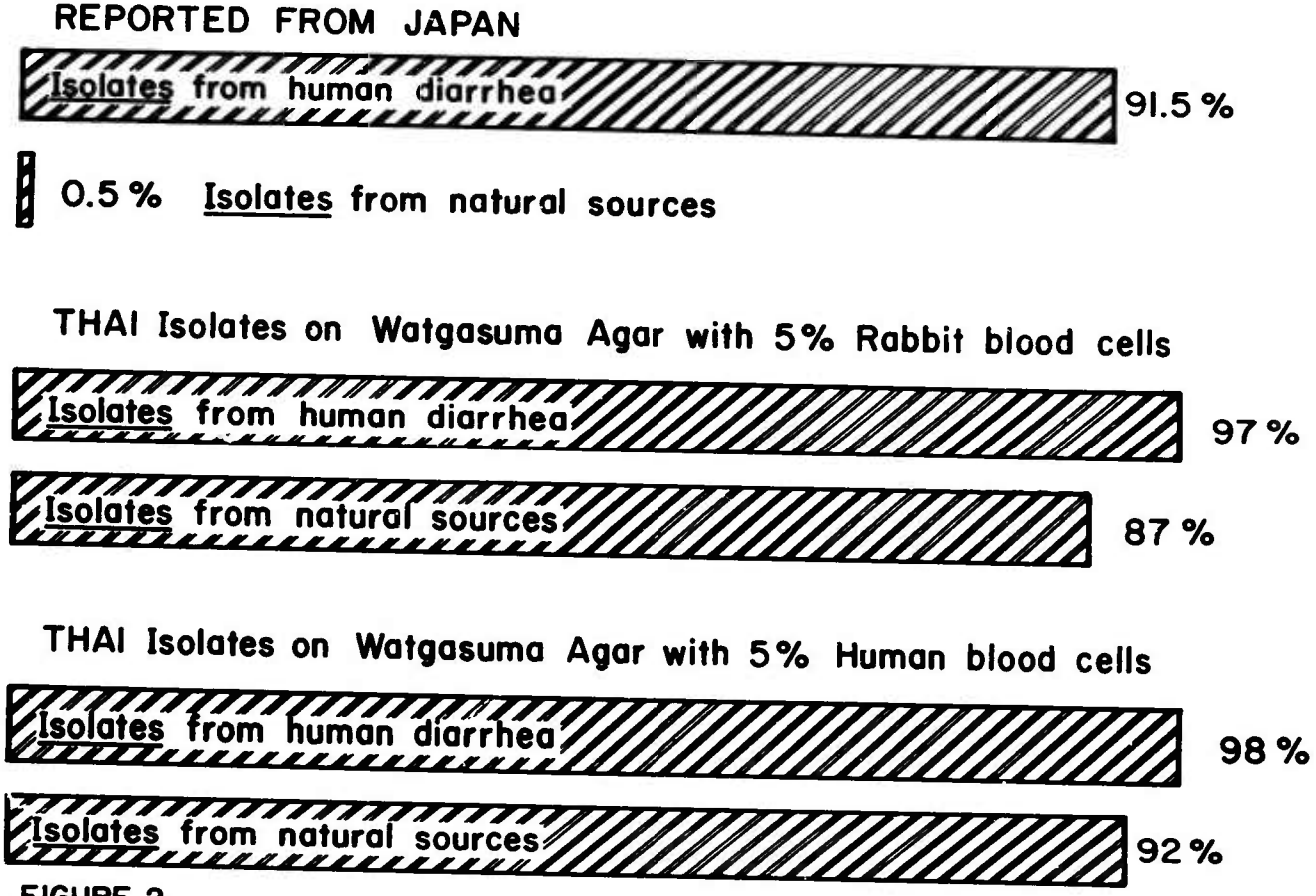

FIGURE 2.

KANAGAWA PHENOMENON OF VIBRIO PARAHAEMOLYTICUS

TABLE 2

Kanagawa Phenomenon of Human and Natural Isolates of V. parahaemolyticus

Organisms from:	Haemolytic on Wagatsuma agar with:			
	Human blood		Rabbit blood	
	Number	%	Number	%
Human sources (128)	126	98.4	124	96.8
Natural sources (140)	129	92.1	122	87.1

TABLE 3

Enteric pathogens isolated from American Nationals with diarrhea from 29 October 1971 to 15 February 1972

Number of Specimens	Number of patients	Salmonella spp.	Shigella spp.	Vibrio para-haemolyticus
71	71	3	1	4

TABLE 4

Sensitivity patterns of Vibrio parahaemolyticus against various antibiotic discs.

Antibiotic		Group	# of strains tested	Percent inhibited
pencillin	10 units	I	67	14.9%
		II	10	50.0%
		III	36	22.2%
		IV	41	17.0%
streptomycin	5 mcg	I	129	35.6%
		II	10	100.0%
		III	98	46.9%
		IV	41	24.3%
tetracycline	10 mcg	All groups	394	100.0%
chloramphenical	10 mcg	All groups	394	100.0%
Neomycin	10 mcg	All groups	394	100.0%
Kanamycin	10 mcg	All groups	394	100.0%
Colimycin	5 mcg	I	187	92.5%
		II	10	80.0%
		III	98	93.8%
		IV	99	89.8%
Ampicillin	10 mcg	I	187	32.6%
		II	10	40.0%
		III	98	44.9%
		IV	99	21.2%

TABLE 4 (Cont.)

Antibiotic		Group	# of strains tested	Percent inhibited
Septrin	25 mcg	All groups	394	100.0%
Furazolidone	150 mcg	All groups	394	100.0%
Gantrisin	1.0 gm	All groups	394	100.0%

DISCUSSION: Data obtained in this laboratory indicate differences in V. parahaemolyticus isolated from Thailand's oceans and those organisms from Japan. Japanese bacteriologists continue to base pathogeny on the Kanagawa phenomenon and thus claim that natural sources are non-pathogenic. Our study of Thai natural isolates indicate that these strains are haemolytic and indistinguishable from those found in diarrheal stools. Our work with the infant rabbit does appear to have some indication that natural isolates may be somewhat less pathogenic (possibly less invasive) than isolates from human diarrhea. This data is not however, as dramatic as the Japanese haemolysis data.

One preliminary experiment conducted in this laboratory may indicate that the haemolytic character of this bacteria may change after several successive passages through animals. Another finding, that 100% of the non-haemolytic strains are sensitive to streptomycin while less than 50% of the haemolytic strains exhibited this sensitivity, may lead to another possible model of pathogenicity.

Certain characteristics of the oceans from which the organisms are obtained may be responsible for some of the differences. Some of our findings suggest that the strains we isolate near populated areas are from fecal contamination. If the organism is physiologically altered by passing through an animal and then dumped back into the ocean, many of our findings would be explained. We are currently examining this and other theses.

Vibrio parahaemolyticus in Pediatric Outpatient Diarrhea Patients

Principal Investigators: M. Talmage M'cMinn, CPT, MSC
Michael W. Benenson, CPT, MC
Richard A. Grossman, LTC, MC

Associate Investigator: Chiraphun Duangmani, M.D.

OBJECTIVE: To determine the frequency of isolation of V. parahaemolyticus from diarrhea patients seen in a pediatric outpatient department.

DESCRIPTION: A large proportion of Bangkok pediatric outpatients present with diarrhea as the major or exclusive symptom. The large majority of these children are treated symptomatically; stool cultures to determine the etiologic agent are usually not attempted. In an attempt to determine whether V. parahaemolyticus might be etiologically related to at least some of these diarrhea episodes, all pediatric patients with diarrhea as the major symptom seen at the Bangkok Children's Hospital outpatient clinic between 27 January and 15 March 1971 were included in the survey. The survey was ended when 100 patients were studied. Age (within 8 months) and sex-matched controls were selected for each diarrhea patient. Control diagnoses were usually upper respiratory infections; patients with G-I complaints were not selected. A group of 14 pediatric hepatitis patients seen during this time, who were being investigated as part of a study of Hepatitis-Associated-Antigen (HAA), were also included in this survey. All patients were interviewed, using a standardized questionnaire form, and rectal swabs were obtained which were cultured for V. parahaemolyticus.

PROGRESS: Table 1 presents the distributions by age and sex of the diarrhea patients and controls. Ninety percent of the patients were under 6 years of age and V. parahaemolyticus was not isolated from any of these 180 children. One isolate was obtained from an 11 year-old male patient with diarrhea and a 7 year-old male control. Both isolates were untypeable. In addition, one isolate (type K-30), in an 8 year female, was obtained from the 14 hepatitis cases followed.

Diarrhea patients and controls both gave a similar history of fish ingestion: 3/4 ate fish at least once a month; 1/2 ate fresh fish; 1/3 ate

salt-water fish; and less than 5% ever ate shellfish. No child gave a history of eating uncooked fish. In addition, fish sauce with peppers is a universal flavoring agent at all Thais meals, but the sauce is derived from cooked fish. Unlike the situation in various parts of Thailand, in Bangkok the use of various shellfish (mostly shrimp) pastes, which may be made from uncooked or partially cooked ingredients, is not common. The results of this survey indicate that there is no evidence that V. parahaemolyticus is associated with clinical diarrhea in young children, at least in the dry months January, February, and March.

Table 1

Age and Sex Distribution of Diarrhea Patients and Controls and V. parahaemolyticus Isolates

	Diarrhea Patients				Controls			
Age	Male	Female	Total	V. para.	Male	Female	Total	V. para.
1	29	11	40	0	28	11	39	0
2	15	11	26	0	15	11	26	0
3	4	11	15	0	5	12	17	0
4-5	7	2	9	0	7	1	8	0
6-12	7	3	10	1*	7	3	10	1*
TOTAL	62	38	100	1	62	38	100	1

* Untypeable strains.

The Infant Rabbit as a Model of Pathogenicity for Vibrio parahaemolyticus

Principal Investigators: Udom Lexomboon, M.D., Ph.D.
Chiraphun Duangmani, M.D
M. Talmage M'cMinn, CPT, MSC

OBJECTIVE : To determine if the infant rabbit could be used as a model for testing the possible pathogenicity of various strains of Vibrio parahaemolyticus.

BACKGROUND : The haemolytic activity of Vibrio parahaemolyticus on a special human or rabbit blood agar (Wagatsuma agar) is the primary biochemical property currently being used to determine the virulence of this organism. It is generally recognized that this organism does not produce a disease in laboratory animals resembling that in man. This laboratory has attempted to demonstrate pathogenicity of V. parahaemolyticus in adult rabbits, mice, monkeys, and gibbons and has not been able to obtain consistent results in any of these animals. The infant rabbit model has been used successfully in some pathogenic studies of V. cholera and preliminary investigations in this laboratory indicated that it was free of Coccidioides spp. which had interfered with our work in adult rabbit intestinal loops.

DESCRIPTION: A preliminary study indicated that the stomach contents of the infant rabbit markedly affected the response of the animal to inoculation of the halophilic V. parahaemolyticus. In feeding experiments, it was noted that in animals fed one ml. of an isolate obtained from a human diarrhea patient, the response was more pronounced after the animal's stomach had been purged with normal saline. This stomach washing technique often resulted in trauma to the small animal and we were often unable to insure the dosage of bacteria fed to the animal.

To overcome the disadvantages of such procedures, an intra-intestinal inoculation technique was developed. Infant rabbits, weighing 100-150 grams, were anaesthesized with ether and a small incision was made in the washed anterior abdominal wall. The ileum was withdrawn through the sterile incision. One ml. of test material was then carefully injected into the lumen of the ileum. The intestine was not tied nor traumatized.

A 27 gauge needle was used to inject the test material and extreme care was taken to insure that no leakage of the inocula occurred. After carefully returning the intestine to the peritoneum and closing the abdominal wall, the animals were deprived of food and water and closely observed for death, diarrhea, or any illness. Animals were sacrificed at 24 hours after the procedure. Cultures for V. parahaemolyticus were then made from the heart blood, small intestine, and large intestine. Necropsy was performed on all animals. A group of three animals was used for each test material. Controls were injected with sterile, fresh media.

Preparation of the Inocula: Selected stock cultures were cultured on thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates. A single colony was picked and inoculated into 20 ml. of brain-heart-infusion broth (BHIB) with 3% NaCl. This culture was incubated for 16-18 hours at 37C. One ml. of the broth culture containing approximately 10^7-10^8 cells was used in the intra-intestinal injection. Cell free filtrates of used media were prepared by picking one typical colony of V. parahaemolyticus and inoculating it into 15 ml. of BHIB with 3% NaCl and incubating at 37C for 16-18 hours. The cells were then removed by centrifugation and membrane filtration at 4C. The cell free supernatant was then checked for sterility and dialyzed against a saturated sucrose solution for 24 hours at 4C. The final concentration of the dialyzed solution was one-fifth of its original volume.

RESULTS: Twenty strains of V. parahaemolyticus isolated from patients with acute gastroenteritis and 9 strains obtained from natural sources (sea water, sand, sea foods) were used as cell broths to test for pathogenesis in infant rabbits. Data obtained from our experiments are presented in Table 1.

Control animals inoculated with BHIB with 3% NaCl failed to demonstrate any of the characteristics noted in the table. Diarrhea (frequent, watery stools) occurred in 30.3% of those animals inoculated with isolates from human diarrheal stools while only 15.1% of those animals inoculated with natural isolates demonstrated any symptoms of acute diarrhea. Animals inoculated with human isolates had large amounts of fluid and gas in the large intestine in 83.3% of the animals tested and in the small intestine in 36.4%. Almost one half (48.5%) of the animals inoculated with natural isolates had fluid in the large intestine and 18.2% had small intestines grossly distended with fluid and gas.

Table 2 shows the results of postmortem cultures onto TCBS agar from the infant rabbits. Cultures from the peritoneum revealing V. parahaemolyticus were used as evidence of leakage of the inocula from the intestine. These animals are not included in this report. All animals that died had positive heart blood cultures of V. parahaemolyticus. These data indicate at those isolates of the organism from human diarrhea appear to be more adapted to survival in the animal intestine than those from natural sources.

Table 3 shows the results of intra-intestinal inoculation with concentrated cell-free used broth. It is clear that this filtrate, in which V. parahaemolyticus was grown for 16-18 hours, contains some toxic substance(s) which caused 50% of the inoculated animals to have positive symptoms of acute diarrhea. 80% of the animals inoculated with filtrate from human isolates of the organism had gross fluid and gas in the large intestine. 17 of the 35 animals had fluid and gas in the small intestine. All postmortem cultures of heart blood, small and large intestine were negative, as were specimens of the inocula that were cultured at the time of injection. While we note that the broth control produced gas in the large intestine of 4 animals, the reaction was not so pronounced nor as frequent as with the used broth.

DISCUSSION : After numerous attempts to demonstrate pathogenicity of V. parahaemolyticus in a variety of ways, these results indicate that the infant rabbit ileum may be an acceptable model for additional research. Japanese investigators, basing their work on haemolysis on Wagatsuma media (the Kanagawa phenomenon), have suggested that isolates from natural sources are not pathogenic. In our hands, the Kanagawa phenomenon has been rather universal in all strains isolated in Thailand (see elsewhere in this report). This has left us without an acceptable method of distinguishing pathogenic and non-pathogenic strains, an important factor in any epidemiological study we may undertake. Attempts to demonstrate pathogenicity by use of the Di Test in adult rabbits were extremely erratic due to the rather universal contamination of the rabbits with Coccidioides spp. We continue attempts to feed the organism to monkeys and gibbons but usually cannot even recover the organism from the stool. No primate has ever developed any symptoms of diarrhea.

These data appear to demonstrate that V. parahaemolyticus isolated

from natural sources does not have the pathogenic properties that isolates obtained from human gastroenteritis specimens have. Results of cultures after intra-intestinal inoculation of the organisms suggest that natural isolates may not be as invasive as isolates from diarrheal stools. Additional studies into these findings continue. These data raise epidemiological questions regarding the source of human infection that will have to be answered by additional studies.

TABLE 1

Observations of Infant Rabbits Inoculated with V. parahaemolyticus Cells

Source of Organism	Animals Tested	Observation			
		Death *	Diarrhea	Fluid in Large Int.	Fluid in Small Int.
Human isolate(20)	66	14	20	55	24
Natural isolate(9)	33	6	5	16	6

* within 24 hours

TABLE 2

Results of Postmortem Culture of Infant Rabbits Inoculated with V. parahaemolyticus Cells

Source of Organism	Animals Tested	Positive Cultures From:		
		Heart blood	Small intestine	Large intestine
Human isolates (20)	66	16	59	64
Natural isolates (9)	33	6	14	24

TABLE 3

Observations of Infant Rabbits Inoculated with Cell-free Concentrate of V. parahaemolyticus cultures

Source of organism	Animals Tested	Observation			
		Death *	Diarrhea	Fluid in Large Int.	Fluid in Small Int.
Human isolate (6)	35	1	17	28	4
Natural isolate (2)	6	0	3	4	0
Broth control	15	0	0	4	0

The Etiology of the Non-Luetic Soft Chancre

Principal Investigators: M. Talmage M'cMinn, CPT, MSC
Sp5 Alan T. Evangelista, B.Sc.

Associate Investigator: Chiraphun Duangmani M.D.

OBJECTIVE: To determine the bacterial etiology of penile lesions resembling non-luetic soft chancre.

BACKGROUND: Soft chancre or ulcus molle is the second most common venereal disease among US troops operating in Southeast Asia. The etiology of this disease is uncertain and treatment is occasionally misdirected because of this lack of bacteriological definition. The purpose of our study is to determine what bacteria are present in these lesions and to attempt to determine the causative agent.

DESCRIPTION: Specimens are obtained from penile lesions by means of scraping the floor of the lesion with a large gauge wire loop. Occasionally, neglected ulcers must be washed to remove necrotic tissue before the edge and bottom of the lesion is scraped with the wire loop. A routine questionnaire is completed at the time of the examination and venous blood is obtained for syphilis serology. 151 of 181 subjects included in this report were adult American males between the ages of 19 and 52 while the remainder were male members of the Thai military forces. Histories obtained at the time of examination indicated that none of the US personnel had treated themselves with antibiotics, while 65% of the Thai patients admitted prior treatment with non prescribed drugs.

Lesions ranged from vesicular eruptions with no indication of bacterial infection through the classical 2-10 mm wide, slightly indurated, tender, deep ulcer described as the "soft chancre", to large fulminating lesions with marked destruction of tissue. Many patients presented with multiple lesions. We classified 34 lesions as "herpetic-like" which were characterized by painful multiple vesicular eruptions with a history of similar vesicular lesions that healed spontaneously. 68% of the patients with herpetic-like lesions reported prior lesions of a similar nature.

We had found, from previous work that excellent growth of numerous organisms occurred on Eugonoagar plates with 25% fresh rabbit blood. This media was inoculated, as were Thayer-Martin media, sheep blood agar, desoxycholate agar, chopped meat broth, and fresh rabbit blood clot tubes, and were incubated at 37C. Experience had revealed that inoculation of blood clot tubes made of the patients blood did not add to recovery of any additional organisms. The Eugonoagar +25% rabbit blood plates, the sheep blood agar plates, and the Thayer-Martin media were incubated under increased CO_2 tension. Subsequent inoculation of another Eugonoagar plate with 25% fresh rabbit blood from the rabbit clot tube was made after overnight and 48 hour incubation. Three smears were made: one for immediate darkfield examination, another for gram stain, and the thrid for additional studies with special stains for cytology and presence of bacteria. No unusual cytology indicating viral involvement was ever observed in these smears.

RESULTS: During the period April 1971 - April 1972, 181 penile lesions were examined and cultured in this laboratory. Table 1 presents the bacteriological findings from these cultures. Five of the 181 darkfield examinations revealed spirochetes and four serological examinations (RPR Card Test screen) were reactive. Only 7 patients were submitted for virology studies, determined by the nature of the lesion. Of these 7 patients with closed vesicles resembling herpetic lesions, 3 were found to harbour Herpes progenitalis.

Three of the "Haemophilus spp." we recovered from penile ulcers were sent to the diagnostic laborstories of the Center for Disease Control in Atlanta, Ga., in the United States. There, based on the inability of the organism to grow without haemolysis on rabbit blood and their need for factor V (phosphopyridine nucleotide; DPN; NAD) and no need for factor X (haemin), the isolates were classified as Haemophilus parainfluenzae. These isolates were identical to the other Haemophilus spp. that we isolated from penile ulcers.

It is interesting to note that 3 isolates of Neisseria gonorrheae were isolated from penile lesions. All of these isolates of N. gonorrheae came from uncircumcised males and two of the lesions were on the shaft of the penis. One of these three males had gonococcal urethritis while the other two denied any symptoms of gonorrhea. This finding may represent self-contamination with urethral exudate or may indicate

infection of a pre-existing lesion with N. gonorrheae derived from an infected female.

Subcutaneous inoculations of pure cultures of the Haemophilus spp. into adult rabbits failed to produce any chancroid-like ulcers. Other pure cultures of bacteria isolated from penile lesions in our laboratory produced small self-limiting lesions but never anything resembling the penile ulcers from which they were isolated. Lesions produced by subcutaneous inoculation of the Haemophilus spp. were never positive on subsequent culture for the Haemophilus bacillus. Rabbits inoculated subcutaneously with mixtures of Haemophilus spp., diptheriods, and non-haemolytic streptococcus did produce ulcers resembling the typical soft chancre. We were able to recover all three of the bacteria inoculated on subsequent culture.

This laboratory has developed some additional biochemical test media that may enable additional characteristics of the fastidious Haemophilus organisms to be enumerated. By adding various amounts of NAD and/or haemin to sugar fermentation media and to other test media, we have begun to notice consistent growth patterns of the organisms recovered from the lesions in our study. This work continues and is somewhat dependent upon the quantity of Haemophilus isolates obtained.

DISCUSSION: While the etiology of soft chancre is usually defined in medical texts, a search of recent literature reveals that few workers have been successful in recovering the Ducrey bacillus. No adequate schema for its identification exists. To date, our data indicate that many penile lesions are infected with normal skin and/or vaginal flora. Many lesions are obviously herpetic eruptions that are characterized by multiple eruptions, pain, and recurrence. Patients presenting with this type of lesion usually reveal histories of past bacterial infections after neglect of the original lesion. Extreme care in this laboratory in obtaining thorough specimens and careful inoculation and incubation of a wide variety of media have not resulted in isolating a single organism that resembles the Haemophilus ducreyi bacillus that is generally defined as the causative agent for soft chancre.

TABLE 1

Bacteriological Findings of 181 Penile Lesions

	Sum	%
Haemophilus spp.	5	2.75
Staphylococcus aureus	83	45 .65
S. epidermidis	84	46.20
Micrococcus spp.	3	1.65
diptheroids	78	42.90
Neisseria gonorrheae	3	1.60
Streptococcus faecalis	20	11.00
beta haemolytic streptococcus	6	3.3
alpha streptococcus	34	18.7
gamma streptococcus	81	44.55
Escherichia coli	14	7.7
Klebsiella spp.	2	1.1
Enterobacter aerogenes	5	2.75
Enterobacter cloacae	2	1.1
Proteus spp.	5	2.75
Paracolobactrum spp.	4	2.20
Pseudomonas aeroginosa	3	1.65
Alcaligenes spp.	1	.55
Herellea spp.	1	.55
Clostridium perfringens	2	1.1
Candida albicans	1	.55

Neisseria Gonorrheae Infections of the Premenarcheal Female

Principal Investigator: Udom Lexomboon, M.D., Ph.D.

Associate Investigators: M. Talmage M'cMinn, CPT, MSC
Chiraphun Duangmani, M.D.
Yachai Ampaipast, B.Sc.

OBJECTIVE: To determine the frequency of recovery of Neisseria gonorrheae from Thai premenarcheal females seen at Bangkok, Thailand.

BACKGROUND: Traditionally, Neisseria gonorrheae has been predominantly associated with vulvovaginitis in premenarcheal females. In the United States, however, reports from Lang in 1955, and Heller, et al, in 1966, have indicated that gonorrhea in such patients was not as frequent as had been expected. Heller, Joseph, and Davis in 1969, reported no findings of N. gonorrheae in 50 patients with vulvovaginitis ranging in age from 12 days to 13.5 years. These findings and such rarity could be attributed to the rapid and efficient treatment of gonorrhea in the adult population.

With an increase in the frequency of N. gonorrheae infections in Thailand reported by the Minister of Health in Dec 1971, and the frequent recovery of strains of the gonococci that display "decreased sensitivity" to penicillin (see elsewhere in this report), this laboratory proceeded to conduct a survey of young Thai patients with vulvovaginitis. An extensive search of the literature revealed that there was no report dealing with vulvovaginitis in premenarcheal children in Thailand. The purpose of this study was to determine the frequency of recovery of N. gonorrheae from patients presenting with vulvovaginitis to the Children's Hospital in Bangkok, Thailand. A case report is presented.

DESCRIPTION: All girls between birth and 14 years of age presenting to the out-patient clinic at Children's Hospital in Bangkok, Thailand, with vulvovaginitis during the study period were examined by the principal investigator. Cotton swabs were obtained from the vagina and urethra of these female patients and immediately inoculated onto fresh sheep blood agar plates and onto haemoglobin-free Thayer-Martin

TABLE 1

Bacteriological Findings from 47 Children with Vulvovaginitis

Organisms Isolated	No. Isolated	% Isolated
Neisseria gonorrheae	20	42.5
Staphylococcus aureus	6	12.5
S. epidermidis	24	51.0
alpha haemolytic streptococcus	11	23.4
non-haemolytic streptococcus	2	4.2
Diplococcus pneumoniae	1	2.1
Micrococcus spp.	6	12.7
diphtheroids	14	29.8
Bacillus spp.	1	2.1
Escherichia coli	16	34.0
E. aerogenes	2	4.2
E. cloaca	2	4.2
Proteus mirabilis	2	4.2
P. morganii	4	8.5
Haemophilus spp.	1	2.1
Shigella flexneri 3	1	2.1

TABLE 2

Bacteriological Findings from 10 Patients with no vulvovaginal complaints

Organism Isolated	No. Isolated	% Isolated
Staphylococcus aureus	1	10
S. epidermidis	9	90
beta streptococcus (not group A)	1	10
alpha streptococcus	4	40
gamma streptococcus	5	50
Streptococcus fecalis	2	20
Micrococcus tetragenous	2	20
diptheroids	7	70
Candida spp. (not C. albicans)	1	10
Escherichia coli	4	40
Paracolobactrum aerogenes	1	10
Herellea spp.	1	10

media. The plates were incub ated at 37C in CO_2 jars overnight and then suspected colonies were picked for further identification by Gram-stain, oxidase test, and biochemical fermentation tests. Attempts were made to recover and identify other bacteria from both the sheep blood agar plates and the Thayer-Martin plates. Forty-seven girls with vulvovaginitis, whose ages ranged from 2 months to 9 years, were examined. Subjects with vulvovaginitis were characterized by inflammation of the vulvovaginal area w' h purulent discharge. Few of them complained of vulva pain, frequen. urination, dysuria, or itching. The child's general health was not affected. Ten girls with ages ranging from 6 months to 13 years were examined and cultured as controls. None of the control subjects presented with urogenital complaints and all of them were normal on examination of the vulva and vagina.

RESULTS: The results of bacteriological cultures of the 47 patients wi.h vulvovaginitis are presented in Table 1. Findings from the 10 controls are presented in Table 2.

Shigella flexneri 3 was isolated from the vaginal discharge of a one year old girl who presented with vulvovaginitis after having symptoms of dysentery for a few days. There was no complaint of dysentery at the time of examination.

Of the 20 patients with proven N. gonorrheae infections, three had no admitted history of any family member with gonorrhea. Histories obtained from the parents of the other 17 revealed that either the father or mother had recent symptoms of acute gonorrhea. All of the patients with gonorrhea infections slept with their parents. Only 5 of the girls had a history of using the same towels after bathing that their parents had used. One four year old with a positive vaginal culture for N. gonorrheae admitted to "sex-play" with an eight year old boy who had a urethral discharge. We were unable to obtain a culture from this boy.

CASE REPORT: A three year old girl was seen at the out-patient clinic of Children's Hospital on 1 July 1971 complaining of a pur ilent discharge from the vagina for the past three days. Her general condition was within the normal limits. The girl's father, a 29 year old traveling salesman, had a history of gonorrheal urethritis in 1969. The patient's mother stated that he was free from any symptoms at the time of the daughter's examination. The 29 year old mother had a history of chronic leukorrhea

of one year duration. She had not taken any medication for this condition. The infant slept with her mother and usually shared the same towel with the mother after bathing.

Vaginal cultures from the patient and her mother on the initial visit yielded a heavy growth of N. gonorrheae. The patient and her mother responded well after a single intramuscular injection of 1 million units of clemizole penicillin (megacillin).

The same patient again developed a purulent vaginal discharge on 12 October 1971, 3.5 months later. The cultures of the urethra and vulvovaginal sites failed to reveal N. gonorrheae. The mother positively denied any symptoms of leukorrhea or the common use of towels or any other articles. The vulvovaginal discharge of the patient disappeared rapidly after a single injection of megacillin.

On 3 February 1972, this same girl had another attack of vulvovaginitis. The vaginal culture revealed a heavy growth of N. gonorrheae. The mother and father denied any symptoms in either of them of acute gonorrhea. A vaginal culture of the mother failed to reveal any N. gonorrheae. We could not obtain cultures of the father. The symptoms of vulvovaginitis of this girl again rapidly disappeared after treatment with megacillin.

DISCUSSION: Lang in 1955, indicates that towels and linens may serve as transmitting agents of gonococcal vulvovaginitis, a "highly contagious" disease. He reports that gonococcal vulvovaginitis usually results from contact with an infected adult. Cohn, et al, in 1940, report that intermediate objects are of little importance in the transmission and that sexual contact is the usual mode of infection. Nelson, in 1964, states that gonococcal disease is moderately contagious and can be introduced to the vulva by personal objects contaminated with N. gonorrheae organisms. He advises that upon diagnosis of N. gonorrheae vulvovaginitis, personal clothing and towels should be separated from the remainder of the family until the patient is cured of the infection and the commonly used articles are thoroughly cleaned. It may be reasonably assumed that the mode of transmission of N. gonorrheae in the premenarcheal children examined in our study was via person-to-person contact, but without sexual contact (except in the one instance where "sexual-play" was admitted).

In Thailand, personal cleanliness is an important social habit that is stressed to children. This desirable habit often means that children will bathe several times a day. In the lower classes, the towels are commonly used by other family members and may seldom become dry. While we have not been able to obtain cultures from these damp towels, the thesis that the diplococcus could remain viable in the damp folds and thus be transmitted to the female children cannot be ignored.

These findings indicate that N. gonorrhoae infections in vulvovaginitis is common in Thailand. The causative diplococcus must be cultured for, and specific, directed antibiotic therapy administered against this increasingly "resistant" organism.

Serum Antibiotic Levels Associated With Gonorrheae in Females

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Associate Investigators: Chirpahun Duangmani, M.D.
Prakit Kanchanavatee, M.D.
Godom Areeya, M.D.

OBJECTIVE : To determine the relationship between patients with serum antibiotic levels and frequency of Neisseria gonorrheae infections among such patients.

BACKGROUND: In the SEATO Medical Research Laboratory Annual Report from April 1970 - March 1971, a report was made regarding the isolation of Neisseria gonorrheae from females. Included in this report were data regarding serum antibiotics in samples obtained from these promiscuous females. By using tube dilution techniques on sera from 56 randomly selected females, we observed that one half of these subjects had serum antimicrobial activity. Twenty three of the 56 subjects (41%) had proven N. gonorrheae infections. Of these 23, 17 had serum antimicrobial activity and 6 had no serum antimicrobial activity. These data appeared to indicate that patients taking non-prescribed drugs were more susceptible to gonorrhea than those without measureable serum antibiotics.

After those preliminary findings this laboratory proposed a study of the affect of clandestine use of non-prescribed antibiotics among promiscuous females. In this country patients can obtain antibiotics without prescriptions from physicians, and it is common for promiscuous females to treat themselves without ever seeking medical advice. Upon questioning these females we discovered that the regimen of medication was usually quite naive with patients reporting taking "one red pill a day" or "one shot that hurt a lot".

DESCRIPTION: Subjects for this study were selected at the VD Control Clinic on Monday, Wednesday, and Thursday from 0830 - 1130 hours for 1 week and on the same days from 1300 - 1500 hours in the following week. Every fifth subject presenting for examination was selected for

inclusion in this investigation, Subjects came from two groups. One group included females presenting with symptoms and/or complaints of gonorrhea and the other consisted of females with no complaints but presenting for periodic examination. Our study combined these two groups. The subject was asked to complete a questionnaire regarding her place of work, her age, and the length of time that she had been working in her profession.

All subjects received pelvic examinations and cervical specimens were obtained for bacteriological studies. Ten ml. of venous blood was obtained. Routine bacteriological examination was conducted on cervical specimens to identify N. gonorrheae. Sera was tested by tube dilution techniques against Staphylococcus aureus (ATCC 6538P), Bacillus cereus (ATCC 11778), and Sarcina lutea to determine antimicrobial activity. While these biological assays could not determine the presence of specific antibiotics, they did permit positive indication of serum antimicrobial activity. The test organisms were routinely tested against known serum with no antibiotics to ensure their lack of sensitivity to normal serum proteins.

RESULTS: Five hundred and one specimens from 501 subjects were examined. Table 1 indicates the findings in this study. 176 subjects had bacteriological evidence of gonorrhea infections. 325 of the subjects had no bacteriological evidence of gonorrhea infections. Correlation between gram strains taken at the examinations and cultures was good. 114 of these subjects (22.8%) had serum antibiotic levels that were measured with our techniques while 387 of the subjects had no indication of serum antibiotic levels.

There was no significant difference, by Chi square test, between those patients with infection and serum antimicrobial activity and those with infection and no serum antimicrobial activity. ($X^2 = 1.030$, df = 1, $0.50 > p > 0.30$)

While we had previously suspected that the clandestine use of non-prescribed antibiotics might result in less competition from the normal vaginal flora to N. gonorrheae and thus make the patient taking these drugs more susceptible to such infections, these findings fail to support that thesis. However, it should be noted that the non-directed use of such drugs apparently does not protect the female from infection with N. gonorrheae.

TABLE 1

Comparison between N. gonorrheae infected females and serum antimicrobial activity

		Bacteriological evidence of gonorrhea	
		Yes	No
Serum antimicrobial activity	Yes	35	79
	No	141	246

Minimum Inhibitory Concentration (MIC)
of Penicillin G & Oxytetracycline to Neisseria Gonorrheae

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

Associate Investigator: Panyasri Benjadol, M.Sc.

OBJECTIVE: To determine the MIC of penicillin G and Oxytetracycline to Neisseria Gonorrheae in Thailand.

DESCRIPTION: Randomly selected isolates of N. gonorrhea were used to determine the minimum inhibitory concentrations of potassium penicillin G and oxytetracycline to those strains. The antibiotic was mixed with Thayer-Martin chocolate agar without antibiotics and slightly turbid suspensions of the selected strains in brain heart infusion broth were placed on the agar surface with platinum loops.

Isolates were selected from promiscuous females seen at the Ban Chiwi VD Control Clinic in Bangkok, from the US Army 5th Field Hospital in Bangkok, and from the Thai Army Hospital in Bangkok. All isolates from the Ban Chiwi Clinic were from females, all from the US Army Hospital were males, and all but on efrom the Thai Army Hospital were from male patients. None of the strains selected were specifically picked from so called 'resistant' cases. Table 1 and 2 present the MIC's of the two drugs tested to N. gonorrheae.

DISCUSSION: It is noted that the World Health Organization indicates that "less sensitive strains" of N. gonorrheae are inhibited by concentrations of penicillin ranging from 0.05 to 0.125 IU/ml. Our study indicates that 87.2% of the few strains studied in this laboratory demonstrate a decreased sensitivity to penicillin. A study done in 1968 by the VD Control Laboratory of the Thailand Department of Health shows that 47% of 181 strains tested at that time had penicillin MIC's of 0.128 or greater.

This report indicates that the frequency of recovery of "less than sensitive strains" of N. gonorrheae is increasing. Prompt, vigorous treatment

with adequate serum concentrations of the appropriate antibiotic is indicated with patients infected with gonorrhea. Cultures of suspected material should be accomplished and antibiotic sensitivities carefully determined in order to ensure use of the proper drug.

TABLE 1

Minimum Inhibitory Concentrations of penicillin to randomly selected strains of Neisseria gonorrheae.

	Isolates from:		
	Ban Chiwi 27 patients	US Army Hospital 34 patients	Thai Army Hospital 34 patients
	unit/ml	unit/ml	unit/ml
Range	0.1-0.8	0.05-1.6	0.1-1.0
Mean	0.34	0.85	0.6
Median	0.2	0.8	0.6

TABLE 2

Minimum Inhibitory Concentrations of oxytetracycline to randomly selected strains of Neisseria gonorrheae.

	Isolates from:		
	Ban Chiwi 27 patients	US Army Hospital 34 patients	Thai Army Hospital 34 patients
	mcg/ml	mcg/ml	mcg/ml
Range	0.2-1.6	0.8-2.4	0.8-3.6
Mean	0.86	1.3	1.5
Median	0.8	1.2	1.2

Clinical Evaluation of Co-trimoxazol (Trimethroprim/Sulfamethylthiazole) and Furazolidone in Treatment of Shigellosis in Children

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Pethai Mansuwan, M.D.*

Associate Investigators: Chiraphun Duangmani, M.D.
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OBJECTIVE : To determine the efficacy of Co-trimoxazol in the treatment of Shigellosis in pediatric patients.

BACKGROUND: Co-trimoxazol is a recently developed drug consisting of 5 parts sulfamethylthiazole and 1 part trimethroprim** that has demonstrated synergistic properties against a variety of bacteria. This drug and furazolidone have been shown to be very effective against a majority of Shigella spp. in vitro, however, in vivo studies are lacking. The purpose of this study is to compare Co-trimoxazol (Trimethroprim 80 mg + Sulfamethylthiazole 400 mg) with furazolidone in the treatment of Shigella diarrhea in children.

DESCRIPTION: One hundred sixty-six patients with gastroenteritis who visited the out-patient clinic of Children's Hospital, Bangkok, during March through August 1971 were available for this study. Patients were assigned to be treated with Co-trimoxazol or furazolidone randomly. Clinical and bacteriological observations were made on the initial visit and also on every alternate day for a period of 7 days. Patients were excluded from the study if their stool cultures did not confirm the diagnosis of shigellosis.

Only 33 patients in the Co-trimoxazol group and 30 patients in furazolidone group were proven to be Shigella infections and completed the scheduled visits. These two groups were generally comparable in regard to age, sex, duration of illness and the severity of the disease.

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** "Bactrim"; Roche and "Septrin"; Burroughs Wellcome

Approximately 50% of the children were younger than 2 years and the majority of the patients were seen within a few days after onset of the disease. Shigella flexneri was the most common pathogen isolated from each group.

RESULTS: Results indicate that Co-trimoxazol is superior to furazolidone by virtue of the shorter clinical symptoms and bacteriological responses to the treatment without any failure. See Table 1 and 2.

TABLE 1

Bacteriological Response to Antimicrobial Therapy
in 63 Shigellosis Patients

Drugs	Total Number of Patients	Days until Negative Shigella					
		2	3	4	5	6	7 or over
Co-trimoxazol, (trimethroprim 80 mg+Sulfamethylthiazole 400 mg)	33	22	9	2	-	-	-
Furazolidone	30	11	6	5	1	-	8

TABLE 2

Clinical Response to Antimicrobial Therapy
in 63 Shigellosis Patients

Drugs	Total Number of Patients	Clinically Improved (days after therapy)						
		1	2	3	4	5	6	7 or over
Co-trimoxazol, (trimethroprim 80 gm+Sulfamethylthiazole 400 mg)	33	15	16	1	1	-	-	-
Furazolidone	30	4	9	5	4	1	-	7

Anaerobic Bacteriology

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

Associate Investigators: Panyasri Benjadol, M.Sc.
Kanchana Leelasiri, B.Sc.

OBJECTIVE : To determine the importance of anaerobic bacteria in clinical infections.

BACKGROUND: During the past year, this laboratory has established a capability for identifying strict anaerobic bacteria. The use of pre-reduced-anaerobically-sterilized (PRAS) media and the training of two technicians in the use and manufacture of this specialized media has enabled the recovery and identification of numerous organisms that would have otherwise been overlooked in a routine laboratory.

This media, first developed by Dr. R.E. Hungate in 1950 and subsequently modified for use in the clinical laboratory by Dr. W.E.C. Moore in 1966 enables the most fastidious anaerobic bacteria to grow. By employing these techniques in various biochemical media, identification of the organisms can be accomplished. By carefully excluding any atmospheric oxygen from contact with the media, more than twice the number of organisms can be recovered from clinical specimens than by use of standard methods utilizing the Brewer anerobic jar or fluid thioglycolate media. Once recovery of the fastidious organisms is accomplished, antibiotic sensitivity profiles can be established using prereduced agar plates and directed antibiotic therapy can be attempted by the clinician. Moore has noted that many anaerobic bacteria are resistant to certain antibiotics and has indicated the need for more efficient culture methods and accurate sensitivity data.

While our research has not yet demonstrated any of the anaerobic bacteria to be the cause of any disease process (except for tetanus), it is important to note that many lesions have been examined and discovered to harbour these fastidious organisms. We believe that specific treatment directed towards eliminating these organisms, be they the causative agent or opportunists, results in more rapid healing. McDonald has demonstrated

the synergistic properties of several of the organisms that we have recovered from lesions with mixed flora. Table 1 shows that from 194 specimens examined, we recovered 237 anaerobic species of bacteria and 491 various species of aerobic bacteria. It would be unwise to assume that these anaerobic organisms were of no importance in the lesions that we cultured.

Table 2, 3 and 4 present the identification of anaerobic bacteria identified using the techniques employed in this laboratory.

Use of PRAS techniques has enabled the rapid recovery of Clostridium tetani from numerous umbilical specimens. During such recovery, it was noted that numerous lesions were insignificant and had been ignored before clinical symptoms of tetanus had appeared. A search of the literature revealed that while some authors, like B.P. Davis, et al in their 1967 text, Microbiology, state that spores may remain dormant in healed wounds, no one had presented any data regarding this phenomenon. This laboratory proceeded to design experiments to demonstrate the inability of the Cl. tetani spores to germinate in non-traumatic wounds.

Our experiments in working with Cl. tetani had indicated that most lesions harbouring the tetanus bacillus also contained several other species of bacteria. There was never a pure culture of Cl. tetani from the 60 specimens examined for bacteria. Tetanus spores require an environment with a low Eh (oxidation - reduction potential) for germination. In non-traumatic wounds or in wounds without a simultaneous pyogenic infection, the normal vascularity would remain intact and maintain a normal tissue Eh. It was postulated that non-traumatic injection of a pure suspension of Cl. tetani spores would not result in germination of the spores and that clinical tetanus would not occur in these animals.

Spores of Cl. tetani were obtained by drying 2 week old broth cultures of Cl. tetani in sealed, sterile petri dishes. After 3 weeks, the dried material was rendered into a powder and checked by way of Gram strain and inoculation on appropriate media for purity. Counts were made of the spore suspension.

White mice were inoculated IM, with 0.1 ml of spore suspension using a 27 gauge hypodermic needle. Every effort was made to insure a non-traumatic inoculation. Duplicate inoculations were made into mice that had recently received tetanus antitoxin. None of the inoculation sights showed any sign of trauma nor did any mice develop clinical tetanus after 28 days of observation.

Subsequent inoculations of coagulase positive Staphylococcus aureus into the leg muscle of mice inoculated 14 days prior with Cl. tetani spores resulted in clinical tetanus and death of 2 of seven animals. Tetanus occured in these animals 4 days after the Staph. aureus inoculation. The remaining 5 animals were sacrified 30 days after spore injection and had no signs of tetanus. Staph. aureus inoculation of mice protected with antitoxin prior to spore inoculation produced a typical lesion but no tetanus.

These findings indicate that the spores of Cl. tetani can remain dormant in healthy tissue for as lon g as 28 days without germination. The presence of the spores was demonstrated by subsequent inoculation with a pyogenic bacteria that caused tissue necrosis and enabled germination of the dormant spore.

TABLE 1

Anaerobic Specimens
(April 1971 - March 1972)

Hospitals	Source (lesion)	No. of specimens	Anaerobic Organism Isolated				Aerobic org.
			Clostridium	Bacterioides	Cocci	Gram-post-rod (non-spore former)	
Children's Hosp.	Umbilical swab	60	68	25	45	1	268
	Miscellaneous *	23	18	13	11	1	85
Chulalongkorn Hosp.	Corneal ulcer	29	-	-	-	4	-
	Umbilical swab	15	6	2	3	-	42
	Wounds	34	6	8	3	-	58
	C.S.F.	7	-	-	-	-	-
	Miscellaneous *	23	2	5	6	2	34
Other (Army Hosp., Rama Hosp.)	Miscellaneous *	3	2	3	2	-	4
	Total	194	102	56	70	9	491

* Note Table 4

TABLE 2

CHILDREN'S HOSPITAL

Source	Anaerobic Organism Isolated			
	Clostridium	Bacteroides	Cocci	Gram-post-rod (non-spore former)
Umbilical (60)	C. tetani 18 C. perfringens 14 C. sordellii 8 C. bifermentans 10 C. sporogenes 4 C. sphenoides 2 C. tertium 2 C. subterminale 2 C. novyi A 2 C. histolyticum 1 C. paraputrificum 1 C. innocuum 1 C. limosum 1	B. fragilis 18 B. terebrans 3 B. melaninogenicus 1 F. fusiforme 2	Peptococcus magnus 17 P. prevotii 16 P. asaccharolyticus 6 Peptostreptococcus - intermedius 1 - anaerobius 2 Veillonella - alcalescens 1 - parvula 2	Eubacterium lentum 1
Miscellaneous (23) *	C. tetani 4 C. perfringens 6 C. bifermentans 2 C. cadaveris 2 C. sphenoides 1 C. sporogenes 1 C. novyi A 1 C. capitovale 1	B. fragilis 4 B. corodens 3 B. oralis 1 F. necrophorus 3 F. fusiforme	Peptococcus magnus 2 P. prevotii 2 P. asaccharolyticus 2 Peptostreptococcus - intermedium 1 Veillonella - alcalescens 1 - parvula 3	Propionibacterium - anaerobium 1

* note Table 4

TABLE 3

CHULALONGKORN HOSPITAL

Source	Anaerobic Organism Isolated			
	Clostridium	Bacteroides	Cocci	Gram-post-rod (non-spore former)
Corneal ulcer (29)	-	-	-	P. propionicum 2 P. granulosum 2
Umbilical 1609	C. tetani 2 C. perfringens 1 C. bifermentans 1 C. sphenoides 1 C. capitovale 1	B. fragilis 1 B. ferebrans 1	Peptococcus intermidius 1 P. asaccharolyticus 1 Peptostreptococcus - anaerobius 1	
Wound (34)	C. perfringens 1 C. sphenoides 2 C. novyi A 1 C. bifermentans 1 C. limosum 1	B. fragilis 6 F. necrophorus 1 F. fusiforme 1	Peptococcus magnus 2 P. asaccharolyticus 1	Eubacterium lentum 1
Miscellaneous (26) *	C. sordelli 1 C. perfringens 1	B. fragilis 2 B. corodens 1 B. melaninogenicus 1 F. necrophorus 1	Peptococcus magnus 3 P. asaccharolyticus 2 P. prevotii 1	Bifidobacterium spp. 1 Eubacterium lentum 1

* Note Table 4

TABLE 4

OTHER HOSPITAL

Source	Anaerobic Organism Isolated			
	Clostridium	Bacteroides	Cocci	Gram-post-rod (non-spore former)
Miscellaneous (3) *	C. perfringens 2	B. fragilis 2 B. terebrans 1	Peptococcus prevotii 1 P. asaccharolyticus 1	-

* Identification of miscellaneous specimens

Miscellaneous (Chulalongkorn Hosp.):-

Pleural effusion
Cervical swab
lesion of mouth
Burns
Hemoculture
Peritoneal fluid

Miscellaneous (Children's Hosp.):-

Hemoculture
Pleural fluid
Swab from ear
Swab from teeth
Wounds

Miscellaneous (Other):-

Hemoculture
Wound
Stool

Diarrheal Diseases

Principal Investigator: Joe D. Worsham, SFC

Associate Investigator: Chiraphun Duangmani, M.D.

OBJECTIVE: To monitor the occurrence of enteric pathogenic bacteria in diarrheal diseases in Thailand.

BACKGROUND: This laboratory was originally established to study diarrheal diseases in Thailand. It has become recognized and respected for the completeness of identification of enteric organisms, both by biochemical means and by serological techniques.

With the discovery of Vibrio parahaemolyticus as a probable cause of diarrhea in Thailand, certain aspects of SMRL's routine survey for enteric pathogens was de-emphasized. In August of 1971, it was determined that the routine survey of nursery employees at Children's Hospital in Bangkok could be conducted by the laboratory at that facility. This relieved some of the work load of the enteric identification laboratory and permitted more work to be done on research projects.

This laboratory continues to maintain a thorough and accurate capability to isolate and identify enteric pathogens.

RESULTS: Tables 1 through 4 indicate the finding in the enteric identification laboratory during the past year.

TABLE 1

Enterobacteriaceae Isolated From Patients With Acute Diarrhea In Thailand

Month	Thai National					American National				
	No. of Specimens	No. of Patients	Salmonella	Shigella	E. coli	No. of Specimens	No. of Patients	Salmonella	Shigella	E. coli
Apr	235	84	6	1	86	3	3	-	1	-
May	178	75	1	2	59	5	5	1	-	3
Jun	275	87	2	1	87	2	2	-	-	-
Jul	331	101	2	1	122	3	3	-	-	2
Aug	140	74	- *	1	53	3	3	-	-	1
Sept	28	28	5	2	2	-	-	-	-	-
Oct	29	28	1		5	3	3	-	-	-
Nov	26	26	-	-	8	-	-	-	-	-
Dec	25	25	-	-	8	4	4	-	-	2
Jan	16	16	-	1	6	-	-	-	-	-
Feb	38	35	6	1	25	-	-	-	-	-
Mar	26	26	1	2	14	1	1	-	-	-
Total	1,347	606	24	12	475	24	24	1	1	8

* stopped getting specimens from Children's Hospital

TABLE 2

Salmonellae Species Isolated From Patients With Acute Diarrhea In Thailand
(1 April 1971 - 28 March 1972)

Species	Thai National				American National			
	Children	Adults	Unknown	Total	Children	Adults	Unknown	Total
S. derby	3	1	-	4	-	-	1	1
S. weltevreden	1	-	-	1	-	-	-	-
S. typhesa	5	1	-	6	-	-	-	-
S. anatum	1	1	-	2	-	-	-	-
S. poona	7	-	-	7	-	-	-	-
S. ndolo	-	1	-	1	-	-	-	-
S. lexington	1	-	-	1	-	-	-	-
S. meleagridis	1	-	-	1	-	-	-	-
S. stanley	1	-	-	1	-	-	-	-
Total	20	4	-	24	-	-	-	-

TABLE 3

Shigellae Species Isolated From Patients With Acute Diarrhea In Thailand
(1 April 1971 - 28 March 1972)

Species	Thai National				American National			
	Children	Adults	Unknown	Total	Children	Adults	Unknown	Total
Sh. flexneri 2	5	-	-	5	-	-	-	-
Sh. flexneri 3	-	1	-	1	-	-	-	-
Sh. flexneri 4	1	-	-	1	-	-	-	-
Sh. flexneri 6	1	-	-	1	-	-	-	-
Sh. sonnei form 1	2	-	-	2	-	1	-	1
Sh. dysenteriae 4	1	-	-	1	-	-	-	-
Sh. dysenteriae 7	-	1	-	1	-	-	-	-
Total	10	2	-	12	-	1	-	1

TABLE 4

Pathogenic Escherichia coli Isolated From Patients With Acute Diarrhea In Thailand
(1 April 1971 - 28 March 1972)

Serotypes	Thai National				American National			
	Children	Adults	Unknown	Total	Children	Adults	Unknown	Total
055 : B5	2	-	-	2	-	-	-	-
026 : B6	1	-	-	1	-	-	-	-
0127 : B8	30	1	-	31	-	-	-	-
0119 : B14	64	1	-	65	-	-	1	1
0112 : B11	1	-	-	1	-	-	-	-
0125 : B15	6	-	-	6	-	-	-	-
0126 : B16	149	-	-	149	-	-	-	-
0128 : B12	2	-	-	2	-	-	-	-
025 : B19 : B23	26	-	-	26	-	-	-	-
086 : B7	4	-	-	4	-	-	-	-
0111 : B4	4	-	-	4	1	-	-	1
334 a	137	2	-	139	-	2	3	5
B2C	44	-	-	44	1	-	-	1
B7a	1	-	-	1	-	-	-	-
Total	471	4	-	475	2	2	4	8

Mycotic Diseases

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

OBJECTIVE: To monitor the occurrence of pathogenic fungi in Thailand.

BACKGROUND: This laboratory continues to conduct routine examinations for pathogenic fungi. This practice ensures the presence of properly trained personnel to manufacture appropriate media and to be able to identify various species of fungi. Maintenance of stock cultures also permits this laboratory to train technicians from other medical facilities in Thailand.

RESULTS: During the past year we have examined 244 specimens submitted for fungus isolation. 29 non-pathogenic fungi were identified and 38 pathogenic species were recovered.

Table 1 presents data indicating the source of the specimens and the pathogenic organisms isolated.

TABLE 1

Specimens Received & Examined for Fungus
30 March 1971 - 30 March 1972

Sources of specimens	Total No examined	No. of non-pathogenic fungi	Number of Positive cultures
Skin	28	13	2 Candida albicans 2 Microspotum gypseum
Ear	7	4	1 Microspotum canis
Hair	2	1	
Cerebrospinal fluid	6		
Joint fluid	1		2 Candida albicans
Nail	5	1	1 Trichophyton rubrum
Scalp	2		1 Candida albicans
Finger	5	1	1 Microspotum gypseum
Body (Trunk, face, arms, legs)	4	1	1 Candida albicans
Foot	5		
Hand	4	1	1 Candida albicans
Toe	2	1	
Throat swab	6		2 Candida albicans
Bronchial Washing	1		
Vagina	37		3 Candida albicans
Sputum	8	1	5 Candida albicans
Gastric Washing	1		1 Candida albicans
Urine	1		
Biopsy	1		
Male genital organs	2		
Thoracentesis	1		
Female perineum	99		11 Candida albicans 1 Microspotum gypseum
Unidentified specimen	16	5	2 Microspotum canis 1 Candida albicans
Total	244	29	38

Mycobacterium Tuberculosis

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

OBJECTIVE : To monitor the occurrence of Mycobacterium tuberculosis in Thailand.

DESCRIPTION: The Department of Bacteriology maintains facilities with special equipment and media for the recovery and identification of acid-fast bacteria. Two technicians are trained in the necessary techniques of Mycobacterium sp. isolation.

While no active research is being conducted regarding acute tuberculosis at present, our laboratory facility has performed a valuable service to local hospitals in Thailand.

RESULTS: The results of this service are presented in Table 1.

TABLE 1

Specimens Received & Examined for
Mycobacterium Tuberculosis & Other Species

Type of Specimens	Total No. Examined	Number of Negative Cultures	Number of Positive Cultures
Sputum	137	135	2 Mycobacterium species
Gastric Washing	47	44	3 M. tuberculosis
Urine	35	35	
Cerebrospiral fluid	17	16	1 Mycobacterium species
Pleural fluid	7	7	
Joint fluid	4	3	1 M. tuberculosis
Abdominal fluid	2	2	
Thoracic fluid	3	3	
Aspiration from neck	3	3	
Stool	1	0	1 M. tuberculosis
Lymph node	2	2	
Bone marrow	2	2	
Biopsy	7	6	1 M. tuberculosis
Lung tissue (monkey)	4	4	
Unidentified specimens	3	3	
Throat swab	1	1	
Granuloma	1	1	
TOTAL	276	267	9

Bacterial Diseases of Domestic Animals Native to Thailand

Principal Investigators: Dennis O. Johnsen, MAJ, VC
Markpol Tingpalapong, DVM
Alexander De Paoli, MAJ, VC
Robert L. Hickman, MAJ, VC

Associate Investigators: SFC Joe D. Worsham
SFC Alson R. Hickerson
Prayot Tanticharoenyos, DVM
Jerm Pomsdhit

OBJECTIVE: The objective of this report is to present zoonotic information of public health comparative medical, and veterinary significance.

DESCRIPTION: This information has been compiled from the results of routine clinical and necropsy examination of laboratory animals and native domestic species that are examined upon request or incident to other laboratory studies.

PROGRESS: During September 1971, an apparent outbreak of leptospirosis occurred among the sentry dogs at the Nakorn Panom Air Force Base. At least 5 dogs died during a 2 month period and a number of others became sick. Clinical signs of illness in the dogs consisted of depression and anorexia; occasionally, emesis and icterus were reported. Haematological examination of affected dogs revealed that there was a neutrophilic regenerative left shift. Hemotonia was not reported. Progressive atoxia and signs of CNS disturbances developed in dogs that succumbed to the disease SMRL was requested by the base veterinarian to provide assistance in the diagnosis of this problem. The clinical signs and pathological lesions in the liver and kidneys of two dogs from which tissues were obtained suggested a diagnosis of leptospirosis. Specimens were collected from dogs at the base for both bacterial isolation and serological examination on 10 and 30 September. Urine specimens were inoculated intraperitoneally into weanling hamsters. After two weeks, if hamsters did not die before, a kidney biopsy was made and the tissue taken was inoculated into Fletcher's media. Two blind passages were made in Fletcher's media before the specimen was considered

negative for leptospira. The urine of 30 dogs was examined in this manner, but no isolates were obtained. Serological examination showed that approximately 20% of the dogs had antibodies to two leptospira serotypes present in high titer. These serotypes were L. hebdomadis and L. hyos. Antibodies to L. canicola and L. ballico were also found in lesser titer. In two cases antibody increases in paired sera confirmed that infection with L. hebdomadis or L. hyos serotypes had occurred between the time the two serum samples had been drawn. During the time that illness was seen in the dogs, an animal handler also became ill and was hospitalized with clinical signs similar to those observed in dogs. It was not possible to confirm serologically that this individual had leptospirosis. Improved methods of sanitation and rodent control were instituted in the sentry dog kennels and there have been no further reports of similar illness occurring among the dogs.

Serum samples were collected from pigs at 3 farms in Thailand and examined for the presence of Leptospira and Brucella antibodies. The location of these farms and the clinical problems they experienced are more fully described in the report concerning Japanese encephalitis infection in animals. There were no leptospiral antibodies found in any of the more than 100 serum samples examined. From these findings it appears that leptospirosis has not been involved in the swine reproductive problems that have occurred in these areas.

Serum samples collected from the pig farms and examined for Leptospira antibodies were also checked for evidence of Brucella infection. Although antibodies were found in pigs at each of the three farms, pigs at the Kasetsart University pig farm in Tubkwang Saraburi province had a significantly higher incidence of antibody than the other two areas located at Kanchanaburi and Nong Khai. Of 151 pigs sampled, 24 had Brucella plate agglutination titers of 1:50 or greater. Attempts have been made to isolate Brucella by inoculation of whole blood from several reactor animals (those with titers of 1:100 or higher) into guinea pigs but no isolations have been made. It is not possible to conclude at this time whether or not brucellosis is a significant problem among these pigs or not.

Distribution and Ecology of Ectoparasites of Vertebrates in Southeast Asia.

Principal Investigators: Joe T. Marshall, Ph.D.
H. Elliot McClure, Ph.D.
Panita Lakshana, B.Sc.
S. Wongsathuaythong, M.B., Dr. Med.*

Associate Investigators: M. Nadchatran**
Inkam Inlao
Nongnuj Maneechai

OBJECTIVE: To assemble information on the systematics, geographic and seasonal distribution and the host-parasite relationships of the ectoparasites of vertebrates in Southeast Asia.

DESCRIPTION: Ectoparasites are removed from mammals, birds and other vertebrates collected in selected study sites and in connection with various disease studies in Thailand and elsewhere in Southeast Asia. The ectoparasites are preserved, sorted into major groups and identified at SMRL or submitted to specialists abroad for identification. Aliquots of collections used for inoculation of test animals are given priority in these identifications. Studies on the taxonomy and ecology of the various vertebrate hosts of these ectoparasites are also conducted.

PROGRESS:

1) Distribution and Systematics of trombiculid mites. During the period of this report chiggers from many species of hosts collected by the Migratory Animal Pathological Survey (MAPS) were submitted to SMRL for identification. These chiggers were collected from the following provinces:- Chiangmai , Nan, Nakhon Ratchasima, Nakhon Sawan, Petchabun and Phitsanulok. One new species of the genus and subgenus Leptotrombidium was found in the collections from Chiangmai. One additional new record for Thailand (Trombicula (Sasatrombicula) kukongensis Chen & Hsu, 1963) was collected from Hipposideros bicolor in Pak Thong Chai district, Nakhon Ratchasima province.

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2) Survey of house-dust mites in Thailand. House-dust mites are one of the most common causative agents of bronchial asthma and allergic rhinitis in Thailand. A study was undertaken to determine which species are actually common or native in house dust. Samples of house-dust were collected (most frequently from edges of mattresses), weighed and examined for mites. A total of 412 collections of house dust mites were made from the following 15 provinces : Ayuthaya, Chanthaburi, Chon Buri, Krungthep (Bangkok), Lop Buri, Nakhon Pathom, Nakhon Ratchasima, Ratchaburi, Samut Prakan, Samut Sakhon, Suphan Buri, Surat Thani, Thon Buri, Trang and Udon Thani. Mites belonging to 9 known families and 5 unidentified families were found in these collections (Table 1). The largest number of dust samples containing mites were collected from Bangkok and Thonburi. Only single samples of dust were collected in Chanthaburi and Udon Thani and these contained no mites. Dust samples containing the highest concentrations of mites were collected in Thon Buri (3665 mites/gm) and Bangkok (3485 mites/gm). Mites belonging to the family Pyroglyphidae were found in 92 of the 95 positive collections and made up the largest part (95.6%) of the collections. Percentages of mites of the family Cheyletidae, Glycyphagidae and Acaridae in these collections were 2.1%, 1.6% and 0.4%, respectively. Mites of other 10 families constituted only minor and perhaps insignificant fractions of the whole population. Four species of Pyroglyphid mites were present in these collections: Dermatophagoides chelidonis, D. farinae, D. pteronyssinus and Malayoglyphus intermedius. Of these four species D. pteronyssinus was the most frequently collected species, and D. farinae the next most commonly encountered.

Table 1. Distribution of Dust Mites in Studied Provinces.

Provinces	Family of Dust Mites													
	Bdellidae	Cheyletidae	Raphignathidae	Tarsonemidae	Acaridae	Glycyphagidae	Listrophoridae	Pyroglyphidae	Haplochthoniidae	Family A	Family B	Family C	Unknown D	Unknown E
Ayutthaya					+	+		+						
Chanthaburi														
Chon Buri		+						+						
Krungthep	+	+			+	+	+	+	+	+	+	+	+	+
Lop Buri								+						
Nakhon Pathom		+				+	+	+			+			
Nakhon Ratchasima						+		+						
Ratchaburi								+						
Samut Prakan		+				+		+						
Samut Sakhon								+						
Suphan Buri		+						+						
Surat Thani		+						+						
Thon Buri		+	+	+	+	+		+	+					
Trang						+		+						
Udon Thani														

Survey to Estimate Significance of Certain Zoonotic Diseases and Their Military Importance in Thailand.

Principal Investigators: Bruce O. Coles, Maj, USAR (Attached)
Markpol Tingpalapong, D.V.M.

Coordinators: Dennis O. Johnsen, Maj, VC
Robert L. Hickman, Maj, VC
Prayot Tanticharoenyos, D.V.M.

Period of Survey: 22 May - 6 June 1972

OBJECTIVE: The objective of this survey was to determine the principal sources of information from which data concerning the incidence of certain zoonotic diseases may be obtained and to collect and evaluate as much of the same as could be accomplished in the time period allowed.

BACKGROUND: A review of the literature revealed that little if any work has been done to determine the significance of zoonotic diseases in Thailand. A study conducted by USOM in cooperation with the RTG Department of Livestock 1967 - 1969 in Northeast Thailand showed that in Changwat Loei, out of a sample of 2,689 cattle and buffalo bled only 28 head were positive and 69 head suspects for Brucellosis. A slightly more than .01% of reactors. This was the result of a pilot survey reported in USOM AD/AG Memo dated 2 August 1968. An extensive survey in Konkaen, Udorn, and Ubol provinces followed. The exact results have not been made available, however, it was reported that the incidence of brucellosis ran only slightly higher than in the pilot study. The conclusion drawn from this study is quoted "The results of the brucellosis testing show a very low incidence in the areas tested. This should not adversely affect the early stages of intensified livestock production. However, there is enough brucellosis present that when animals are concentrated on feed lots, serious losses due to abortion will be constant threat." This is the type of information that is absolutely necessary for planning livestock programs.

METHOD: A list of zoonotic diseases known to be present in Thailand was drawn up. Those selected for the study were; Foot & Mouth Disease, Brucellosis, Tuberculosis, Leptospirosis, Rinderpest, Hog cholera, Rabies, Anthrax, Encephalitis, Hemorrhagic septicemia and Trichinosis.

A performa was designed for interviewing. This included headings as follows: species infected; areas of occurrence; reported incidence,

control program if applicable; economic and public health significance and the source of information for each disease listed.

Interviews in Bangkok included the Royal Thai Department of Livestock, FAO, College of Veterinary Medicine Chulalongkorn University and the Division of Agricultural Economics of the Ministry of Agriculture.

Time permitted only a selected sample of interviews from the field. The Northeastern Region of Thailand and part of the Central Plain was chosen for the field survey. The Northeast Region contains 40.5 & 55.9% of the kingdom total number of cattle and buffalo respectively and a high percentage of hogs. This corresponds to 1/3 of the total human population of the Kingdom in the same region. See table II. Source DAE.

Main livestock stations, forage crops centers, and other research centers were selected within the regions and key personnel were interviewed.

Table I Estimated Livestock Population in Thailand 1971 - DAE* Reported Animal Survey of Cattle, Buffalo, and Swine.

Region	Cattle	Puffalo	Swine
Northeast	1,892,108 40.5%	3,195,834 55.7%	1,403,466 27.3%
Central	1,552,795 33.3%	1,652,206 28.8%	2,352,197 45.8%
South	694,495 14.9%	230,593 4.0%	810,792 15.8%
North	527,571 11.3%	655,921 11.4%	563,792 11.0%
Total for Kingdom	4,666,966	5,734,500	5,133,244

* Division of Agriculture Economics.

RESULTS & DISCUSSION.

The diseases investigated are summarized categorically below:

FOOT & MOUTH DISEASE.

Species	Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Significance	Control Program	Total Sources
Cattle	80/1,196,863	4/28 [2]	฿9,240 [3]	Q.V. [4]	16
Buffalo	41/168,228	2/15	-	V.E.	5
Swine	21/383,522	2/20	฿2,000	V.E.	12

From the table above it is noted that a very low incidence of Foot and Mouth Disease has been reported in the areas surveyed.

Note that there were four areas reporting FMD among cattle in the 28 area sampled. The individual cases were not concentrated. This indicates that there were no major outbreaks and implies that swift and effective suppressive measures were taken. The same situation exists in the case of Buffalo and Swine affected with FMD. The principal areas reporting FMD in all three species were Udorn and Konkaen. Saraburi reported only 5 cases among 1,400 head of cattle at the Thai Danish Dairy Farm (introduced from the outside by newly purchased animals) and Muak Lek reported 20 cases among swine, all on the Livestock station. The Organization of Foot and Mouth Disease at Pakchong reported 26 cases in cattle, one in swine and 20 in buffalo for all regions excluding the free zone located in the far south of Thailand.

Foot Note:

1) i.e. 80 cases from a total sample of 1,196,863 head

2) i.e. four general producing areas out of 28 as defined by the one interviewed.

3) i.e. the total value of known losses due to death, does not account for other economic loss such as "downtime" or inefficiency of draft animals affected.

4) i.e. Q = quarantine; V.E. = voluntary elimination by owner. All tables are in this pattern and represent the period of 1 Jan - 31 Dec 71. C.E. = Completely eradicated; V = Vaccination.

BRUCELLOSIS.

Species	Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Signi- ficance	Control Program	Total Sources
Cattle	34/196,863	10/28	฿33,000	V.E.	16
Buffalo	*/168,228	*/15	*	V.E.	5
Swine	20/383,522	2/20	฿38,000	V.E.	12

The findings of the survey of reported incidence of Brucellosis shows only slightly higher rate than what was reported in 1968 by the USOM team in Loei Province. A difference of .17% versus .01% in the Loei survey. In cattle, abortion is the most frequently observed symptom. In swine, abortion and arthritis are seen most frequently. The concensus of opinion of authorities interviewed in the field was that the farmers do not recognize brucellosis in cattle since most of the Bos indicus (native Indian stock)showing positive to serologic test do not show the abortion syndrome. There is a greater incidence of abortion in swine, these same farmers do believe brucellosis is exists in this species. The same is true in Bos taurus (European stock) for the same reasons. The first interview reported in annex B supports this opinion.

It appears from the interviews that most buffalo and many of the native cattle are not being tested in the villages.

The Forage Crop Station at Pakchong reported 10 of the total of 34 reported in 1971. These ten were from a total of 650 animals tested.

* None reported.

1. Leptospirosis, 2. Rinderpest, 3. Encephalitis, 4. Anthrax, 5. Trichinosis.

Species	Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Signi- ficance	Control Program	Total Sources
Cattle	*/196,863	*/28	*	(4) V (1) C.E. (2) C.E.	16
Buffalo	*/168,228	*/15	*	(1) Q.V. (2) C.E. (4) V.	5
Swine	*/383,522	*/20	*	(1) V.E.	12

The five diseases listed at the top of the table have not been reported from any of the areas surveyed for the year of 1971.

However, provincial hospitals have seen encephalitis, trichinosis and even anthrax as reported by reliable sources outside this survey.

The eating habits of Northeastern Thai people are conducive to transfer of these disease from animal to man, i.e. frequent consumption of uncooked or in adequately cooked meat.

TUBERCULOSIS.

Species	Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Signi- ficance	Control Program	Total Sources
Cattle	23/196,863	4/28	฿151,000	V.E.	16
Buffalo	*/168,228	*/15	*	V.E.	5
Swine	*/383,522	*/20	*	V.E.	12

* None reported.

T.B. in cattle was reported from Tab Kwang, Muaklek, Pakchong and Konkaen. A total of 23 cases were reported from a sample of 4,780 head tested in these four areas. It appears that Buffalo and swine are not generally being tested for T.B. The control measures seem to be grossly inadequate when left to a voluntary elimination basis. The characteristic of infecting heterologous hosts is a main factor complicating control of tuberculosis.

RABIES.

Species	Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Signi- ficance	Control Program	Total Sources
Cattle	16/196,863	5/28	฿43,600	V	
Buffalo	*/168,228	*/15	*	V	
Swine	*/383,522	*/20	*	V	

Additional information from MEDCAP, at Udorn Air Base including 61 villages within a 10 miles radius of the base. A total of 20,895 dogs were immunized with Rabies vaccine, 1,200 human exposures, 1,439 suspected dogs and heads sent to the diagnostic laboratory, SEATO Med Lab, 62 of these cases were positive. This infectious disease affected mostly dogs, but all mammals including human are susceptible.

HEMORRHAGIC SEPTICEMIA.

Species	Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Signi- ficance	Control Program	Total Sources
Cattle	147,196,863	10/28	฿135,140	V	
Buffalo	459,168,228	7/15	฿686,300	V	
Swine	25/383,522	3/20	฿ 26,600	V	

*None reported

Hemorrhagic septicemia was quite widespread. In ten areas of the 28 surveyed there were cattle affected. Seven areas of 15 surveyed had a relatively high incidence. The distribution of the disease was wide. Total economic loss was also heavy relative to total population of livestock in the Northeast region. The chief method of control was by inoculation of a Pasteurella bacterin.

HOG CHOLERA.

Reported Incidence/ Total Population	Area Occurrence/ Total Area	Economic Significance	Control Program	Total Sources
44/383,522	3/20	฿23,150	Q.V.	12

The number of incidence of hog cholera seems quite low to the investigators. Since it is a common practice of villagers to slaughter and consume ailing animals, it is believed that the number of cases in the villages and not reported may be much greater. Out of 44 cases reported, there were 29 cases from Kon kaen.

Table II. Serum JE Titer of the Horses in Tapra Animal Breeding Section in August 1970.

Name	Titer	Name	Titer	Name	Titer
Ngam-ngnae	320	Wang-ngen	640	Khab-rong	160
Kraw-thong	160	Wah-thamnieb	320	Chintana	160
Harn-yow	40	Sa-makki	40	Ngam-chamrieng	40
Ga-grabaad	320	Klin-pikul	160	Kah-archa	80
Sia-sala	80	Kamchai	320	Khen-kham	80
Wat-dhaporn	640	Hor-muad	80	Rong-long	320
Chand-dhib	40	Cham-nancha	80	Chan-dhari	80

At the 3rd animal breeding section in Tapra, twenty horses out of 100 died from a disease of unknown etiology. Treatment did not alter the clinical source of the disease whose clinical signs were suggestive of encephalitis to the breeding section veterinarians (See Annex B, interview #2). The data in table I shows serum JEV titers on horses at the Tapra section as determined by the Japanese Virus Institute. Since JEV infections in domestic animals are widespread in Thailand, the significance of these titers in relation to the disease experienced in horses there is unknown.

ANNEX A

LIST OF PERSONS INTERVIEWED

1.	Mr. Supote Sinives	M. S. Oregon State University Livestock Officer Tab Kwang Livestock Station Saraburi Province.
2.	Dr. Chamnean Satayapunt, D.V.M.	Tab Kwang Student Training Farm, Saraburi.
3.	Dr. Knud Vinther, D.V.M.	Thai-Danish Dairy Farm Muak Lhek, Saraburi.
4.	Dr. Pracal Smitinondana, D.V.M.	Organization of Foot & Mouth Disease Laboratory, Pak-chong, Korat.
5.	Mr. Komchakr Pichaironna-rongsongkram	M.S. Alanata University, P.I. Forage Crop Station, Pak-chong, Korat.
6.	Mr. Anan Chinvala	M.S. Agronomy Taphra Livestock Station Officer Konkaen.
7.	Dr. Sarmart Charanyanont, D.V.M.	Veterinarian In-charge Taphra Livestock Station, Konkaen.
8.	Dr. Manit Shanitwong, D.V.M.	Veterinarian Local Practitioner Konkaen.
9.	Lt. Vichian Uchamrut	3rd Animal Breeding Center Taphra Konkaen.
10.	C. C. McLeod	Farm Advisor, Borabu Pasture and range development center, Mahasarakarm N.Z. Columbo Plan.
11.	Mr. Prasiti Sanseha	Animal Husbandry Officer Mahasarakarm Livestock Station Mahasarakarm.

12.	Mr. Nipon Chantarapoh	M.S. Panjab Agr. University Chief, Dept. Animal Science Konkaen University Konkaen.
13.	Dr. Michael M. Albersmeyer	Chief, Vet Services 432nd USAF Dispensary Udorn Air Force Base Udorn.
14.	Mr. Prakarn Virakul	B. Sc. Agr. Ban-Naka Agriculture Economic Center Nongkai.
15.	Mr. Narong Sai-tong	B.Sc. Agr. Chief, Demonstrated Farm Srichiengmai, Nongkai.
16.	Mr. Vech Home-Wong	Provincial Vet Nongkai.
17.	Mr. Kanchai Tar-Horm	Chief of Animal Breeding & Selection Center, Nongkai.
18.	Mr. Arome Limpananont	Acting Chief of Animal Breeding & Selection Center, Udorn.
19.	Mr. Paktra Lert-Lum	Regional Vet Udorn
20.	Dr. Sanan Ratana-o-lan, D.V.M.	M. S. Cornell Univ. Regional Vet Korat.
21.	Mr. Preecha Chaiburut	Increasing Protein Production Center Panomsarakarm Chacherngsao.
22.	Prof. Tieng Tansanguan	Dean of Vet Faculty Chulalongkorn University Bangkok.
23.	Dr. Chua Wongsongsarn, D. V.M.	Acting Director, Department of Livestock Development Bangkok.

24.	Mr. Sawaeng Thongsoot	Farms Representative Borabu, Mahasarakarm.
25.	Dr. C.P. Pilai	Regional Vet Officer F.A.O. Bangkok.
26.	Dr. Somnuk Sriplung	Agriculture & Economic Section Ministry of Agriculture Bangkok.
27.	Dr. Nuam Settachan	Director Korat Provincial Hospital Korat.
28.	Dr. Nopadon Thongsotit	M. D. Konkaen Provincial Hospital Konkaen.

ANNEX B

SUMMARY OF OPINIONS

May 26, 1972: Khun Supote Sinives, M.S., Oregon State, Livestock Officer, Tabkwang Livestock Station. Farmers in and around Tabkwang are not convinced that Brucellosis exists. Cows tested and found positive still bear live calves. Especially this is true of native cows (Bos indicus) which are those most commonly raised. Abortions are quite rare, thus the conclusion drawn by farmers.

Bos taurus (European Cattle) on the other hand are quite sensitive to Brucellosis and have a high incidence of the disease. Those tested and found positive are also those which have aborted calves.

The respondent reported that of a sample of Bos indicus tested in Tak, Chanwat, 60% were positive without abortion symtoms.

May 28, 1972: Lt. Vichient Uchamrut, 3rd Animal Breeding Section, Taphra, Konkaen, 20% of the horses show high titer on Japanese Encephalitis Serological test. Disequilibrium of the head, neck and light reflex of the pupil is slow, muscular tremor, cool on extremities, monthly normal temperature rectal (38.5°C).

Stallions are put paddocks with 20 mares and come in after one day with these symptoms. In addition, some will hit their head on the wall and show hyperephidrosis. Predisposing cause may be weakness or decrease in body defence from breeding and sudden changes in environment.

The treatment given affected horses at this station, are intramural and intradural injections of antibiotics. However, animals treated have not responded to these treatments. Ultimately all of affected horses have died within 5 days.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 045, Bacterial and mycotic diseases of man and animals

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2. Publication

Lexomboon, U., Mansuwan, P., Duangmani, C., Benjadol, P., and M'cMinn, M.T. "Clinical Evaluation of Co-trimoxazol and Furazolidone in Treatment of Shigellosis in Children, Brit. J. Med. "in press".

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6467	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY CAT	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A811	00	046
b. CONTRIBUTING				
XXXXXXXXXXXX	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Parasitic Infections of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 003500 Clinical Medicine; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMING 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 — PREVIOUS: 72	3.6	162.0
FISCAL YEAR — CURRENT: 73	1.5	90.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Altstatt, COL L. B.

TELEPHONE: 984-4523

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Wilks, LTC N. E.

NAME: Iber, MAJ P. K. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U)Parasitic Diseases; (U)Malaria;(U)Host Parasite Relationships;(U)Gnathostomiasis (U)Immunodiagnosis; (U)Pathophysiology; (U)Antimalarial drugs; (U)Dirofilariasis

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 of parasites of military importance in Southeast Asia providing estimates of the risk and consequences of infections with these parasites and describing effective control measures.

24. (U) Prevalence estimates for a given parasite are made in populations of interest by serological techniques and/or by isolation and identification of the organism in clinical specimens. The disciplines of clinical medicine, veterinary medicine, medical entomology, epidemiology and parasitology are utilized to identify life cycles and the variables which influence transmission, clinical course and chemotherapy.

25. (U) 71 07 - 72 06 Preservation of parasitized erythrocytes for re-invasion of malaria parasites in an in vitro system was successful for up to 1 year. Studies on the susceptibility of the gibbon to Strongyloides continue; the proteolytic enzymes of E. histolytica continued to be studied; radio-active isoleucine uptake test for malaria growth measurement continues to be improved; the screening test for antimalarial drugs in monkeys continues successfully; drug screening for gnathostomiasis has failed to produce an effective compound; and the immunodiagnosis of parasitic infections continue as diagnostic as well as research technology using commercially prepared antigens; an immunodiagnostic test for gnathostomiasis shows promise. For technical reports see Walter Reed Army Institute of Research Progress Report, 1 Jul 71 - 30 Jun 72.

*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 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 046, Parasitic infections of man and animals

Investigators.

Principal: Norman E. Wilks, LTC, MSC; Peter K. Iber, MAJ, MSC; Dennis O. Johnsen, MAJ, VC; Alexander De Paoli, MAJ, VC; Svasti Daengsvang, Med.D.; Katchrinnee Pavanand, M.D.; Vithune Yuthasastr-Kosol, M.D.; Pirom Phisphumvidhi, B.Sc.

Associate: Norman E. Wilks, LTC, MSC; Peter K. Iber, MAJ, MSC; Alexander De Paoli, MAJ, VC; Robert L. Hickman, MAJ, VC; Dennis O. Johnsen, MAJ, VC; Markpol Tingpalapong, DVM; Prayot Tanticharoenyos, DVM; William L. O'Brien, SP6; Prasan Kaewsritong, B.Sc. (M.T.); Prasit Sookto.

Assistant: Barnyen Permpanich, R.N.; Phaibul Sirichakwal, B.Sc.*; Paisarl Yingyourd, B.Sc.; Rapee Machimasatha, B.Sc.; Prasit Sookto; Nipon Chuanak.

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* Resigned 12 September 1971

Viability of Plasmodium falciparum Frozen in Liquid Nitrogen in the Presence of Dimethyl Sulfoxide.

Principal Investigators: Katchrinnee Pavanand, M.D.
Peter K. Iber, MAJ, MSC
Norman E. Wilks, LTC, MSC

Assistant Investigators: Barnyen Permpanich, R.N.
Nipon Chuanak
Prasit Sookto

OBJECTIVE: To determine a simple and reliable method for long term preservation of plasmodium parasitized erythrocytes for both in vitro and in vivo uses.

DESCRIPTION: The in vitro culture technique[1] developed in this laboratory offers an extensive approach to further investigation of parasite metabolism and response to drugs. The source of intact parasites for these studies is limited to fresh blood specimens from patients and is seasonally dependent. The glycerol preservation of parasitized cells which has been recommended[2] was found inapplicable for in vitro cultures. Several different attempts have been made to preserve and retain the viability of intact parasites with least hemolysis upon thawing after long-term storage. Different concentrations of dimethyl sulfoxide were added to washed parasitized cells to determine the optimum protection against damage due to freezing and thawing. A similar technique was used to preserve and store simian and rodent malaria for in vivo studies. Viability of frozen P. falciparum infected cells was studied in vitro by radiochemical and morphological methods.

PROGRESS: Fresh blood specimens were obtained from patients admitted to Cholburi and Somdej Sri Racha Hospitals and kept at 4°C before being processed. A control in vitro culture was made on an aliquot of each specimen. Heparinized blood was washed twice with Tyrode's solution, centrifuged at 1000 rpm in a cold centrifuge. A solution of DMSO in Tyrode's solution was added to washed packed erythrocytes. Final concentrations of DMSO in cell suspensions were 8, 12 and 15 percent by volume. An aliquot of 0.5 - 1 ml of cell suspension was transferred into plastic tubing and immediately stored in liquid nitrogen. Frozen cell suspensions were thawed rapidly in a solution of 5% glucose in isotonic saline at 42°C. To obtain a good recovery, an instant thawing process was achieved by agitation of frozen cells in the prewarmed thawing solution. Least hemolysis was seen in specimens containing 12% DMSO. Thawed cells were washed and suspended in culture medium containing C^{14}-isoleucine[3]. Growth of parasites in vitro is demonstra-

ble by an increase in the incorporation of C^{14}-isoleucine. Morphology of the parasites was studied in serial stained slides made at the same time. (In this study, no foetal cells were included in tne culture medium since the parasite growth was studied radiochemically).

Two blood specimens stored in the presence of 12% DMSO for a period of 12 months were studied in vitro. Despite the existence of hemolysis, the remaining intact parasites resumed their viability in vitro and infectivity in vivo. In both specimens, a two-fold increase in parasitized cells was seen after a 72 hr. culture period, (Patient P. from 0.6% to 1.4% and Patient B 0.16% to 0.3%). The C^{14}-isoleucine incorporation rate showed a good correlation with the stages of parasite growth; i.e. radioisotope protein incorporation was observed up tc schizogony with no further incorporation being observed until merozoites penetrated new red blood cells. At this time, the incorporation resumes at a rate relative to the increased parasitemia created by repenetration.

Infectivity of frozen simian and rodent malarias was demonstrable by infection in susceptible laboratory animals. A specimen of P. knowlesi, stored for a period of 8 months, was injected into rhesus monkeys and produced a patent parasitemia on the third day. Similar results were observed with P. berghei in mice.

SUMMARY: A simple method for long term preservation of intact plasmodium species is described. P. falciparum infected erythrocytes preserved in the presence of 12% DMSO and kept frozen in liquid nitrogen for a period of 12 months resumed viability in vitro. The infectivity of simian and rodent malarias was also well preserved by this method. Patent infections were demonstrable in susceptible laboratory animals.

Malaria Drug Screening Study

Principal Investigator: Dennis O. Johnsen, MAJ, VC

Associate Investigators: Alexander De Paoli, MAJ, VC
Markpol Tingpalapong, DVM
Prayot Tanticharoenyos, DVM
Robert L. Hickman, MAJ, VC

OBJECTIVE: The purpose of this study is to determine in macaque monkeys both the maximum tolerated dose and the antimalarial efficacy of drugs being developed for the treatment of malaria.

DESCRIPTION: The maximum tolerated dose of a drug is determined by carefully observing monkeys as they receive increasing doses of the test drug. These clinical observations are supplemented by laboratory tests that serve as additional indicators of toxic effect. The therapeutic effectiveness of a drug is measured by how well it eliminates or reduces parasitemia in monkeys infected with simian malaria. Various doses of the test drug are given until a maximum to minimum therapeutic range is established. All monkeys are necropsied at the conclusion of testing to determine if spontaneous diseases were present that might influence the results of the test.

PROGRESS: During the period of time beginning 1 January, 1971 and ending 31 December, 1971, 313 monkeys were purchased for use in this program. Of this total, 56 monkeys were local Macaca fascicularis (irus) and 257 were Macaca mulatta purchased in New Delhi, India. Prior to 1 January, 1971, 94 Macaca fascicularis ~~that~~ were on hand and could not be used when the decision was made to employ rhesus monkeys in the program. Six others were used as parasite donors and 58 were lost as a result of spontaneous diseases that made them unsuitable for use in the program. As of, 31 December, 60 monkeys remained on hand for use in tests to be run during early 1972. During calendar year 1971, 24 drugs were tested for toxicity (T1 test) and 12 complete tests for therapeutic effectiveness (T2 tests) were begun. These tests included the testing of seven "known" drugs that were supplied for initial testing and standardization.

Comparative Pathophysiology of Strains of E. histolytica.

Principal Investigators: Norman E. Wilks, LTC, MSC
Pirom Phisphumvidhi, B.Sc.

OBJECTIVE: To investigate the comparative invasive traits of strains of E. histolytica in SE Asia to determine whether differences in proteolytic enzyme activity account for some strains colonizing in the liver rather than producing the classical colonic ulceration with typical amebic dysentery.

DESCRIPTION: As previously reported (Annual Report 1970-1971) enzymes studied are aminopeptidase using glycine as a standard; dipeptidase using glycylglycine as a standard; pepsin; trypsin; carboxypeptidase; hyaluronidase; hydrolase using casein, hemoglobin and gelatin as substrates. Axenic strains of amebae are cultivated in a monophasic media which permits harvesting of relatively clean populations. Attempts are made to culture amebae from colonic lesions, amebic abscesses and from cyst passers without symptoms.

PROGRESS: Several attempts to adapt isolates of E. histolytica to the monophasic media have been unsuccessful, and enzymatic studies have been restricted to the axenic strains HK-9, HLT10 and HLT12. HK-9 is a strain isolated by W.W. Frye from proctoscopic material several years ago and is a common culture to many laboratories. HLT10 was isolated directly from the pus of a liver abscess in August, 1970, by Dr. L.T. Wang from a patient in Taiwan who suffered from dysentery as well as multiple liver abscesses. HLT12 was also isolated by Wang in November of the same year from a liver abscess. The homogenates of these strains were prepared by washing the parasites 3 times in physiological saline, counting them in a hemacytometer and the homogenizing them in an ice bath. Following centrifugation at 12,000 rpm for 10 minutes the supernate was used for the enzyme assays. The activity of enzymes was measured by spectrophotometric analysis.

The enzyme activities determined for these three strains differed only in the hyaluronidase activity. As shown in Table I, this enzyme was detected to be of low level activity in strains HK-9 and HLT10, and was absent in HLT12.

The World Health Organization Expert Committee report No. 421 suggests that amebae cultured in vitro in the presence of cholesterol, or if the hosts are given high doses of this steroid by mouth, it is possible to convert non-invasive strains isolated from asymptomatic human subjects into

invasive strains indistinguishable from those isolated from patients with amebic dysentery. To test this, the strains cultured in this laboratory were placed in media containing 0.05 mg cholesterol per ml. When analyzed for enzymactic activity the only difference detected was in the hyaluronidase activity (Table I). Strain HK-9 advanced from weak activity (±) to a strong positive (+), and where it had been absent in strain HLT12 the enzyme was readily detected after the introduction of the cholesterol.

Hamsters 4 weeks old were inoculated directly into the liver with the HK-9 strain cultured both in the presence and absence of cholesterol. After two weeks the animals were examined for abscess formation. No lesions were present in those hamsters which had received the amebae not exposed to cholesterol, and one abscess was found in one hamster which received the culture with cholesterol. Microscopic examination of this abscess revealed E. histolytica. The numbers of hamsters were small (4 in each group) and no significant conclusions can be drawn from this attempt. Further studies to elucidate this suggested enhancement of pathogenicity are in progress.

SUMMARY: Enzyme analyses of three strains of axenically cultured E. histolytica showed differences only in hyaluronidase activity. This enzymatic activity was enhanced in two strains by culturing the amebae in the presence of cholesterol. An amebic abscess was produced in a hamster after exposing the amebae to cholesterol.

Table I

Enzyme Activity of Axenic E. histolytica Cultures

Proteolytic Enzyme	Cultivated in Diamond's Media			Cultivated in Diamond's Media with Cholesterol		
	HK-9	HLT10	HLT12	HK-9	HLT10	HLT12
Aminopeptidase	+	+	+	+	+	+
Dipeptidase	+	+	+	+	+	+
Hyaluronidase	±	±	-	+	±	+
Pepsin	+	+	+	+	+	+
Trypsin	+	+	+	+	+	+
Carboxypeptidase	+	+	+	+	+	+
Hydrolase:						
Casein	+	+	+	+	+	+
Haemoglobin	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+

Immunodiagnosis of Parasitic Infections

Principal Investigator: Norman E. Wilks, LTC, MSC

Associate Investigators: William L. O'Brien, SP6
Prasit Sookto
Prasan Kaewsritong, B.Sc. (M.T.)

OBJECTIVE: To employ commercially prepared antigens and experimentally developed antigens in the SAFA test and the IHA test for screening patients with suspected amebiasis, filariasis, malaria and gnathostomiasis.

DESCRIPTION: Under contract with the R&D Command, Parke-Davis and Co. has produced two antigens which have been standardized in preparation and in the resulting nitrogen content per milliliter of fluid. One has been prepared from axenic cultures of E. histolytica, the other from D. immitis. The antigens may be used in both the SAFA and IHA test systems. An antigen has been prepared at WRAIR intended for use in the diagnosis of P. falciparum infections, and an antigen is being developed in the SEATO Medical Laboratory for the detection of patients with gnathostomiasis. It is intended to test these antigens for specificity and sensitivity with sera from a population with a broad spectrum of infection and immunity, and to provide a reference diagnostic capability in support of other U.S. installations in SE Asia.

PROGRESS: A disparity in test results with the Parke-Davis antigen for the diagnosis of amebiasis by the SAFA test was reported in the last annual report (1970-1971). This has been rectified and the antigen has produced good results over the past reporting period. The SAFA and IHA now provide nearly equivalent results. Positive results have been confirmed locally by the demonstration of the amebae.

Serum samples were obtained in Vietnam from 1339 newly arrived troops and from 1102 soldiers who were departing that command. These sera were screened for filariasis and malaria with the following results:

	Number positive	
	Arriving	Departing
Filariasis	38	25
Malaria	4	14

The incidence of positive SAFA results for malaria were anticipated, but the increased number of positive findings for filariasis among arriving troops was not. There is the possibility that personnel with past Vietnam experience were among the arriving group. They may have also had inapparent infections with Dirofilaria immitis in the past. SAFA tests for amebiasis were not performed.

An attempt to develop an antigen to be used in a SAFA test for gnathostomiasis continues. During the reporting period fresh larvae from infected mice produced a weakly reactive fraction which gave significantly higher fluorescent responses in 3 of 5 suspected cases of gnathostomiasis. These three continue to experience the symptoms associated with migrating gnathostome larvae. One of the positive sera was from a patient who was also strongly positive for filariasis. Symptoms of both infections were clinically present.

A recent trip to a town 100 km west of Bangkok where there is an abbatoir noted for its pork production yielded over 100 adult Gnathostoma hispidum. These worms were frozen in liquid nitrogen and brought to SMRL for antigen preparation, a process underway at this time.

SUMMARY: The SAFA test has yielded satisfactory results in the detection of amebiasis and filariasis which are comparable to those obtained by IHA. The test for malaria has not produced conclusive results yet. A reactive fraction for the detection of gnathostomiasis has been obtained and additional antigenic extracts are in preparation.

Chemptherapy of Gnathostomiasis.

Principal Investigator: Professor Svasti Daengsvang, Med.D.

Assistant Investigators: Phaibul Sirichakwal, B.Sc.*
Paisarl Yingyourd, B.Sc.
Rapee Machinmastha, B.Sc.

OBJECTIVE: To determine the effect of oral administration of drugs on white mice infected with Gnathostoma spinigerum larvae. The drugs selected have been used effectively in the treatment of certain helminthic diseases but have yet to be used in treating gnathostome infections.

DESCRIPTION: (1) Bithionol or Bitin, 2, 2'-thiobis (4.6-dichlorophenol) oral administration.
Previously, (Annual Progress Report 1971) oral administration of Bithionol to a small number of adult white mice, each weighing 23-38 grams, infected with Gnathostoma spinigerum larvae (40 mg/kg body weight) showed little or no effect on the parasite. During this reporting year 1972, repeated studies were undertaken by oral administration of the drug in distilled water, in 10% ethanol and in 0.5% methocel (methyl cellulose) to adult white mice infected with G. spinigerum larvae. Dosage, 40 mg/kg body weight every other day. Autopsies were performed on mice after completing the experiment. An illuminated examination box and microscope were used to determine the presence of worms in the muscles and visceral organs.

(2) Thiabendazole (MK-360), 2-(4-Thiazolyl benzimidazole) oral administration.
A preliminary study on the effect of Thiabendazole chemotherapy in distilled water with or without 0.05% hydrochloric acid on induced gnathostomiasis in white mice by oral administration was reported in the Annual Progress Report 1971. No effect on the parasite was observed. During 1972 repeated studies were undertaken by oral administration of the drug in distilled water and in 0.05% hydrochloric acid to more adult white mice infected with G. spinigerum larvae.

Eleven white mice were infected orally with G. spinigerum fully developed larvae in cyclops of which 7 were treated by 14 doses of 50 mg/kg body weight of the drug in distilled water and 4 used as controls. Seventy-one white mice were infected each with 5 G. spinigerum advanced third-stage larvae obtained from other infected mice, of which 10 were treated by 14 doses of 50 mg/kg body weight of the drug in 0.05% hydrochloric acid and 5 used as controls, 37 were treated by 14 doses of 100 mg/kg body weight of the drug in distilled water and 19 used as controls. Autopsies were performed after completing the experiment.

*Resigned in September 1971

(3) Niridazole (Ambilhar, Ciba), 1-(5-nitro-2-thiazolyl)-2-imidazolidinone or Ciba 32, 644-Ba oral administration.
Previously, (Annual Progress Report 1971) Niridazole was prepared fresh at 1 mg/ml distilled water for oral administration at a daily dosage of 25 mg/kg body weight. During this reporting period continuation of this experiment and a new study on additional mice with increased concentration of the drug in distilled water at a daily dosage of 50-100 mg/kg body weight for 10 days were undertaken. In total, 132 adult white mice were orally infected with G. spinigerum larvae; 93 were treated with 25, 50 and 100 mg of the drug per kilo body weight daily for 10 doses, and 39 were used as controls. Autopsies were performed.

(4) Banocide (Hetrazan) or Diethylcarbamazine citrate (1-diethyl-carbamyl-4-methylpiperazine dihydrogen citrate) oral administration.
Diethylcarbamazine administered per os is rapidly absorbed and excreted in various forms in the urine within 1-2 days. This drug rapidly eliminates circulating microfilariae and tends to act more slowly on the adult worms of Wuchereria bancrofti. Large doses of Hetrazan killed the adults and developing stages of Dracunculus medinensis (Faust, Russell and Jung 1970). The drug usually destroys the microfilariae of Onchocerca volvulus in the skin within a few days but has little effect upon the adult worms (Hawking 1958). Banocide is now being studies to determine its effectiveness by oral administration at a daily dosage of 6 mg/kg body weight for 15 doses in distilled water and administered to 39 adult white mice infected orally with 5 G. spinigerum advanced third-stage larvae. Twenty infected white mice were used as controls. Autopsies were performed

(5) Niridazole (1-(5-nitro-2-thiazolyl)-2-imidazolidinone or Ciba 32, 644 Ba) combined with Hetol (1,4-bis-trichloromethylbenzol) oral administration.
Hetol has been used for treating cattle infected with liver flukes with satisfactory results (Enigk and Duwel 1960). Yokogawa et al. (1965) successfully treated Clonorchis sinensis infections in animals with Hetol (Hoechst). The combination of Niridazole 100 mg and Hetol 50 mg/kg body weight in distilled water was orally administered, daily for 10 doses, to 40 experimental white mice after each being infected with 5 G. spinigerum advanced third-stage larvae. Ten infected white mice were used as controls. Autopsies were performed.

(6) Banocide (Hetrazan) or Diethylcarbamazine citrate combined with Bithionol (Bitin) oral administration.
The combination of these 2 drugs was prepared in distilled water for oral administration to white mice after being infected with 5 G. spinigerum advanced third-stage larvae at a daily dose of Banocide 10 mg and Bithionol 50 mg/kg body weight for 15 doses.

PROGRESS: (1) Bithionol or Bitin. Table 1 summarizes the results of chemotherapy on induced gnathostomiasis in adult white mice by oral administration every day for 20 doses of Bithionol in distilled

Table 1. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with 15 fully developed larvae in cyclops by oral administration of Bithionol (bitin), 2, 2'-thiobis (4, 6-dichlorophenol). The dosage is 40 mg/kg body weight every day for 20 doses.

Dose of Bithionol per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in distilled water					
20	54	32 (59)	114 (14)	livers and/or body muscles	Autopsies 19-25 days after the last dose.
20 doses distilled water (control - no drug)	20	10 (50)	38 (13)	" "	Autopsies 19-26 days after the last dose.
Drug in 10% ethanol					
20	59	39 (66)	195 (22)	livers and/or muscles	Autopsies 1-25 days after the last dose.
20 doses 10% ethanol (control - no drug)	23	15 (65)	43 (12)	" "	" "

(continued)

Dose of Bithionol per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in 0.5% methocel (methyl cellulose) 20	42	33 (79)	130 (21)	livers and/or body muscles	Autopsies 5-26 days after the last dose.
20 doses 0.5% methocel (control-no drug)	22	17 (77)	69 (21)	" "	Autopsies 7-26 days after the last dose.

water, in 10% ethanol and in 0.5% methocel (methyl cellulose). The results of autopsies on the treated and control white mice after completing the treatment showed (a) drug in distilled water: found in 54 treated and 20 control mice 14% and 13% G. spinigerum living advanced third-stage larvae respectively, (b) drug in 10% ethanol: found in 59 treated and 23 control mice 22% and 12% G. spinigerum living advanced third-stage larvae respectively, (c) drug in 0.5% methocel: found in each group of 42 treated and 22 control mice 21% G. spinigerum living advanced third-stage larvae. The drug appears to have no therapeutic effect on the infected mice.

(2) Thiabendazole. The findings of the repeated studies of chemotherapy on white mice infected with G. spinigerum larvae treated by oral administration of this drug in distilled water and in 0.05% hyrochoric acid at dosage of 50 mg/kg body weight and in distilled water at dosage of 100 mg/kg weight showed no significant difference between the treated mice and controls (Tables 2 and 3). The drug appears to have no therapeutic effect on the infected mice.

(3) Niridazole (Ambilhar, Ciba). Daily oral administration of Niridazole in distilled water to white mice infected with G. spinigerum advanced third-stage larvae at dosages 25 mg, 50 mg and 100 mg/kg body weight for 10 doses showed no effect on the parasite. In a total of 10 treated mice administered 25 mg/kg body weight and 53 treated mice given 50 mg/kg body weight found on autopsies after completing the treatment 17% and 23% living advanced third-stage larvae respectively, compared with 17% living larvae found in the 25 controls. Another 30 infected white mice treated with 100 mg/kg body weight showed on autopsies 51% advanced third-stage larvae compared with 54% of the larvae found in 14 control mice (Tables 4 and 5). This drug appears to have no therapeutic effect on the infected mice.

(4) Banocide (Hetrazan) or Diethyl carbamazine citrate. The findings showed no significant difference between the treated mice and controls. The 39 treated mice yielded a total of 133 (68%) living advanced third-stage larvae in the livers and/or body muscles. The 20 control mice examined showed 67 (67%) living advanced third-stage larvae also in the livers and/or body muscles (Table 6). It therefore appears to have no therapeutic effect in infected mice.

(5) Niridazole (1-(5-nitro-2-thiazolyl)-2-imidazolidinone or Ciba 32,644 Ba) combined with Hetol (1, 4-bis-trichloromethylbenzol). The findings on autopsies of the experimental mice showed no significant difference between the treated mice and controls (Table 7). The 40 treated mice found in a total of 130 (65%) living advanced third-stage larvae in the livers and/or body muscles. The daily oral administration of combined Hetol and Niridazole for 10 doses showed no therapeutic value in the infected mice.

Table 2. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with fully developed larvae in cyclops by oral administration of Thiabendazole (MK-360), 2-(4-Thiazolyl benzimidazole). The dosage is 50 mg/kg body weight every day for 14 doses.

Dose of Thiabendazole per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in distilled water 14	7	7 (100)	48 (46)	livers and/or body muscles	Autopsies 23-39 day after the last dose.
14 doses distilled water (control-no drug)	4	4 (100)	31 (52)	" "	Autopsies 40 days after the last dose.

water, in 10% ethanol and in 0.5% methocel (methyl cellulose). The results of autopsies on the treated and control white mice after completing the treatment showed (a) drug in distilled water: found in 54 treated and 20 control mice 14% and 13% G. spinigerum living advanced third-stage larvae respectively, (b) drug in 10% ethanol: found in 59 treated and 23 control mice 22% and 12% G. spinigerum living advanced third-stage larvae respectively, (c) drug in 0.5% methocel: found in each group of 42 treated and 22 control mice 21% G. spinigerum living advanced third-stage larvae. The drug appears to have no therapeutic effect on the infected mice.

(2) Thiabendazole. The findings of the repeated studies of chemotherapy on white mice infected with G. spinigerum larvae treated by oral administration of this drug in distilled water and in 0.05% hyrochoric acid at dosage of 50 mg/kg body weight and in distilled water at dosage of 100 mg/kg weight showed no significant difference between the treated mice and controls (Tables 2 and 3). The drug appears to have no therapeutic effect on the infected mice.

(3) Niridazole (Ambilhar, Ciba). Daily oral administration of Niridazole in distilled water to white mice infected with G. spinigerum advanced third-stage larvae at dosages 25 mg, 50 mg and 100 mg/kg body weight for 10 doses showed no effect on the parasite. In a total of 10 treated mice administered 25 mg/kg body weight and 53 treated mice given 50 mg/kg body weight found on autopsies after completing the treatment 17% and 23% living advanced third-stage larvae respectively, compared with 17% living larvae found in the 25 controls. Another 30 infected white mice treated with 100 mg/kg body weight showed on autopsies 51% advanced third-stage larvae compared with 54% of the larvae found in 14 control mice (Tables 4 and 5). This drug appears to have no therapeutic effect on the infected mice.

(4) Banocide (Hetrazan) or Diethyl carbamazine citrate. The findings showed no significant difference between the treated mice and controls. The 39 treated mice yielded a total of 133 (68%) living advanced third-stage larvae in the livers and/or body muscles. The 20 control mice examined showed 67 (67%) living advanced third-stage larvae also in the livers and/or body muscles (Table 6). It therefore appears to have no therapeutic effect in infected mice.

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Table 3. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with 5 advanced third-stage larvae obtained from other infected white mice by oral administration of Thiabendazole (MK-360), 2-(4-Thiazolyl benzimidazole).

Dose of Thiabendazole per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in 0.05% HCL (50 mg/kg body weight) 14	10	10 (100)	42 (84)	livers and/or body muscles	Autopsies 5 days after the last dose.
14 doses 0.05% HCL (control-no drug)	5	5 (100)	23 (92)	" "	Autopsies 1-5 days after the last dose.
Drug in distilled water (100 mg/kg body weight) 14	37	37 (100)	117 (63)	" "	Autopsies 12-29 days after the last dose.
14 doses distilled water (control-no drug)	19	19 (100)	61 (64)	" "	Autopsies 7-30 days after the last dose.

Table 4. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with 5 advanced third-stage larvae obtained from other infected white mice by oral administration of Niridazole (Ambilhar, 1-(5-nitro-2-thiazolyl) 2-imidazolidinone or Ciba 32, 644 Ba). The dosage is 100 mg/kg body weight every day for 10 doses.

Dose of Niridazole per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in distilled water					
10	30	28 (93)	76 (51)	livers and/or body muscles	Autopsies 3-29 days after the last dose.
10 doses distilled water (control-no drug)	14	13 (93)	38 (54)	" "	Autopsies 29 days after the last dose.

Table 5. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with 15 fully developed larvae in cyclops by oral administration of Niridazole (Ambilhar, 1-(5-nitro-2-thiazolyl) 2-imidazolidinone or Ciba 32, 644 Ba). The dosages are 25-50 mg/kg body weight every day for 10 doses.

Dose of Niridazole per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in distilled water (25 mg/kg body weight) 10	10	10(100)	25 (17)	livers and/or body muscles	Autopsies 33-34 days after the last dose.
Drug in distilled water (50 mg/kg body weight) 10	53	39 (74)	185 (23)	" "	Autopsies 7-25 days after the last dose.
10 doses distilled water (control-no drug)	25	17 (68)	62 (17)	" "	Autopsies 1-34 days after the last dose.

Table 6. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with 5 advanced third-stage larvae obtained from other infected mice by oral administration of Banocide (Hetrazan) or diethylcarbamazine citrate (1-diethylcarbamy 1-4-methylpiperazine dihydrogen citrate). The dosage is 6 mg/kg body weight every day for 15 doses.

Dose of Banocide per mouse	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in distilled water 15	39	39 (100)	133 (68)	livers and/or body muscles	Autopsies 1-18 days after the last dose.
15 doses distilled water (control-no drug)	20	19 (95)	67 (67)	" "	Autopsies 15-19 days after the last dose.

Table 7. Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae after each being infected with 5 advanced third-stage larvae obtained from other infected white mice by oral administration of Niridazole (Ambilhar) combined with Hetol (1, 4-bis-trichloromethyl-benzol). The dosage is 100 mg Niridazole combined with 50 mg Hetol/kg body weight in distilled water everyday for 10 doses.

Dose of Niridazole combined with Hetol	No.mice treated	Autopsy findings			Remarks
		No.mice positive with advanced third-stage larvae (%)	No.advanced third-stage larvae found (%)	Organs infected	
Drug in distilled water 10	40	38 (95)	130 (65)	livers and/or body muscles	Autopsies 9-19 days after the last dose.
10 doses distilled water (control-no drug)	10	9 (90)	26 (52)	livers and/or body muscles	Autopsies 5-19 after the last dose.

(6) Banocide (Hetrazan) or Diethylcarbamazine citrate combined with Bithionol (Bitin). This experiment on mice infected with G. spinigerum advanced third-stage larvae is still in progress.

SUMMARY: Oral administration of Bithionol, Thiabendazole, Niridazole, Banocide, and Niradazole combined with Hetol seems to have no therapeutic value in experimentally induced gnathostomiasis in white mice. The oral administration to mice infected with G. spinigerum advanced third-stage larvae with combined Banocide and Bithionol is now in progress.

Studies of New Experimental Hosts Life Cycles and Modes of Transmission of Gnathostomes.

Principal Investigator: Professor Svasti Daengsvang, Med.D.

Assistant Investigators: Paisarl Yingyourd, B.Sc.
Rapee Machimasatha, B.Sc.

OBJECTIVE: To locate new experimental host animals susceptible to Gnathostoma spinigerum, G. hispidum and G. doloresi and to determine the life cycle of G. vietnamicum.

DESCRIPTION: Young fresh water shrimps (Macrobrachium rosembergi) and adult M. mirabile (Kemp) from the Bangkok area were obtained and tested as possible paratenic hosts of G. spinigerum. Advanced third-stage larvae from infected laboratory white mice were fed four M. rosembergi and maintained in fresh water.

Pieces of white mice flesh that had been infected with 55 gnathostome larvae were also fed to the four M. rosembergi. Three M. mirabile were fed 24 gnathostome larvae. The 2 groups of tested shrimps were autopsied 6-22 days and 3-16 days respectively after commencement of the experiment. Pieces of mice flesh infected with 16 G. hispidum advanced third-stage larvae were fed to a four week old chicken. The chicken was autopsied 31 days after the feeding. A study was undertaken to determine whether fresh water fish would act as a possible intermediate host for G. vietnamicum. Many cyclops infected with 16 G. vietnamicum fully developed larvae were placed into a beaker containing 2 adult small fighting fish (Trichopsis vittatus). The fish were examined 21 and 60 days later.

In order to determine the natural infection, other shrimps, chickens and small fish obtained from the same areas were autopsied for the presence of gnathostomes.

PROGRESS: The results of experimental feeding of G. spinigerum advanced third-stage larvae obtained from experimental white mice and fed to fresh water shrimps were as follows:

Number of shrimps	Larvae fed and source	Autopsy results
4 Macrobrachium rosembergi	55 larvae from white mice	Neg.at 6-22 days
3 M. mirabile (Kemp)	24 larvae from white mice	Neg.at 3-16 days
17 M. rosembergi	None (controls)	Negative
279 M. mirabile (Kemp)	None (controls)	Negative

It appears fresh water shrimps can not act as paratenic hosts for G. spinigerum, but in as much as so few shrimps were studied, future studies should be expanded.

Advanced third-stage larvae of G. hispidum obtained from white mice and fed to one chicken and the control were negative, but repeated studies using more animals will be performed later.

The results of feeding G. vietnamicum fully developed larvae in cyclops to small fighting fish showed, after 21 days, one living larva in the stomach wall of the fish which was of the same morphology and size of larvae found in cyclops. The controls were negative. This result indicates that the larva in cyclops could not develop in the fish. Repeated studies using more animals are planned. Studies on G. doloresi were not conducted during the reporting period.

SUMMARY: Fresh water shrimps and a chicken were found on autopsies to be negative for gnathostome infection after the experimental feeding with G. spinigerum advanced third-stage larvae to the former, and G. hispidum larvae to the latter. One of two experimental fresh water fish was found to contain in its stomach wall, 1 living G. vietnamicum undeveloped larva on autopsy 21 days after being fed with the fully developed larvae in cyclops.

Strongyloidiasis in the Gibbon

Principal Investigator: Alexander De Paoli, MAJ, VC

Associate Investigators: Norman E. Wilks, LTC, MSC
Dennis O. Johnsen, MAJ, VC

OBJECTIVES: The study was initiated to:

1. Determine the susceptability of gibbons to S. stercoralis from man.

2. Compare the natural disease with that produced by the human parasite.

DESCRIPTION: Previous studies have shown that strongyloidiasis is a major cause of death in the gibbon colony. Clinically and pathologically the disease in the gibbon is similar to that reported in man. The nematode responsible for the gibbon disease is morphologically similar to S. stercoralis in man. Its relationship to the human parasite, however, has not been determined.

PROGRESS: A study is presently under way to evaluate the above objectives.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 046, Parasitic infections of man and animals

Literature Cited:

References:

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Publications:

1. Daengsvang, S. 1971. Infectivity of Gnathostoma Spinigerum Larvae in Primates. The Journal of Parasitology, Vol. 57, No. 3, p. 476-478.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6468	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. GROUP SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A811	00	047
b. CONTRIBUTING				
XXXXXXXXXX	CDOG 114(f)			

11. TITLE (Procede with Security Classification Code)

(U) Metabolic Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine; 010100 Microbiology; 012900 Physiology

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 PREVIOUS 72	2.0	90
FISCAL YEAR CURRENT 73	4.0	180

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Johnsen, MAJ D. O.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide each with Security Classification Code)

(U)Veterinary Medicine; (U) Gibbon; (U)Leukemia; (U)Encephalopathy; (U)Hepatic Failure; (U)Hyperammonia

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

23. (U) To identify and characterize metabolic diseases of man and animals in Southeast Asia that may complicate or mimic infectious diseases of military importance.

24. (U) The contribution, either wholly or in part, of metabolic disturbances resulting from malnutrition, concurrent infectious disease, or toxic agents, is evaluated in both field and laboratory studies. Clinical, biochemical, serological, pathological, and epidemiological methods are employed in this evaluation. Animal models are sought which, duplicate features of these diseases processes by virtue of unique physiological characteristics or susceptibility to spontaneous or induced diseases.

25. (U) 71 07 - 72 06 Granulocytic leukemia has been transmitted experimentally from an adult gibbon in the terminal stages of this disease to an infant produced in the gibbon breeding program. A study of hospitalized malaria patients indicated that hepatic dysfunction was probably not related to the occurrence of cerebral malaria. Attempts to reproduce Reye's syndrome in cynomolgus monkeys by oral administration of salicylates were inconclusive. For technical reports see Walter Reed Army Institute of Research Progress Report, 1 Jul 71 - 30 Jun 72.

* (applicable 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 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAMS IN S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 047, Metabolic diseases of man and animals

Investigators.

Principal: Alexander DePaoli, MAJ, VC
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Gibbon Menstrual Cycle & Breeding Study

Principal Investigator: Markpol Tingpalapong, DVM

Associate Investigators: Robert L. Hickman, MAJ, VC
Dennis O. Johnsen, MAJ, VC
Prayot Tanticharoenyos, DVM

OBJECTIVE: To develop methods of breeding and rearing gibbons in the laboratory environment.

DESCRIPTION: In this study the reproductive cycle of the female gibbon and semen of the male gibbon are characterized and related to other physical parameters of breeding performance with the ultimate goal of obtaining reproduction of the gibbon in a laboratory environment. This basic information is utilized in the application of artificial insemination techniques as well as natural mating methods.

PROGRESS:

Natural Breeding Study.

Eight gibbon breeding pairs were kept in the large outside breeding cages continually. These breeders were selected from among mature gibbons kept indoors. Breeding potential was evaluated in these animals by regularity of the reproductive cycle in the female, good physical appearance, and normality of semen in the male as evaluated by gross and microscopic examination. Females were palpated rectally each month to detect pregnancy. Enlargement of uterus was found to be detected as early as seven months prior to the delivery in pregnant animals (B-27). There were 4 babies born during the period of this report. Pertinent data concerning these births is listed in Table 1.

Table 1

Delivery Date	Female Number	Male Number	Baby Number
May 4, 71	B-37	S-76	Pc-8
July 26,71	B-4	B-8	Pc-9
Oct 12,71	B-59	B-2	Pc-10
Oct 15, 71	B-11	B-12	Pc-11

The time of ovulation of the female gibbon has been determined by direct observation of the ovaries at certain times of the female reproductive cycle. Laparotomies were performed in three animals at varying periods of times after the commencement of periodic bleeding in the females. The results of these examinations are shown in Table 2. Such examinations done previously in gibbons at periods of time between 11 and 16 days following the commencement of bleeding showed no evidence of ovulation. Thus, it appears that ovulation in the gibbon occurs shortly after the time bleeding commences as in the dog and other lower mammalian species.

Table 2

Species	I.D.#	Date	Hours after beginning of bleeding	Observations
H. lar	B-66s	Jun 29,71	45	The left ovary contained 3 mature follicles; there was evidence of follicular rupture.
H. lar	B-14s	Jul 1,71	72	The left ovary contained a hemorrhagic focus, apparently the result of follicular rupture.
H. piliatus	B-85	Jul 12,71	60	Enlarged fimbria, horn and body of uterus, a hemorrhagic focus found on the surface of the left ovary; an atretic follicle also present.

To test this information concerning the time of ovulation sixteen female gibbons were inseminated artificially 44 times according to the schedule shown in Table 3. The females were inseminated at 48, 72, and 96 hours after bleeding commenced. Fourteen male gibbons served as semen donors. Semen was collected from these animals a total of 53 times. Three kinds of semen diluents were used: 3% Sodium Citrate 10 times, isotonic saline 24 times, and homologous serum from the semen donor 13 times. There have been no pregnancies detected in animals that have been bred artificially.

Table 3

Hrs. after bleed	Female data																Total
	S2	S10	S20	S27	S70	S78	S92	S96	B14s	B66s	B35	B88	P2	P5	P7	DZ1	
48	1	-	1	1	1	-	1	5	1	2	1	1	1	-	-	-	16
72	1	1	1	-	-	1	-	3	1	3	3	-	-	-	3	-	17
96	2	-	-	-	-	1	-	1	1	-	4	-	-	1	-	1	11

	Male data														Total
	S5	S18	B25	S28	S65	B83	S98	S102	B39	B40	B56	B61	P16	DZ2	
#times semen collected	1	2	1	2	1	2	15	1	1	2	7	1	2	14	52

Pregnancy testing was done in all females that did not bleed within 1 month after insemination. Pregnosticon planotest (Organon) was evaluated for pregnancy testing in the gibbon. A positive test for pregnancy in this test is based on the presence of chorion gonadotrophin (HCG) in the urine. When HCG is present in the urine test an agglutination reaction occurs between HCG antibody reagent and this hormone. Rectal palpation was used to confirm the result of the urine test.

Table 4

	Artificially bred females				Naturally bred females	
♀ #	S27	S10	S20	DZ1	B59	B11
Agglutination	+	-	-	+	-	-
Rectal palpation	-	-	-	-	+	+
Delivered young	-	-	-	-	+	+

In addition, gibbons B-59 and B-11, which were pregnant animals bred naturally, were control animals for the urine test; the uterus of both animals was enlarged on palpation and each delivered a baby a few months later. The results of comparing the test for HCG and rectal palpation for pregnancy determination in the gibbon are shown in Table 4. Necropsies were performed on both S27 and DZ-1 when they died from other causes and both were found to be non pregnant. Thus it appears that this particular urine test is not reliable when used for pregnancy testing in the gibbon.

Salicylate Toxicity in Monkeys

Principal Investigator: Allen M. Glasgow, MAJ, MC

Associate Investigator: Robert L. Hickman, MAJ, VC

OBJECTIVE: The etiology of Reye's syndrome is unknown. One possible cause that has been suggested is the ingestion of salicylate. In man, salicylate intoxication is clinically very similar to Reye's syndrome. This study was an attempt to produce Reye's syndrome in young monkeys by administering salycilate.

DESCRIPTION: The study was to be conducted in 2 parts; one acute, involving administration of 150 mg of salicylate per kg. body weight every 6 hours for 48 hours to fasted animals, and one chronic, involving the administration of 40 mg. of salicylate per kg. body weight every 8 hours for 10 days to unfasted monkeys. Blood, spinal fluid and liver biopsies were to be collected before, during and after the study. All animals were to be observed regularly for clinical signs of illness and were to be euthanized and necropsied 8 hours after the last treatment. Control animals were to be administered sodium chloride in equivalent amounts and handled similarly.

Detection and evaluation of Reye's syndrome was to be based on clinical observations; changes in blood glucose, CO_2, SGOT, SGPT. and prothrombin time; alterations in spinal fluid glucose and glutamine levels; and histopathologic changes in the liver, kidneys, heart and brain.

PROGRESS: The acute phase of the study was conducted with 5 Macaca fascicularis, 3 receiving salicylate and 2 sodium chloride. In this preliminary trial, clinical, biochemical and pathological changes occurred but they could not be satisfactorily differentiated from the changes observed in the control animals which presumably were due to the prolonged fasting (96 hours). Before the study could be modified and repeated, the principal investigator departed. No further investigation is contemplated.

SUMMARY: The results of a preliminary study of the possible relationship between acute salicylate intoxication and Reye's syndrome were inconclusive.

Leukemia in the Gibbon

Principal Investigator: Alexander De Paoli, MAJ, VC

Associate Investigators: Walter W. Noll, MAJ, MC
Dennis O. Johnsen, MAJ, VC

OBJECTIVE: The objective of this study is to determine the incidence of leukemia in the gibbon colony, characterize clinically and pathologically this disease in the gibbon, and to evaluate its transmissability.

DESCRIPTION: Gibbons in the SMRL animal colony are screened periodically to detect developing cases of leukemia. Gibbons in which leukemia has been detected are placed under close observation and clinical, hematological, and pathological methods are employed to characterize the development of the disease. Necropsies are done on gibbons that die and tissues from them have been inoculated into gibbons and other laboratory animals to determine if the disease is transmissible.

PROGRESS: Two additional cases of granulocytic leukemia were detected in the gibbon colony during the report period. Clinical, hematological, pathological studies have shown the 2 cases are similar to those observed in earlier cases. A bone marrow suspension from one of the gibbons was inoculated intraperitoneally into 2 baby gibbons, 2 stumpy tailed monkeys, mice, hamsters, and guinea pigs. No changes suggestive of leukemia have occurred in any of these animals except for one gibbon. One baby gibbon died 3 months after inoculation as a result of a laboratory accident. The second young gibbon developed a disease which was identical to that observed in the spontaneous cases of granulocytic leukemia seven months after it was inoculated. A manuscript describing the above events is now being prepared for publication.

Gibbon Growth and Development Study

Principal Investigators: Markpol Tingpalapong, DVM
Dennis O. Johnsen, MAJ, VC
Dirck L. Brendlinger, MAJ, VC

Associate Investigator: Prayot Tanticharoenyos, DVM

OBJECTIVE: The production of gibbons from the gibbon menstrual cycle and breeding program has offered a unique opportunity to measure certain parameters of growth and development in animals where birthdates are known. The purpose of this study is to relate distinctive development features to the age of these young gibbons so that the age of animals with unknown birthdates may be accurately determined.

DESCRIPTION: Growth and development is measured by body weight, dentition, whole body radiographs, and sexual development evaluated at quarterly intervals.

PROGRESS: Skeletal radiographs have been made quarterly on gibbon Pc-1 at 39, 42, 45 and 48 months of age. No readily detectable skeletal changes, such as closure of epiphyseal plates in bones that are being checked, occurred between the age of 3 and 4 years. Between 36 and 48 months of age Pc-1 gained 0.7 kg. to reach a final weight in December, 1971 of 4.9 kg. Significant developments in dentition occurred during this time. They were:

Age	Dental Formula			
	Incisors	Canines	Premolar	Molar
36 mos	$\frac{1\ 2}{1\ 2}$	$\frac{(1^*)}{(1\ )}$		$\frac{(1)\ (2)\ 1}{(1)\ (2)\ 1}$
39 mos	$\frac{1\ 2}{1\ 2}$	$\frac{(1)}{(1)}$		$\frac{(1)\ (2)\ \ 1}{(1)\ (2)\ 1\ 2}$
42 mos	$\frac{1\ 2}{1\ 2}$	$\frac{(1)}{(1)}$	$\frac{\ }{1}$	$\frac{(1)\ 1\ 2}{(1)\ 1\ 2}$
45 mos	$\frac{1\ 2}{1\ 2}$	$\frac{(1)}{(1)}$	$\frac{1\ 2}{1\ 2}$	$\frac{1\ 2}{1\ 2}$
48 mos	$\frac{1\ 2}{1\ 2}$	$\frac{(1)}{1}$	$\frac{1\ 2}{1\ 2}$	$\frac{1\ 2}{1\ 2}$

* circled figures are jevenile teeth.

Hepatic Function in Cerebral Malaria

Principal Investigators: Allen M. Glasgow, MAJ, MC
Edward Colwell, LTC, MC
Chalard Tirabutana, MD
Ravivan Intraprasert, MD

OBJECTIVE: To evaluate the possibility that the encephalopathy of cerebral malaria is secondary to hepatic dysfunction.

BACKGROUND: It was proposed that hepatic dysfunction may explain the encephalopathy of cerebral malaria because:

1. Abnormalities in hepatic function are known to exist in malaria;

2. Previous experience with Reye's Syndrome has shown that severe hepatic dysfunction may be present despite the relative absence of hepatic abnormalities as detected by light microscopy;

3. Certain features of the cerebral pathology are similar in Reye's syndrome and cerebral malaria;

4. During a previous study a child with cerebral malaria was found to have marked abnormalities in hepatic function including an elevated venous blood ammonia.

DESCRIPTION: Patients with uncomplicated malaria or cerebral malaria admitted to Chantaburi Provincial Hospital were studied on admission. The test for venous blood ammonia was performed immediately. Reducing substances, SGOT, SGPT and bilirubin were measured in frozen sera.

PROGRESS: The results of these tests are given in Table 1. It seems unlikely from these findings that hepatic dysfunction accounts for the encephalopathy in any of our patients with cerebral malaria.

Table 1

NUMBER	AGE	SEX	SERUM SUGAR mg/100 ml	SGOT (Sigma Units)	SGPT (Sigma Units)	BILIRUBIN (mg/100 ml)	AMMONIA (Normal<0.1 um/ml)
Uncomplicated Malaria							
1	28	M	100	14	22	0.7	.023
2	18	M	164	34	21	0.6	.068
3	31	M	98	31	21	1.0	.049
4		M	97	73	36	2.1	.128
5		M	119	24	16	0.4	.096
6	15	M	99	98	39	1.1	.050
7	28	M	145	23	16	0.8	.049
8	21	F	93	25	15	1.0	.049
9	30	F	97	75	28	0.5	.035
10	20	M	113	25	16	0.7	.062
Cerebral Malaria							
1	31	M	96	36	19	1.3	.052
2	17	F	117	84	41	1.3	.046
3	15	M	92	71	28	5.2	.041
4	32	M	63	55	34	1.7	.062
5	27	M	96	44	31	0.7	.038
6	16	M	220	27	25	0.0	.046
7	18	M	114	24	23	0.3	.024
8	29	M	149	31	23	4.5	.032

Laboratory Animal Disease in Thailand: Its occurrence and importance to comparative medicine

Principal Investigator: Dennis O. Johnsen, MAJ, VC

Associate Investigators: Alexander DePaoli, MAJ, VC
Robert L Hickman, MAJ, VC
Kwanyuen Lawhaswasdi, DVM
Markpol Tingpalapong, DVM
Prayot Tanticharoenyos, DVM

OBJECTIVE: The objective of this study is to detect and investigate spontaneous metabolic and infectious diseases of laboratory animals for the purpose of recognizing and developing animal models for research studies as well as to define and improve the health of laboratory animals maintained in Thailand.

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: the disease screening program conducted in the laboratory animal breeding colony, the recurring clinical and laboratory examination of animals housed in the laboratory including those procedures performed during the quarantine of newly purchased animals, complete post mortem examination of each animal that dies in the colony, and the development of standards for operation and quality control that are indicated by the resulting findings. When indicated by the findings, experimental studies are initiated to explore the problems that occur in detail.

PROGRESS: There was little evidence of spontaneous disease among laboratory rodents during the report period. The annual production of mice, hamsters, and guinea pigs has been maintained at levels comparable to that of previous years, as have indicators of production efficiency such as the conception rate and yield per female. The number of small rodents necropsied and the distribution of gross pathological lesions according to the organs system where they were most frequently observed is shown in Table 1. Gross pathological lesions usually consisted of lobar pneumonia, regional enteritis, and cystic

ovaries in the case of guinea pigs. Bacteria isolated from either the respiratory or digestive systems of mice, hamsters, and guinea pigs as part of the disease screening program are shown in Table 2. Randomly sampled retired breeders from the mouse colony were screened for the presence of antibodies to 12 latent murine viruses. This survey was the result of concern that viruses thought to be isolated in the field in suckling mice might actually be murine viruses latent in the mouse colony. Approximately 30 viruses other than Japanese encephalitis virus were isolated from suckling mice which had been inoculated with field specimens collected from the Chiengmai JEV study. The serological testing of the 20 randomly sampled mice revealed that 9 had antibodies to REO 3 virus, 4 had antibodies to CD V111 virus, 12 had antibodies to Sendai virus, 12 had antibodies to Minute Virus of mice, 2 had antibodies to Mouse Hepatitis virus, and 2 had antibodies to Mouse Corona virus. There was no serological evidence of infection with Pneumonia virus of mice, K virus, Polyoma, Ectromelia, Mouse Adenovirus, or Lymphocytic choriomeningitis. The 30 virus isolates themselves were sent to the Division of Veterinary Medicine at the Walter Reed Army Institute of Research for identification. Because there are no mice bred either by conventional or SPF methods that are not already infected with latent murine viruses, the isolates were inoculated into germfree mice to produce "pure" antibody. This antibody was utilized in standard tests to identify the isolates. These tests are currently in progress and have not yet been completed.

A number of spontaneous deaths occurred among primates in the laboratory. Twenty-two deaths occurred among gibbons and 27 among macaques. The causes of these deaths are summarized in Table 3 for gibbons and macaques. Of particular interest are the two additional cases of granulocytic leukemia that developed in gibbons during the report period. Clinical signs and pathological lesions of interstitial and giant cell pneumonia suggest that primary measles virus infection with bacterial complications and attendant high mortality is the most frequently occurring and serious disease seen among rhesus monkeys newly imported from India. Virus isolation procedures to confirm the etiology of this disorder have not been attempted. This is largely a result of the fear that other agents such as Herpesvirus simiae might be recovered simultaneously.. Although the possibility of immunizing monkeys with human measles vaccine has been considered as a prophylactic measure,it has not yet been attempted. Oral administration of vitamin C at 100 mg. per day for a 14 day period appeared to bring about a dramatic decrease in mortality in one room of quarantined monkeys compared to 2 other rooms, but these results could not be duplicated when a more definitive study was done later.

Table 1. Summary of Rodent Breeding Colony Pathology, 1971

Species	Number	Pulmonary pathology	Gastro-Intestinal pathology	Genito-Urinary pathology
Guinea pig	137	35	13	19
Hamster	139	13	7	-
Mice	207	10	4	-

Table 2. Bacterial Isolates Identified in the Animal Disease Screening Program, 1971

SPECIES	NUMBER	LUNGS																FECES				
		Alpha hem. Strept	Gamma Strept	Strep. sp.	Staphylo. epi.	Staph. aureus	Proteus spp.	Proteus mirabilis	Paracolobactrum coliforme	Paracolobactrum intermedium	Mima polymorphia var Oxidans	Mima polymorphia	Enterobacter spp.	Micrococcus sp.	Bacillus sp.	E. coli	Intermedial collform	Hafnia	Proteus sp.	Pseudomonas sp.	Paracolon sp.	Enterobacter	E. coli
Mice	200-	2	3	-	6	16	2	6	1	-	1	1	-	2	7	10	-	-	20	-	-	5	11
Hamster	110	12	-	1	6	15	1	2	1	-	14	-	2	1	4	1	-	-	21	2	2	14	3
Guinea pig	110-	-	-	-	4	5	-	-	1	2	-	2	1	6	-	1	1	2	9	-	2	7	6

Table 3

Summary of Primate Necropsy Findings

Organ System	Lesions	Etiology	Gibbons		Macaques	
			Primary	Secondary	Primary	Secondary
Respiratory	Pneumonia	Viral			4**	
		Bacterial	2*	1	4	
		Inhalation	1	1	1	
	Pulmon. hemo. cong. edema		1	2		3
	Bronchiolitis	Unknown		2		
		Lung mites				22
	Pleuritis					1
Cardiovascular	Pericarditis	Bacterial				1
	Epicardial hemorr.			1		
	Generalized Cong.			4		1
Hemic & Lymph.	Splenitis/ necrosis	Viral		2		2
	Lympho-denopathy			1		1
Digestive	Gastritis				2	
	Enteritis	Bacterial/ mycotic	2	5	6	4
		Strongyloidiosis	1			
		Balantidiasis		2		
		Esophagostomiasis				14
	Peritonitis			1		
	Hepatitis			1	1	2
Urogenital	Nephritis	Interstitial	1	2		
		glomerular				1
		multifocal	1	1		
	Nephrosis			2		3
Endocrine	Adrenalcortic necrosis	Viral				1
	Adrenocortical mineralization					2

* - Diplococcus sp. isolated
** - lesions suggestive of measlesvirus infection

Organ System	Lesions	Etiology	Gibbons		Macaques	
			Primary	Secondary	Primary	Secondary
Nervous	Meningo-encephalitis	bacterial			2*	1
		parasitic		1		
Body as a Whole	Granulocytic leukemia		2			
	Septicemia	Unknown	2			
Musculoskeletal	Myositis	Sarcosporidiosis		1		
Undetermined - not completed			9		7	

* - Diplococcus sp. isolated

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Research

Work Unit 047, Metabolic diseases of man and animals

Literature Cited.

1. Reference

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2. Publications

Johnsen D.O., Wooding W.L., Tanticharoenyos P., Bourgeois C.H. Malignant lymphoma in the gibbon, 1971. Journal of the American Veterinary Medical Association, Vol. 159, No. 3, Pages: 563-566.

Shank R.C., Johnsen D.O., Tanticharoenyos, P., Wooding W.L., and Bourgeois C.H. Acute Toxicity of aflatoxin B. in the macaque monkey, 1971. Tox. & Appl. Pharm. 20:227-231.

Tanticharoenyos P. Preparing macaque monkeys for use as research animals. Proceedings of the 1971 International Committee on Laboratory Animals Asian Pacific Meeting. In Press.

Tanticharoenyos P. Evaluation of the gibbon (Hylobates lar lar) as a laboratory Primate - Use of Gibbons. Proceedings of the 1971 International Committee on Laboratory Animals Asian Pacific Meeting. In Press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6469	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SMSD'S INSTR'S	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A811	00	048
b. CONTRIBUTING				
c. XXXXXXXXX	CDOG 114(F)			

11. TITLE (Provide with Security Classification Code)

(U) Rickettsial Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 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 72	0.1	4.1
	CURRENT 73	0.1	6.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Provide DEAN if U.S. academic institution)

NAME: Altstatt, COL L. B.

TELEPHONE: 984-4523

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Grossman, LTC, R. A.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide 3 to 5 with Security Classification Code)

(U)Rickettsial Diseases; (U)Scrub Typhus; (U)Murine Typhus; (U)Leptothrombidium arenicola

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Provide brief technical paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To define the ecology of rickettsial diseases of military importance in Thailand.

24. (U) Disease occurrence in Thailand is determined by case detection and laboratory methods. The disciplines of clinical medicine, medical entomology, epidemiology, and rickettsiology are used to identify the various components of the ecosystem (e.g. vectors, hosts, reservoirs).

25. (U) 71 07 - 72 06 The execution of this project has now been largely assumed by the Thai Component and our role is one of providing advice and support.

Continue on reverse side if necessary and identify by number

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

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6470	72 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
71 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	3A062110A811	00	109
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE (Proceed with Security Classification Code)

(U) Psychiatry and Behavioral Studies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology; 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 72	1.9	86
	CURRENT 73	2.0	120

19. RESPONSIBLE DOD ORGANIZATION

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

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

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO
ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Altstatt, COL L. B.
TELEPHONE: 984-4523
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Firestone, MAJ M. H. DA
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Psychiatry; (U) Human Behavior; (U) Neurological Diseases; (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 study variables in a Southeast Asia environment that may adversely affect the performance of US military personnel, their dependents, and local nationals, principally through alterations in human behavior.

24. (U) American and Thai psychiatrists, working with trained technicians using systematic observation of human behavior and mental status testing, observe the impact of such diseases as Japanese encephalitis and indiscriminate drug usage upon the immediate and long-term performance of individuals in their natural environment or in some cases in an alien environment.

25. (U) 71 07 - 72 06 A one-year follow-up study of the acute and chronic effects of Japanese encephalitis in survivors is complete. Preliminary data suggest significant residual neurological and psychological deficits. Study of the patterns of drug abuse has been surveyed at a local high school for English speaking students. A long-term study of the patterns of drug abuse and an attempt to develop a predictive test of drug abuse for incoming Army personnel is presently in progress, with emphasis on influencing factors present in the Thai community. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* Available to contractors, upon publication approval.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 109 Psychiatry and behavioral studies

Investigators

Principal: Pien Cheovanich, M.D.; Marvin H. Firestone, M.D., MAJ, MC; Prof. Phon Sangsingkeo, M.D.; Robert J. Schneider, Ph.D., CPT, MSC; Pricha Singharaj, M.D.; Prof. Chira Sitasuwan, M.D.; Arvuth Srisukree, M.D.; Prathan Vutikul, M.D.

Associate: Robert Edelman, M.D., LTC, MC; Richard A. Grossman, M.D., LTC, MC; Suchinda Udomsakdi, M.D.

Assistant: Verl M. Lackey, SFC; Boonarb Panpanya, P.H.N.; Sukree Tumrongrachaniti, R.N.; Chinda Witayarut, P.H.N.

A Follow-Up Study of Japanese Encephalitis in Northern Thailand

Principal Investigators: Marvin H. Firestone, M.D., MAJ, MC
Prof. Phon Sangsingkeo, M.D.
Prof. Chira Sitasuwan, M.D.
Robert J. Schneider, Ph.D., CPT, MSC
Arvuth Srisukree, M.D.
Pien Cheova..ch, M.D.
Prathan Vutikul, M.D.
Pricha Singharaj, M.D.

Associate Investigators: Robert Edelman, M.D., LTC, MC
Richard A. Grossman, M.D., LTC, MC
Suchinda Udomsakdi, M.D.

Assistant Investigators: Boonarb Panpanya, P.H.N.
Chinda Witayarut, P.H.N.
Sukree Tumrongrachaniti, R.N.
Verl M. Lackey, SFC

OBJECTIVES AND DESCRIPTION: For a list of the objectives and description of the study see the previous annual report.

PROGRESS: Progress through the acute stage has been reported in the previous annual report. Complete data collection for the study was accomplished in October 1971. In the ensuing months, the date have been translated into English, and collated in a form compatible with automatic data processing. The data are presently undergoing analysis and should be completed, along with preliminary write-up in July 1972. Final write-up and publication is anticipated shortly thereafter.

SUMMARY: The Japanese Encephalitis project, as described in the 1971 annual report, is nearing completion. Data have been collected and are currently being analysed. Final write-up publication is anticipated during the first quarter of the next fiscal year.

A Study of the Interface between American Soldiers and the Thai Community and Its Effects on Drug Taking Behavior

Principal Investigators: Prof. Phon Sangsingkeo, M.D.
Marvin H. Firestone, M.D., MAJ, MC
Robert J. Schneider, Ph.D., CPT, MSC

Associate Investigators: Boonarb Panpanya, P.H.N.
Chinda Witayarut, P.H.N.
Sukree Tumrongrachaniti, R.N.

OBJECTIVES:

1. To determine what factors particular to relations with Thais appear to predispose toward use or non-use of illicit drugs by the American soldier population.

2. To determine what are the Thai attitudes toward illicit drug usage, and what factors relate to drug use by Thais.

3. To determine on an informal basis, what drugs are generally used by Thais and how this drug use affects American drug usage.

DESCRIPTION: This is an interview study of available and cooperating Thai entertainers, bar-girls, and "teerak" (rented wives) in the Sattahip area. In addition, families living in proximity to bangalows occupied by, or frequented by, American servicemen will be interviewed. Cultural factors influencing tolerance of drug abuse will be investigated, and local sources of drugs will be surveyed to determine which drugs are prescribed for various morbid complaints presented by local Thais. These should provide information bearing on the pressures mitigating against, or predisposing to, drug abuse among American soldiers living in the community. This study is being conducted, utilizing Department of Neuropsychiatry nurses administering semi-structured interviews. Individuals under study are contacted, primarily, through the facilities of the Kilo Sip Health Center, which is the local VD control unit financed by the U.S. military in the Camp Samae San - Utapao vicinity.

PROGRESS: Data collection began 15 January 1972. Questionnaires have been revised and refined. Data collection will terminate by the end of April, 1972. Ordering and analysis of the data for write-up will be completed during this fiscal year.

SUMMARY: Data are currently being collected which should provide information bearing on the influences of the local Thai community on drug abuse among American servicemen. Data collection will be completed at the end of April, 1972, with analysis to be completed shortly.

Prediction of Drug Abuse in Servicemen - Phase I

Principal Investigators: Marvin H. Firestone, M.D., Major, MC
Robert J. Schneider, Ph.D., Captain, MSC

Assistant Investigator: SFC Verl M. Lackey

OBJECTIVE: To develop an instrument which will aid in the prediction of chronic drug abuse among American soldiers in Thailand.

DESCRIPTION: Although the extent and prevalence of illicit drug use in Thailand remains essentially indeterminate, actions of armed forces command indicate its considerable concern with the problem of drug abuse. One important aspect of any complete drug abuse program for the military should be the isolation of high risk individuals prior to their assignment to areas where drugs of abuse are easily obtained. To differentiate individuals into "high risk" or "low risk" categories requires the knowledge of the etiology of and the predisposition to drug abuse, and a description of how the chronic drug user differs from the non-user. Knowledge of the influencing and etiological factors could provide the information necessary for categorizing individuals as indicated.

The available literature concerning drug abuse indicates that there has been no attempt to empirically differentiate the chronic or heavy user from the non-user of amphetamine, barbiturate, marijuana, hallucinogen, and heroin, for prediction of chronic drug use. Many studies have, however, suggested that there may be important ways in which these individuals differ. Only two available studies have presented statistically derived "profiles" of the drug user. Both studies utilized factor analysis and suggested that there might be a "general orientation toward drug use" or a "general drug-taking predisposition." The isolation of a series of factors which differentiate the chronic or heavy drug user from the non-user could aid in the prediction of the likelihood of becoming a chronic drug abuser. A finding of such a differentiation must, however, include a follow-up study of labeled individuals to assess accuracy of prediction.

The present study is a structured interview study of a population of soldiers, made up of chronic drug users and non-drug users. Those questions whose answers correlate with chronic or heavy drug use (e.g., are answered differently by the chronic user and non-user) will

be isolated. These questions will be combined into a final questionnaire which, in a future study (Phase II), will be administered, along with urinalyses, to an incoming population of troops. In the follow-up study, a prediction will be made for each soldier of the probability that he will chronically abuse certain drugs during his tour in Thailand. He will be followed for one year during his stay in Thailand to assess the accuracy of the predictions. All questions will be selected via statistical procedures, based on the present study.

If no statistically valid differentiation between chronic drug user and non-user is found, then Phase II will consist of a prospective long-term cohort study of incoming troops, with the emphasis on monitoring their behavior vis-a-vis drug usage, with the same psychological test battery administered early in their Thailand tour, at critical periods during their tour, and at the end of their one-year tour.

PROGRESS: Starting 1 January 1972, interviewing was begun. It is expected that the data collection phase will terminate by the end of August 1972, and the final questionnaire will be completely formulated in September. At this time, incoming troops will be surveyed, predictions made, and the follow-up begun.

SUMMARY: A method to predict drug use in a soldier population is described. This involves developing a series of questions which are answered differently by the drug user and non-user, and assuming that anyone who responds like a drug user has high probability of using drugs during his tour.

Data collection has begun; this study will be completed in September, 1972. The success of this study will determine whether such a method of prediction can be utilized in a planned future study of prediction of chronic drug abuse.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 109 Psychiatry and behavioral studies

Literature Cited

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6463	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. OVRD'N ILSTR'N	8B. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A811	00	310
b. CONTRIBUTING				
c. SUPPORTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Etiology of Infectious Hepatitis (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 003500 Clinical Medicine

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

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 72	1.5	35
	CURRENT 73	1.5	35

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Ginsberg, MAJ A. L. DA
NAME:

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, preceded by 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 diseases.

24. (U) Methods for the detection of Australia antigen and antibody were evaluated. These methods were utilized to study various populations of soldiers and to ascertain if gamma globulin prevented serum and infectious hepatitis.

25. (U) 71 07-72 06 Radioimmune assay methods for the detection of Australia antigen and antibody were found to be significantly more sensitive than available methods. The prevalence of antigen and antibody in recruit blood donors was 1 and 5 percent respectively. A 20 percent prevalence of antibody was found in soldiers arriving in Korea and a 30 percent prevalence upon departure. Gamma globulin prophylaxis was shown to be effective in the prevention of both serum and infectious hepatitis. Some evidence was found that suggested the titer of Australia antibody was not the important factor in providing protection against serum hepatitis. For technical reports see Walter Reed Army Institute of Research Annual Progress Reports, 1 July 71 - 30 Jun 72.

* Available to contractors upon military'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 3A062110A811, MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 310, Etiology of Infectious Hepatitis

Investigators.
Principal: COL Marcel E. Conrad, MC
Associate: MAJ Allen L. Ginsberg, MC

Description.

New methods of testing serum specimens for Australia antigen (HBAg) and antibody (HBAb) were evaluated to ascertain if they were more sensitive and reliable for use in blood banks. A sensitive method was found and was used to study serum specimens obtained from patients with hepatitis in a recently performed double blind study among soldiers in Korea to evaluate whether human serum gamma globulin was effective in the prevention of both serum and infectious hepatitis. This method was modified to permit the detection of various subtypes of HBAg. Studies were initiated to ascertain if the titer of HBAb in gamma globulin preparations was important in the prevention of serum hepatitis. Studies have been initiated in various military populations to quantify the prevalence of both HBAg and HBAb.

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.

A simple radioimmune assay method was utilized and evaluated to detect Australia antigen (HBAg) in serum specimens from normal military blood donors and soldiers with endemic viral hepatitis. This method detected more than twice the number of HBAg positive specimens among both groups of subjects than by other available methods. Furthermore, it detected all positive specimens found by agar gel diffusion, immunoelectroosmophoresis, complement fixation, and passive hemagglutination inhibition methods. The radioimmune assay method showed that about one percent of screened military blood donors and twenty-five percent of soldiers with icteric endemic hepatitis had HBAg in

their serum. In these patients with hepatitis, we had previously shown that the prophylactic administration of human serum gamma globulin decreased the incidence of both HBAg positive and negative endemic icteric hepatitis. The utilization of the radioimmune assay method provided convincing evidence that an injection of gamma globulin could protect against HBAg positive icteric endemic hepatitis for about six months. In collaboration with WRGH, a study has been initiated to ascertain if gamma globulin will provide protection against transfusional hepatitis and if the HBAb titer of the gamma globulin is a factor in providing protection. This study is being performed in patients undergoing cardiac bypass surgery. These patients receive either a placebo injection or an inoculation of a gamma globulin with a high titer of HBAb or low titer of HBAb.

Australia antigen (HAA) can be typed as ad or ay by agar gel diffusion (AGD) by showing precipitin lines of identity and spurs of partial identity with specimens of known subtype. Since only 50% of HAA specimens detected by complement fixation and 20% of specimens detected by radioimmune assay (RIA) are positive by AGD, only a small proportion of all HAA positive sera can presently be typed. We have used a solid phase RIA (Ausria- Abbott Laboratories) to type HAA specimens that are negative by AGD. The RIA is performed using a pool of guinea pig anti-HAA labeled with I-125 and polypropylene test tubes coated with nonradioactive anti-HAA. The steps in the procedure are as follows: Antigen containing serum is added to the antibody-coated tube and incubated for 16 hours. The contents are aspirated, the tube washed with a tris buffer, and I-125 labeled anti-HAA is added. After incubation at room temperature for 1½ hours, the contents are aspirated, the tube is again washed, and the radioactivity of the tube is quantified. The antibody used to coat the tube as well as the iodinated antibody contained more anti-ad activity than anti-ay activity. Known ay and ad specimens were serially diluted in normal human serum and assayed. Different levels of tube saturation and different dilution curves for the two major types of HAA could be demonstrated when counts per minute (cpm) were plotted against dilution. Twenty-four sera previously typed by AGD were coded, and the level of tube saturation (cpm) and the pattern of the dilution curves were delineated. Using this method, all 24 specimens were accurately typed. Sera from 52 American soldiers stationed in Korea who developed HAA positive icteric hepatitis were then examined. Only 11 were positive by AGD, of which only 7 (13%) could be typed. In contrast, all 52 were positive and 33 (64%) could be typed by RIA. Twenty-eight (54%) were ad, 5 (10%) were ay, and 19 (36%) could not be typed. Solid phase RIA methodology allows the typing

of many HAA positive specimens that cannot be typed by other methods.

A simple method utilizing solid phase radioimmune assay was used to detect antibody to Australia antigen (HBAb). The method was found to be approximately ten times as sensitive as the hemagglutination method and as sensitive as technically more difficult radioimmunoprecipitation methods for detection of HBAb. It shows that about five percent of screened military blood donors in recruit populations have HBAb. Troop populations arriving in Korea have an HBAb prevalence of 20 percent upon arrival and about 30 percent upon completion of their military tour.

In collaboration with Dr. Joseph Beggs of Northwestern University, plasma has been obtained from volunteers in the Illinois State Penitentiary who developed hepatitis after oral administration of serum from the MS-1 Willowbrook pool. This plasma has been shown to be infectious in subsequent volunteer experiments. The proven infectious material has been provided to laboratories attempting to isolate the etiologic agent of infectious hepatitis. In addition, these specimens are distributed to investigators attempting to develop an antigen-antibody system for infectious hepatitis similar to HBAg-HBAb and for animal experiments used to develop a system that will replace human volunteer studies. Double blind studies performed in collaboration with Presbyterian-St. Luke's Hospital in Chicago indicate that the latter goal is feasible with certain species of marmosets.

Conclusions and Recommendations.

A simple solid phase radioimmune assay method was found to detect at least twice as many carriers of HBAg than currently used methods. Improvements in this method are needed to make it more practical for blood banking. Using this method and serum specimens from a double blind study in Korea, we were able to show that gamma globulin prophylaxis prevents the occurrence of endemic icteric HBAg positive and negative hepatitis. The finding of an increased titer of HBAb in the lots of gamma globulin used in the Korean study suggest that it may be the HBAb that provides protection in the HBAg positive hepatitis. Further studies are needed to ascertain if this hypothesis is correct and if lots of gamma globulin can be made that will provide protection against transfusional hepatitis. A sensitive method for subtyping HBAg was developed. The prevalence of HBAb in various troop populations is being quantified. An experimental animal model for HBAg negative hepatitis was found.

Project 3A062110A811, MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 310, Etiology of Infectious Hepatitis

Literature Cited.

Publications.

1. Conrad, M.E.: Prevention of HAA positive hepatitis with serum gamma globulin. New Eng. J. Med. 285: 1486, 1971.

2. Conrad, M.E.: Drugs and hepatitis among soldiers. New Eng. J. Med. 286: 1111, 1972.

3. Conrad, M.E.: Endemic viral hepatitis in U.S. soldiers: Causative factors and the effect of prophylactic gamma globulin. Canad. Med. Assoc. J. 106: 456, 1972.

4. A Cooperative Study. Prophylactic gamma globulin for prevention of endemic hepatitis. Effects of U.S. gamma globulin for prevention of viral hepatitis and other infectious diseases in U.S. soldiers abroad. Arch. Intern. Med. 128: 723, 1971.

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PROJECT 3A062110A821
COMBAT SURGERY

Task 00
Combat Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6466	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GDSS'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	8c. LEVEL OF SUM
71 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	3A062110A821	00	120
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(F)			

11. TITLE (Precede with security classification code)

(U) Wound healing of injuries incurred in military operations

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	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 PREVIOUS 72	2.5	125
FISCAL YEAR CURRENT 73	3.0	150

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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NAME: Burleson, MAJ R.; Rosenthal, MAJ R. D.

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with security classification code) (U) Immune Mechanisms; (U) Enhancement; (U) Rejection; (U) Intraocular Foreign Body; (U) Copper; (U) Abscess; (U) Combat Injury

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

23 (U) Better understanding of the immune mechanisms involved in the rejection of allografts and foreign bodies. The ultimate goals are enhancement of skin and bone grafts in combat casualties, clinical acceptance of renal transplantation in the Army Transplant Program, a more successful method of identifying the existence and location of abscesses, especially intraabdominal, better criteria for management of retained intraocular foreign bodies, delineation of the pathophysiology of chloroquine retinopathy.

24 (U) Utilizing the uptake of tritiated thymidine as an indication of DNA synthesis (lymphocyte reactivity) in one-way mixed lymphocyte cultures, a large variety of experiments are underway. Models include transplanted skin, kidney and heart, the latter two in the neck. A variety of antilymphocyte sera are being studied during the course of unmodified rejection in the dog. Utilizing a radiographic scanning technique, a method is being evolved to study localization of various isotopes in the presence of abdominal wall abscesses in rabbits. Copper fragments of various composition are being studied in the rabbit vitreous. The rhesus retina is being examined following daily administration of chloroquine in high doses.

25 (U) 71 07 - 72 06. Fractionation of specific antisera has shown components associated with enhancement. Gallium 67 has been shown to localize in white cells of abdominal wall abscess sufficiently greater than in liver or spleen to be of potential clinical application. Both penicillamine and steroids have been shown to be of questionable practical value in the management of copper fragments in the vitreous. Membranous cytoplasmic bodies similar to those seen only in sphingolipidosis have been observed in the retina of monkeys given chloroquine, thereby affording a clue to the pathophysiology of these diseases. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

(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 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120 Wound healing of injuries incurred in military operations

Investigators.
Principal: COL Robert T. Cutting, MC
Associate: MAJ A. Ralph Rosenthal, MC; MAJ Richard L. Burleson, MC; LTC Brack G. Hattler, MC

1. Prevention of the Inflammatory Response to Intraocular Copper Foreign Bodies.

a. Statement of the problem: To prevent the inflammatory reaction which occurs as a response to the presence of a copper foreign body (CFB) in the vitreous cavity.

b. Background: We have been working on problems concerning the natural history, diagnosis (using atomic absorption spectrophotometric analysis of the aqueous) and therapy of retained intravitreal CFB. We observed that placing either a pure or alloyed CFB against the wall of the vitreous cavity results in an intense inflammatory response with eventual encapsulation within 1 week. It was the purpose of this year's work to attempt to prevent this inflammatory response.

c. Approach to the problem: Two different approaches were taken. The first was to examine the effect of subconjunctival steroid administration on the inflammatory response. The second was to study the response to different alloys of copper.

In the steroid studies, either a pure or alloyed CFB (60% copper, 40% zinc) was inserted against the wall of the vitreous cavity in both eyes of albino New Zealand rabbits. Decadron was administered subconjunctivally to one eye immediately postoperatively and on a daily basis for 8 days in three different doses: .6, 1.2, and 2.4 mgm/day. A control group was given saline subconjunctivally to one eye. Each day the response was observed by indirect ophthalmoscopy.

Different alloys of copper were then investigated to ascertain if the inflammatory response would be altered. The alloy of 90% copper and 10% zinc was used as the control as this was the type of alloy we have studied for 2 1/2 years. The alloys of 90% copper and 10% tin or 10% nickel were compared to the zinc alloy.

d. Results and discussion: In the steroid studies, encapsulation of the CFB was prevented to the same degree in both treatment and control groups regardless of dose of steroid or type of CFB. Usually if the injected eye became encapsulated within 9 days the non-injected eye demonstrated a similar response. The converse was also true.

However, there were occasions when the injected eye became encapsulated while the non-injected eye did not. We, therefore, felt that the subconjunctivally administered steroid was having a systemic effect. Analysis of the effects of the 3 steroid doses reveals the smallest dose studied carried a significant retardation in the percent totally encapsulated in both pure and alloyed CFB. There was a suggestion that increasing doses of steroids over the range studied had increasing effects. In almost every instance when the drug was discontinued the foreign body regardless of composition became totally encapsulated within 1 week.

Steroids were observed to delay the appearance of inflammation in both pure and 60% CFB. When the drug was discontinued inflammation was present within 48 hours regardless of the type of foreign body used. Steroids were no more effective in preventing inflammation in the less reactive alloy than in the more reactive pure copper. Their only significant effect appears to be the slight delay in the appearance of the inflammatory response and in the rate of encapsulation. Steroid therapy does not seem to be able to totally prevent chemical inflammation.

The alloy studies showed that the copper-tin alloy and the copper-nickel behaved exactly like the copper-zinc alloy in its ability to elicit an intense inflammatory response, and the nickel and tin alloys behaved similarly to each other. Hence there appeared to be no difference in the response to the alloyed CFB if the second major component is 10% zinc, tin, or nickel.

e. Conclusions: Steroids of varying dosages administered daily via the subconjunctival route appeared to behave systemically, having similar effects in both the injected and non-injected eyes regardless of the type of foreign body used. Nine days of steroid therapy prevented a significant number of CFBs from becoming totally encapsulated during that time. In addition, the onset of the inflammatory response was delayed and occasionally completely suppressed over the course of therapy.

Alloys of 90% copper and either 10% tin, nickel or zinc were compared to each other and were noted to have similar rates of encapsulation and a similar time course for the appearance of the inflammatory response.

f. Recommendations: Further studies on the natural history, diagnosis and therapy of non-ferrous foreign bodies should be undertaken in the primate vitreous. Finally, a referral center for the management of intravitreal foreign bodies should be considered within the military hospital system.

2. Scintigraphic Detection of Experimental Abscesses.

a. Statement of the problem: Wounded soldiers often develop fevers and the question of intraabdominal abscess arises. A technique

to radiographically detect such abscesses would be an incalculable aid to diagnosis.

b. Background: In the past, multiple approaches to scintigraphic localization have not met with success. New radionuclide and leukocyte labelling techniques permit reassessment of the problem.

c. Approach to the problem: Several methods are being evaluated including the intravenous administration of various isotopes and infusion of labelled autologous leukocytes and tagged hard albumin spherules.

d. Results and discussion: A standard abscess has been produced in the rabbit anterior abdominal wall. Gallium 67 and pertechnitate have been infused. Scintigraphic techniques have been worked out which show that gallium especially shows considerable promise in localizing abscesses. Abscesses at various stages are being studied. A technique for labelling leukocytes with 51-chromium, 67-gallium and 99-technetium has been worked out. It is expected that these studies will have clinical application within 6 months.

The study is continuing.

3. In Vivo and In Vitro Properties of Sensitized Lymphocytes in Transplantation Immunity.

a. Statement of the problem: Although the exact role of the lymphocyte in allograft rejection is not known, a close correlation has been established. Following a lag phase of several days after antigenic stimulation lymphocytes can be found in increasing concentration at the allograft site. Contact between the lymphocyte and its target appears necessary for cell death and under these conditions rejection of the allograft follows.

b. Background: Mechanisms for lymphocyte mediated cell death are not known but various possibilities have been considered and include: (1) a release of cell bound antibody, (2) local component production, (3) damage by enzymes, and (4) the production of other cytotoxic or recruiting factors by activated lymphocytes at the graft site. The latter possibility has attracted particular attention recently with the demonstration of various factors produced by lymphocytes in vitro. Thus, macrophage inhibiting factor (MIF), blastogenic factor, cytotoxic factor, chemotactic factor, and various potentiating factors have all been demonstrated in vitro and postulated to occur in vivo. The one obstacle that has hampered a correlation between the in vitro and the in vivo setting has been the inability to isolate "specifically sensitized lymphocytes." We have recently developed a technique of isolating these cells from the target organ that appears promising.

c. Approach to the problem: Lymphocytes and lymphoblasts were isolated from rejecting canine kidney allografts and studied in vivo and in vitro in comparison with peripheral circulating lymphocytes.

d. Results and discussion: Following a lag phase of several days after antigenic stimulation, lymphocytes can be found in increasing concentration at the allograft site. Contact between the lymphocyte and its target appears necessary for cell death and under these conditions rejection of the allograft follows. Mechanisms for lymphocyte mediated cell death are not known, but various possibilities have been considered and include: (1) release of cell-bound antibody; (2) local complement production; (3) damage by enzymes; and (4) the production of other cytotoxic or recruiting factors by activated lymphocytes at the graft site.

We have developed a method of density gradient separation of activated lymphocytes from an organ undergoing rejection. The model used has been the kidney in the dog. These lymphocytes have been shown to actively produce chemotactic factor. These cells extracted from the rejecting kidney have also been shown to be actively stimulated and to continue to proliferate without any further contact with antigen. This is particularly interesting in view of the fact that at the time that this activity can be shown in the "kidney lymphocytes" no activity can be shown in lymphocytes extracted from peripheral blood. It is felt therefore that the kidney acts as an area for local entrapment of the specifically sensitized cells and, in addition, actively secretes substances that recruit other phagocytes and lymphocytes into the area to assist in rejection of the organ.

The study is continuing.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120 Wound healing of injuries incurred in military operations

Literature Cited.

Publications:

1. Miller, J., Hattler, B.G., Jr., and Johnson, M.C.: The lymphocyte culture and renal allograft rejection. Fed. Proceedings 30: 455, 1971 (Abs).

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3. Miller, J., Hattler, B.G., Jr., Currier, C.B., Jr., Johnson, M.C., and Alexander, J.L.: Canine mixed lymphocyte culture during renal allograft rejection. Surg. Forum 22: 216, 1971.

4. Hattler, B.G., Jr., Karesh, C., and Miller, J.: Inhibition of the mixed lymphocyte culture response by antibody following successful human renal transplantation. Tissue Antigens 1, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6467	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GRP'S INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A821	00	121
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Responses to trauma resulting from military operations

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008800 Life Support 016200 Stress Physiology

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

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 72	3	75
FISCAL YEAR CURRENT 73	4.5	125

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Stress Ulcer; (U) Shock; (U) Ulcerogenesis; (U) Ion Transport; (U) Splanchnic Hemodynamics; (U) Combat Injuries

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

23 (U) Elucidate the pathogenesis of irreversible shock. Study the pathophysiology of stress ulcer, a complication that causes massive gastrointestinal bleeding in 3% of severely wounded with a 40% mortality.

24 (U) Study mesenteric hemodynamics in endotoxin shock with emphasis on species variation. Search for a better model for stress ulcer. Study ion transport across the gastric mucosa and the effects of metabolic inhibitors and hemorrhage on gastric mucosal permeability.

25 (U) 71 07 - 72 06. Intravenous endotoxin given to baboons caused profound hypotension but without changes in mesenteric blood flow or portal pressure as opposed to the dog. Other differences were observed in splenic and hepatic arterial flow and in the response to hemorrhage. Silicone rubber injection techniques confirmed microvasculature architectural differences between species, further questioning the visceral target organ theory of shock. A model has been developed which characterizes directional movements of several ions, their substrate requirements and transport. The gastric barrier to hydrogen ion has been better defined. A sensitive index of mucosal integrity, the electrical potential difference, was monitored together with ionic fluxes before and after a series of metabolic inhibitors and showed that neither extramitochondrial electron transport nor carbonic anhydrase are important constituents of the normal barrier but that intact pathways for glycolysis and AT. formation may be critical in maintaining the normal barrier.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

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Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Responses to trauma resulting from military operations

Investigators.
Principal: COL Robert T. Cutting, MC
Associate: LTC Kenneth G. Swan, MC; LTC Wallace P. Ritchie, Jr., MC; MAJ David G. Fromm, MC

1. Comparison of Mesenteric Hemodynamics in the Baboon and Dog Following Hemorrhagic and Endotoxin Shock.

a. Statement of the problem and background: The splanchnic vasculature has been implicated in the pathogenesis of the irreversible phase of shock. The endogenous catecholamines released by hypotension reputedly cause an intense constriction of these vascular beds and lead to ischemia and ultimate tissue necrosis. A series of studies has been completed that question these traditional concepts.

b. Approach to the problem: Mesenteric hemodynamics were studied in baboons subjected to endotoxic shock and the results compared to the classical canine responses as described above.

c. Results and discussion: The intravenous injection of endotoxin (LD_{80}) caused profound hypotension without a change in either mesenteric blood flow or portal pressure. Thus, there is an obvious species difference between canine and subhuman primate responses to endotoxin. A similar study was done in which hemorrhage was employed to reduce arterial pressure. When baboons were hemorrhaged to an extent that would be lethal to dogs within 24 hours of reinfusing the shed blood, the mesenteric blood flow fell, the gut was ischemic, but the animals survived. The effects of endotoxin on hepatic and splenic arterial blood flow were studied in baboons. The results revealed no change in blood flow to the liver whereas splenic blood flow fell in parallel with arterial pressure.

The study is continuing.

2. Vascular Architecture of the Mesenteric System.

a. Statement of the problem: Since species differences have been demonstrated between canine and subhuman primate models of experimental shock, the question arises as to whether there are anatomical differences in the target organ or small intestine.

b. Background: The dog, but not the monkey, responds to experimental shock regardless of its etiology with ischemic damage to the small bowel. If one compares the hemodynamics of the mesenteric circulation in the two species, one notes that there is a marked decrease in the flow of blood through the intestine of the dog, but not the monkey, during the early phase of shock. This may represent a species difference in terms of the response of the microcirculation in the intestine of the two animals to hypotension.

c. Approach to the problem: The silicone-rubber injection technique was used to describe the effects of endotoxin on canine and subhuman intestinal microvascular architecture.

d. Results and discussion: Endotoxemia causes the following vascular changes in the canine mucosa: a 50% shortening of villi; coiling of subepithelial capillaries and core vessels; congestion of subepithelial capillaries; and hemorrhage through core vessels as evidenced by rubber extravasation. These changes might explain the increased mesenteric vascular resistance and decreased mesenteric blood flow characteristic of canine endotoxemia. The mucosa of baboons treated in the same manner revealed evidence of dilation of subepithelial capillaries, a change that might contribute to maintenance of mesenteric blood flow during endotoxin shock. Epinephrine was observed to cause mesenteric dilation in baboons when infused at low dosage. The same dose causes constriction in the dog mesentery. The study suggests that the elaboration of blood epinephrine during hypotension could cause the vasodilation seen in baboons. Similar studies were conducted on dogs and baboons subjected to hemorrhagic shock. There was no change in mucosal vascular architecture in either species after four hours of hypovolemia. One and one half hours after the shed blood was reinfused, there was evidence of hemorrhage into the cores of the villi in the dog studies. However, there were no changes observable in the baboons studied. This observation might explain in part the survival of baboons and the death of dogs when subjected to experimental hemorrhagic shock.

e. Conclusions: The above series of studies on the pathophysiology of shock in baboons has seriously questioned the concept of the splanchnic viscera being target organs in shock and has demonstrated specific species differences in splanchnic hemodynamics between canine and subhuman primate models of shock.

3. Effects of Metabolic Inhibitors on Gastric Mucosal Permeability in the Dog.

a. Statement of the problem: Hemorrhage from stress ulcers occurs in 3% of moderately and severely wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be ten fold higher.

b. Background: Disruption of the gastric mucosal barrier to Na^+-H^+ exchange has been postulated to be the fundamental defect preceeding the development of stress ulcer disease in man.[1] The subcellular components of this barrier are poorly understood, however.

c. Approach to the problem: A sensitive index of mucosal integrity, the electrical potential difference (PD) was monitored and the net fluxes of H^+, Na^+, K^+, Cl^-, and H_2O were assessed in denervated canine fundic pouches using a standard acid test solution before and after (1) topical sodium iodoacetamide (IOA), which in vitro at this dose inhibits aerobic glycolysis at the 3-phosphoglycerablehyde dehydrogenase level; (2) topical 2,4 dinitrophenol (DNP); (3) sodium triocyanate (SCN), which in vitro inhibits electron transfer at the extramitochondrial cytochrome level beyond the point of elaboration of high energy precussors; and (4) acetazolamide (AZM), a specific carbonic anhydrase inhibitor. Each agent caused "inhibition" of histamine stimulated acid secretion in vivo. Each agent = 30 dogs. The results were compared to those obtained with a known barrier disrupter, topical acetylsalicylic acid (ASA).

d. Results and discussion:

Flux rates and PD (± SEM)/30 min/100 cm^2 mucosal area

	Control	ASA	IOA	DNP	SCN	AZM
PD (mV)	-48±7	-12±3*	-21±4*	-18±5*	-50±6	-52±5
Net H^+ (μEq)	-178±28	-178±48*	-444±24*	-394±45*	-189±24	-206±54
Net Na^+ (μEq)	+295±45	+657±65*	+494±46*	+452±68*	+249±50	+247±26

*p = or < 0.05 versus control

Thus, IOA and DNP disrupt the mucosal barrier whereas SCN and AZM do not.

e. Conclusions:

(1) Neither extramitochondrial electron transport nor carbonic anhydrase are important consituants of a normal barrier.

(2) Intact pathways for glycolysis and ATP formation may be critical in maintaining mucosal integrity.

4. Effect of Hemorrhage on Gastric Mucosal Permeability in the Subhuman Primate.

a. Statement of the problem and background: The concept that stress ulcer disease in man is related to excessive back diffusion of H^+ is based in part on the demonstration of increased Na^+ and H^+ exchange across the gastric mucosa of the rabbit during hemorrhagic shock and following transfusion of shed blood.[1]

b. Approach to the problem: The above findings were reassessed in an animal phylogenetically closer to man, the Macaca mulatta. Under phencyclidine HCl anesthesia, the transmural electrical potential difference (PD) was continuously monitored and the net fluxes of H_2O, H^+, K^+, and Na^+ were assessed using a standard acid test solution in denervated fundic pouches and innervated whole stomachs during (1) a control period; (2) following 2.5 hours of hemorrhagic shock (BP art. = 37 ± 2 mm Hg); and (3) 2.5 hours after reinfusion of shed blood. The results were compared to those obtained following the topical application of a 20 mM sodium taurocholate solution, a known barrier disrupter.

c. Results and discussion:

Flux rates and PD (± SEM)/15 min/50 cm^2 mucosal area

	Control	Hemorrhage	$\bar{p}$ Transfusion	$\bar{p}$ Taurocholate
PD (mV)	-49±2	-52±2	-55±5	-23±4*
Net H^+ (μEq)	-140±19	-136±19	-175±22	-409±42*
Net Na^+ (μEq)	+137±17	+122±13	+124±17	+342±34*

*p = or < 0.05 vs control

The innervated whole stomachs demonstrated essentially similar results.

d. Conclusions: These data indicate that in the subhuman primate, neither hemorrhage of the magnitude indicated nor subsequent transfusion of shed blood are per se associated with significant alterations in gastric mucosal permeability. Thus, if excessive "back diffusion" of H^+ is a principal cause of stress ulcer disease in man, factors other than hypovolemia are responsible.

5. Ion Transport Across Gastric Mucosa.

a. Statement of the problem and background: Stress ulcer and gastric ulcer are associated with a breakdown in the mucosal barrier to hydrogen ion. Currently it is believed that gastric ulcer frequently results from the back diffusion of H^+, but the cause and effect relationship for stress ulcer is less clear. The majority of investigations dealing with the gastric barrier have dealt with the demonstration that various agents disrupt the barrier. However, very few studies have studied the properties of passive permeability ("the barrier") in a systematic manner.

b. Approach to the problem: An in vitro model has been developed for the study of the mucosal barrier and functions of the gastric mucosa in general. This system allows strict control of factors which affect gastric function and allows confident distinction between active and passive ionic movement across the mucosa. Electrical and metabolic

parameters of gastric mucosa can also be monitored in this system. Since there is little precedence for the study of mammalian gastric mucosa in vitro, the initial thrust of this study was designed to measure function, capabilities and factors affecting function of the in vitro preparation. As a result of these studied in vitro model for the study of the barrier has also been developed.

c. Results and discussion: A very durable and reliable in vitro model correlates very well with in vivo studies. Ion transport processes for H^+, Na^+, and Cl^- have characterized in terms of directional movement, substrate requirements and factors which influence their transport. The basic mechanisms of acid production have also been characterized. Normal permeabilities of the luminal and serosal borders have also been determined. While these studies as yet have no direct bearing on ulceration, they have enhanced our knowledge of basic processes of the gastric mucosa and have led to the development of a sensitive model for the study of back diffusion. Thus far, 3 agents known to disrupt the barrier have been studied. Some heretofore unknown effects of Na salicylate, acetyl salicylic acid and Na taurocholate have been found. While all of these agents disrupt the barrier they have different effects on the rates of acid secretion, and sequence of metabolic events leading to barrier disruption.

d. Conclusions: A model which allows definition of the gastric barrier to H^+ has been established and will allow definition of metabolic factors affecting the barrier and gastric secretion of acid in general.

e. Recommendations: The present investigation has opened new areas for the study of functions of the gastric mucosa which should be actively pursued. Further definition of information which has become evident as a result of this study, will eventually allow direct application to clinical problems associated with stomach. Extension of the present project will hopefully be able to pinpoint the biochemical events leading to stress ulcer of the stomach.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Responses to trauma resulting from military operations

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6468	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM / A. WORK UNIT
71 07 01	D Change	U	U	NA	NL	☒ YES ☐ NO	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A821	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

Anesthesia and pulmonary complications of combat injury (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002400 Bioengineering 012900 Physiology 008800 Life Support

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
58 05	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 72	4	100
FISCAL YEAR CURRENT 73	5	125

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS

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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Oxygen Toxicity; (U) Tissue Oxygenation; (U) Pulmonary Insufficiency; (U) Intravascular Microaggregation; (U) Stored Blood; (U) Combat Injury

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 in depth several factors that contribute to or result from defective tissue oxygenation and blood supply following combat injury and shock, such as (a) pulmonary insufficiency and oxygen toxicity, (b) alterations in the oxygen transport characteristics of stored blood, (c) the microaggregates of leukocytes and platelets that develop in stored blood and in vivo following shock and trauma, and (d) the contribution of aspiration, liver damage, hemorrhage, and stress on pulmonary bactericidal activity.
24 (U) The Model T Coulter Counter has been developed to measure the size distribution of particles which develop in blood during a variety of insults plus storage. Experimental models have been developed with the Channing Laboratory of the Boston City Hospital to evaluate pulmonary host defense mechanisms following shock, dehydration, acidosis, aspiration, liver damage, and other influences.
25 (U) 71 07 - 72 06. Tracheal instillation of hydrochloric acid in rats showed minimal changes in arterial oxygen tension, shunting, x-rays, and histology, but decreased bacterial inactivation; changes were greater with blood as an instillate. Alterations in platelet function induced by shock, ATP, prostaglandin E1 and adenosine have been quantitated. Platelet aggregates break up and reform following 40 μ pore filtration but are removed by Dacron wool. Light and electron microscopy showed pulmonary structural changes following common bile duct division which were accompanied by impaired bactericidal activity and depressed oxygen consumption. Wire mesh restraint, acute blood loss, dehydration but not starvation were shown to reduce intrapulmonary inactivation of labeled S. aureus.
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

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Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122 Anesthesia and pulmonary complications of combat injury

Investigators.
Principal: COL Robert T. Cutting, MC
Associate: MAJ Robert T. Solis, MC; LTC John F. Mullane, MC; LTC Edgar O. Yhap, MC; MAJ Laurence J. Krenis, MC; Gary Huber, MD*; CPT Neven A. Popovic, VC

1. Quantitative Studies of Microaggregation in vivo and in vitro

a. Statement of the problem: Embolization of microaggregates of platelets and white cells formed in blood both during storage and after shock and trauma is thought to play an important etiologic role in organ dysfunction following trauma (Swank 1967).[1]

b. Background: Quantitation of microaggregates in vivo has previously been limited to the use of indirect methods such as the screen filtration pressure technique and microscopic observations. Previous work in this laboratory has utilized the Model T Coulter counter to measure the size distribution of particles in stored blood.

Recent work in our laboratory has attempted to modify the Model T procedure to measure acutely induced platelet aggregates in whole blood both in vitro and in vivo.

c. Approach to the problem: Initial work consisted of in vitro studies of the effects of dilutions, anticoagulants, saponin, calcium and inhibitors of platelet aggregations in plasma and in whole blood on the measurement of the size distribution of platelet aggregates. Subsequently, the methods developed were used to measure platelet aggregates induced by hemostasis, infusion of adenosine diphosphate and thrombin. In other studies the filtration characteristics of acutely induced platelet aggregates were compared to those of the microaggregates in stored blood.

d. Results: These studies have shown that the methods utilized can provide quantitative data on the in vivo aggregation of platelets. Quantitative studies of the effects of inhibition of platelet aggregation such as prostaglandin E_1 and adenosine have shown that the method detects alterations in platelet functions. The filtration studies show that platelet aggregates break up with and reform after 40 μ pore mesh filtration but are completely removed by Dacron wool filtrations.

e. Conclusion: These studies provide the first quantitative measurements of platelet aggregates. The use of this technique should

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be of value in correlating in vitro studies of platelet aggregations with in vivo observations. The immediate application of the methods will most likely be in cardiac surgery, where platelet aggregate embolization is thought to be important in the etiology of organ dysfunction following surgery (Ashmore 1972).[2]

2. Liver Damage and Stress Ulcer Formation

a. Statement of the problem: Does septic jaundice influence stress ulcer formation?

b. Background: Multiple abnormalities, such as head trauma, sepsis, pulmonary insufficiency, and acute renal failure, may be present in the patient developing stress ulcers. Recently septic jaundice has also been associated with stress ulcer formation. Extrahepatic sepsis affects both the histology and biochemical function of the liver. Sepsis may also affect liver histology and biochemical function without the development of jaundice. Since chronic liver damage is associated with an increased incidence of gastric erosions and ulcers, abnormal liver function associated with sepsis may be an important variable contributing to stress ulcer formation.

c. Approach to the problem: We investigated the effect of restraint stress on the rat with experimental biliary obstruction. Biliary obstruction in the rat produces both jaundice and marked histological abnormalities of the liver. Since part of the effect of liver damage on the stomach may be due to gastric congestion, splenic weight, portal pressure, gastric wet and dry weight, gastric oxygen consumption in vitro, and gastric luminal pO_2 equilibration were measured. Vagotomy and pyloroplasty were also combined with the common bile duct ligation and division to determine if this procedure would reduce the incidence of observed gastric lesions.

d. Results and discussion: Acute stress superimposed on liver damage secondary to biliary obstruction was associated with an increased number of erosions and ulcers in the acid-producing area of the stomach of rats. Vagotomy and pyloroplasty reduced, but did not abolish, the increase in gastric lesions. The stomach was congested from an increase in portal venous pressure and had an impaired oxygen delivery as reflected by the decreased gastric luminal pO_2. The tissue respiration in vitro of mucosal slices of antrum and fundus was abnormal when stimulated with succinate. This could be due to gastric mitochondrial injury from biliary obstruction.

e. Conclusions: A damaged liver may potentiate the effects of sepsis on the stomach of a severely stressed patient. The gastric congestion that occurs in the patients with Laennec's and biliary cirrhosis may make their stomachs more susceptible to stress.

3. Hypoxia and Stress Ulcer Formation

a. Statement of the problem: Does hypoxia alone or combined with anemia increase the susceptibility to stress ulcer formation?

b. Background and approach to the problem: Since hypoxia and anemia are features of clinical stress syndromes that precipitate acute gastric lesions, we have studied the effects of hypoxia and acute anemia on the stomachs of rats subjected to wire mesh restraint stress for 7 hours. Experimental rats were exposed to 10% oxygen-90% nitrogen for 7, 14 and 50 hours while paired control rats were kept in similar chambers with room air to breath.

c. Results and discussion: Awake arterial pO_2 in 15 rats breathing 10% oxygen for 2 hours was 36.1 mm Hg. Anemia was produced by a double 3.0 ml exchange of blood for plasmalyte solution which reduced the hematocrit from 42.5 to 18.1%. Hypoxia did not influence the number of gastric erosions of ulcers at any of the three periods of exposure. When epinephrine was given 0.4 mg/kg intraperitoneally prior to 7 hours of restraint and hypoxia, the number of gastric lesions was unchanged. When the dose was increased to 0.1 mg/kg, there was a significant increase in gastric erosions for the hypoxic rats and without epinephrine, but the incidence of ulcers was unchanged. Acute anemia reduced the number of gastric erosions but not ulcers. When anemia was combined with 7 hours of hypoxia, the number of restraint induced gastric erosions was still decreased. Decreased arterial partial pressure of oxygen alone or in combination with reduced red cell mass did not adversely affect stress ulcer formation in the rodent restraint model. The effect of epinephrine on gastric blood flow together with decreased arterial partial pressure of oxygen were both necessary to precipitate stress gastric lesions.

d. Conclusions: Circulatory abnormalities appear to be of greater significance in contributing to stress ulcer formation than hypoxia.

4. Effect of Liver Damage on the Lung

a. Statement of the problem: Does partial hepatectomy or liver damage influence pulmonary function and ability to kill bacteria?

b. Background: Impaired liver function is frequently accompanied by hypoxemia, respiratory alkalosis and increased susceptibility to pulmonary infection.

c. Approach to the problem: Rats with liver damage following biliary obstruction subjected to partial hepatectomy were studied for change in pulmonary function and ultrastructure and bactericidal activity of the alveolar macrophages.

d. Results and discussion: Liver damage due to biliary obstruction was associated with splenomegaly and increased portal vein pressure, hyperventilation, respiratory alkalosis, increased arterial ammonia, decreased arterial and venous pO_2, decreased O_2 consumption with normal A-V O_2 content, decreased arterial pO_2 on 100% O_2, lacticacidemia, decreased stomach luminal pO_2 and unchanged luminal pH after 2 hrs. equilibration with 4 cc saline, and a macrocytic anemia that was corrected by folic acid and unaffected by splenectomy. Liver damage had no effect on arterial percent saturation (HbO_2), arterial pO_2 on 12% O_2, in vitro lung compliance, and jugular or femoral vein ammonia. The lung had thickening of the alveolar septal walls with proliferation of collagen. The arterial hypoxia of CBD ligation appears related to intrapulmonary shunting of blood. Abnormal perfusion may have caused the observed decreased O_2 consumption. Hyperventilation may be due to arterial ammonia and hypoxia.

In comparison with controls, common bile duct division and ligation, on light microscopy had hepatic cirrhosis and proliferation of bile ducts and on serial lung sectioning exhibited thickening of alveolar walls with proliferation of alveolar cells. Special stains demonstrated a moderate increase in collagen content of alveolar septa. On electron microscopy, structural and pulmonary changes included swelling and increased vacuolation of endothelial and epithelial cells as well as a pronounced numeral increase in alveolar cells (type II). In many cases, type II cells appeared atypical, with an increase in lipid inclusions. Collagen proliferation was present, but in many cases the superficial basement membrane appeared enlarged and likely contributed to the thickening seen. The over-all picture suggests increase in thickness of air-blood barrier.

Pulmonary antibacterial host defenses were studied in these animals by quantitating in situ bacterial inactivation in the lungs of control and experimental rats 14 hours after an aerosolized challenge of radiolabeled (^{32}P) S. aureus. Controls inactivated 86.3% of the bacterial challenge. Following ligation of the common bile duct, bacterial inactivation was significantly progressively impaired: 61.1% inactivation at 1 week, 37.0% at 2 weeks, and 34.5% at 3 weeks. Following 30% and 60% hepatectomy, bacterial inactivation was significantly reduced to 74.7% and 57.4% respectively, with intrapulmonary bacterial replication exceeding inactivation of the bacterial challenge following 90% hepatectomy. Isolated pulmonary alveolar macrophages harvested from the lungs of animals in both experimental groups had impaired bactericidal activity and depressed oxygen consumption in vitro. In addition, these cells had intracellular toxic vacuolization on electron microscopic examination. These studies demonstrate impairment of intrapulmonary bactericidal activity in vivo and functional, biochemical, and structural evidence of alveolar macrophage injury in vitro following experimental liver impairment in two animals. These experiments also provide a pathophysiologic explanation for the high incidence of pneumonia in the patient with impaired liver function.

e. Conclusion: Liver damage has a direct adverse effect on pulmonary function, ultrastructure and bactericidal activity.

5. Effect of Renal Failure and Metabolic Acidosis on Stress Ulcer Formation

a. Statement of the problem: In the traumatized patient, renal failure is frequently present in the patient with stress ulcer. Wounded soldiers treated at the Renal Dialysis Unit at the 3rd Field Hospital, Saigon, had a 35% incidence of stress ulcers.

b. Background: Acute renal insufficiency has been associated with the development of stress ulcers in patients following trauma and peritonitis. However, sepsis occurred concomitantly in many of these patients, and it is possible that factors related to sepsis rather than renal failure accounted for the stress ulceration. Chronic uremia in the human is associated with gastric erosions and ulcers and decreased gastric acidity.

c. Approach to the problem: The effects of bilateral nephrectomy and metabolic acidosis on the stomach of the rat subjected to restraint stress were studied to determine if these factors would influence stress ulcer formation independent of trauma and infection.

d. Results and discussion: Since the rats died between 40 and 54 hours after bilateral nephrectomy, studies were done at 24 hours when the arterial pH was 7.30 ± 0.08 (S.D.), urea 107 ± 13 mg % and potassium 5.3 ± 0.4 mEq/L. There was no increased incidence of gastric erosions or ulcers with either 7 or 12 hours of restraint. At 36 hours after nephrectomy, arterial pH was 7.25 ± 0.08 in the restrained rat and at 48 hours, the pH was 7.22 ± 0.05. There was no change in gastric luminal pO_2 equilibration but there was a marked increase in 4 hour gastric acid production in both the unrestrained and restrained rat with ARF. The increased gastric acid production in the restrained nephrectomized rat was reduced by prior vagotomy and pyloroplasty. ARF increased gastric tissue water. Five days after drinking 1.6% ammonium chloride solution, arterial pH was 7.19 ± 0.11. There was a significant increase in the number of gastric erosions but not ulcers with restraint. Metabolic acidosis had no effect on gastric acid production or luminal pO_2 equilibration in either the unrestrained or restrained rat. With metabolic acidosis, gastric tissue water increased. Other factors (e.g. sepsis) present in patients with posttraumatic renal failure may be of greater etiological significance in stress ulcer formation. Since metabolic acidosis also occurred in ARF, some factors present with ARF may have mollified the effect of acidosis or a longer period of acidosis may have been needed to increase the incidence of gastric lesions.

e. Conclusion: Prolonged acidosis may adversely effect the stomach.

6. Effect of Acute Blood Loss on Pulmonary Bactericidal Activity

a. Statement of the problem: Does blood loss lead to impaired intrapulmonary bactericidal activity?

b. Background: hemorrhage frequently precedes the development of post-traumatic pulmonary insufficiency.

c. Approach to the problem: The ability of the lungs of adult male Wistar rats to inactivate aerosolyzed, radiolabeled (^{32}P) S. aureus was studied 14 and 38 hours following (1) a blood loss of 2.0 cc/100 gm with no drinking water or food allowed during the period, (2) a period with access to food but not water, and (3) a period with access to water but not food.

d. Results and discussion: The intrapulmonary inactivation of S. aureus was impaired 14 hours following acute blood loss but was impaired in the shams as well at 38 hours. Weight loss and arterial pH, pO_2, and pCO_2 were similar statistically at both time periods for the sham and ABL rats. Because of the impaired intrapulmonary inactivation of S. aureus in the sham rats at 38 hours, the effects of dehydration and starvation were also studied. Dehydration for 14 hours had no effect on bacterial inactivation but at 38 hours, there was a significant impairment. Starvation had no effect on bacterial inactivation at either time. Acute blood loss and dehydration adversely affected the pulmonary host defense mechanisms of normal rats. Both of these factors may be operative in the traumatized patient and contribute to the development of post-traumatic pulmonary insufficiency.

e. Conclusion: Acutely decreased body water impairs the ability of the pulmonary macrophage to kill bacteria.

7. Does Stress Have a Direct Effect on the Ability of the Lungs to Kill Bacteria?

a. Statement of the problem: Does stress have a direct effect on the ability of the lungs to kill bacteria?

b. Background: Following trauma, the lungs may develop pathological changes in the alveolar septum and the incidence of pneumonia is high.

c. Approach to the problem: Acute stress was produced by wire mesh restraint and the ability of the lung to kill bacteria was studied. Separate rats were studied with plaster casts on the chest but had mobility and were able to eat.

d. Results and discussion: The ability of the lungs of 50-100

gm male Wistar rats to inactivate aerosolyzed radiolabeled (^{32}P) S. aureus was studied during and following acute stress induced by immobilization in wire mesh restraint for 14 hours. When stress preceded bacterial challenge, the ability to inactivate S. aureus was unimpaired. When lungs of these rats were examined for stress ulcerations, none were found. However, when the rats were challenged with S. aureus first and then restrained for 14 hours prior to sacrifice, pulmonary bacterial inactivation was decreased. Both groups had similar weight changes and lung water. The rats with acute stress had markedly increased gastric erosions and ulcers. Rats with chest casts also had impaired bacterial clearances but they developed a respiratory acidosis compared to their control.

e. Conclusion: The changes in bacterial clearance with wire mesh restraint would appear not to be due to restriction of the chest cage. Acute stress may have a direct neurohumoral influence on the lung and the alveolar macrophage.

8. Oxyhemoglobin Dissociation Curve and Oxygen Release in the Isolated Canine Hindlimb

a. Statement of the problem: Does the position of the oxyhemoglobin dissociation curve, as altered during blood storage, influence the release of oxygen at the tissue level?

b. Background: The position of the oxyhemoglobin dissociation curve is to a great extent controlled by the interaction of organic phosphates (2,3 DPG) within the red cells, with hemoglobin. A shift to the left of the dissociation curve is usually accompanied by a fall in 2,3 DPG level. This left shift results in an increased affinity of hemoglobin for oxygen and theoretically decreasing the efficiency of oxygen delivery to the tissues. A shift to the right of the dissociation curve theoretically will produce the opposite effect. Storage of RBC for greater than one week has been shown to cause a decrease of red cell 2,3 DPG and an increased affinity of hemoglobin for oxygen.

c. Approach to the problem: Under light pentobarbital anesthesia a unit of blood was drawn from each of 10 dogs into standard ACD plastic bags and stored at 4° C for 21 days. On the 21st day the above procedure was repeated. Following the phlebotomy the left hindlimb was surgically isolated and perfused alternately with blood that was stored for 21 days and with fresh blood.

d. Result and discussion: In each isolated hindlimb perfusion study, the total tissue oxygen uptake was significantly greater during perfusion with fresh blood than during perfusion with banked blood. It would appear that stored blood, with the dissociation curve shifted to the left, releases oxygen less readily than fresh blood. However, humoral factors in banked blood other than the shift in dissociation

curve might possibly play a role in the decreased oxygen release observed.

e. Conclusion: Stored blood released less oxygen to the tissues during perfusion of an isolated hindlimb in the dog, than did fresh blood.

f. Recommendation: This study should be repeated with washed, resuspended RBC to rule out any other mechanism, other than 2,3 DPG changes during storage, that might have played a role in the decreased 0_2 release observed.

9. The Effects of Aspiration of Acid Solutions and of Fresh Blood on Lung Structures, Arterial Oxygenation, and Pulmonary Resistance to Infection

a. Statement of the problem: To study the effects of aspiration of acid solutions and of fresh blood on lung structures, arterial oxygenation, and pulmonary resistance to infection.

b. Background: Pulmonary aspiration of acid gastric contents or blood from maxillofacial wounds is a recurrent, serious problem, complicating the surgical care of wounded soldiers.[3] Aspiration was a principal cause of hypoxemia in 14 of 58 casualties with postoperative arterial desaturation.[4] In addition silent or unrecognized aspiration is believed to have caused 10% to 34% of all anesthetic deaths.[5] Acid aspiration frequently leads to acute pulmonary edema, hypoxemia and a supervening bacterial pneumonia, while fresh blood may coagulate within bronchioles to produce atelectasis and pneumonia.[6]

The dynamics of pulmonary changes after aspiration has been outlined incompletely, but rigorous physiologic studies of arterial blood gases, pulmonary shunting and compliance have not been performed. The ability of the lung to inactivate bacteria is uniquely suited to the study of resistance to pneumonia after aspiration.

c. Approach to the problem: The trachea of Wistar rats lightly anesthetized with ether was surgically exposed and the solution to be aspirated injected under direct visualization. The dosage of aspirant was 2 ml/kg body weight and includes hydrochloric acid in Ringer's solution at pH 1.5, unclotted blood freshly obtained from another rat, and control Ringer's solution. Arterial blood gases and shunt measurements were measured by drawing aortic blood under light ether anesthesia, or from an indwelling carotid artery catheter placed before aspiration. Animals exposed to room air and 100% oxygen. The alveolo-arterial oxygen difference is measured plus the degree of intrapulmonary shunting.

At 15 and 38 hours rats were fluoroscoped and roentgenograms were taken. Whole lungs removed at time intervals after aspiration were inflated with air to prevent postmortem atelectasis, preserved in formalin and stained with H&E for microscopic examination.

Rats were exposed to graded doses of aerosolized radiolabelled staphylococcus aureus in a chamber. At 15 and 38 hours after aspiration the bactericidal activity was measured by determining the ratio of live to total bacteria in the lungs.

d. Results and discussion: Arterial oxygen tension was found decrease slightly in animals given acid and blood, but these changes, together with arterial pH and pCO_2 were not statistically significant. Studies of the degree of shunting showed no change between test and control groups. X-rays at 14 and 38 hours revealed no visible changes in either blood or acid aspirated rats. Microscopic examinations of lungs showed minimal atelectasis and pneumonitis at 48 hours in the acid group, but rats receiving blood had large atelectatic areas and alveolar septal cuffing with hemoglobin inclusion bodies.

Bacterial clearance studies indicate that there is a definite decrease in bacterial inactivation in lungs of animals receiving acid or blood, as compared to control animals.

e. Conclusion: The bacterial inactivation system provided a quantitative approach to the effects of aspiration on resistance to pulmonary infection. However, in this model arterial blood gases, shunt studies, radiography and light microscopy offer little help in measuring the effect of aspiration.

f. Recommendation: Corticosteroids, administered both intratracheally and systemically will be given to determine whether aspirated rats can improve their decreased pulmonary bacterial clearance. Also since interstitial pulmonary edema is a feature of acid aspiration, weight weight/dry weight of excised lungs will be measured after aspiration. If this ratio increases with acid, the effects of diuretics such as ethacrynic acid and furosemide on lung water and bacterial clearance will be studied.

10. Effects of Acepromazine Maleate on Selected Cardiorespiratory Parameters of Dogs

a. Statement of the problem: Many research activities require performance of certain tasks on unanesthetized but yet tractable experimental dogs. However, agents commonly used for tranquilization can cause important long-lasting alterations of various physiological parameters thereby indirectly changing the outcome of experimental procedures that may follow.

b. Background: Acepromazine is a neuroleptic agent used as a tranquilizer or pre-anesthetic medication for dogs, cats and several other species. The drug has been reported to produce suitable tranquilization but literature search did not reveal information regarding its physiologic effects.

c. Approach to the problem: Nine dogs of either sex weighing from 20 to 30 kilograms had polyethylene catheters placed into the femoral vein and artery. The catheters were embedded subcutaneously to their exit in the dorsal lumbar area.

Arterial blood samples were collected immediately prior to and at 15 and 30 minute intervals following administration of 1 mg/kg of Acepromazine.

d. Results and conclusions: Decrease in arterial blood pressure and bradycardia caused by intramuscular injection of Acepromazine Maleate were not accompanied by significant changes in any blood gases or arterial pH. In general, the respiratory changes were minimal suggesting that the drug could be of value in experimental work with dogs.

11. Ultrastructure of the Human Vas Deferens

a. Statement of the problem: Earlier literature had shown that the vas was well innervated, but the question of the type of innervation, i.e., adrenergic or cholinergic, has not been entirely resolved. Histochemical and electron microscopic studies suggest that in several animal species (rat, mouse, guinea pig) vas innervation is primarily by adrenergic fibers.

b. Background: All literature points to the fact that the vas is not a passive tube but an active organ whose function has not been fully investigated in sperm transport and maintenance of fertility. In comparison with work on animals, very little has been done with human vasa deferentia. Increasing appreciation of the vas in clinical work and sterility problems will likely require additional basic research.

c. Approach to the problem: The vasa deferentia were obtained from four patients referred to the Urology Clinic, WRGH for voluntary vasectomy under 1% xylocaine anesthesia.

Thick sections were stained with toluidine blue and prepared for light microscopy. Thin sections were cut and stained with uranyl acetate and lead citrate for electron microscopy.

d. Results and conclusions: The narrow irregular lumen of the vas is surrounded by a thick muscular coat consisting of three layers. Collagen was found throughout the muscular layers and in varying amounts among the individual muscle cells. The vas was well innervated, axons being found in close proximity to muscle cells. The epithelium of the vas is pseudostratified with a basal layer of cuboidal cells and a luminal layer of columnar cells, some of which extend from the basement membrane, while others rest on the low cuboidal cells. When examined with the electron microscope, the columnar cells can be divided into two types by their osmiophilic characteristics. All columnar cells have well developed endoplasmic reticulum and granules usually associated with secretory activity. Long microvilli (stereocilia) extend from columnar cells into the lumen of the vas.

12. Physiologic Effects of Phencyclidine Hydrochloride on the Macaca Mulatta

a. Statement of the problem: Phencyclidine Hydrochloride (Sernylan)[a] is a neuroleptic drug widely used for chemical restraint of various species of subhuman primates. The animals can be rendered tranquilized or anesthetized depending on the dosage administered. The physiological responses of the Rhesus monkey to Sernylan are not completely known or understood as demonstrated by the presence of several conflicting reports in the literature.

b. Background: The main problem appears to be the difficulty in obtaining normal (baseline) physiological values in non-sedated monkeys. Stress associated with physical restraint, fear of unfamiliar surroundings and procedures may be sufficient to mask or amplify a reaction to Sernylan.

c. Approach to the problem: Thirteen Rhesus monkeys weighing from 4 to 7 kg were maintained in primate restraining chairs, with free access to food and water and becoming more accustomed to close human contact.

Two silver wire electrodes (F-P) were surgically placed on the dura of each hemisphere of the brain several weeks prior to the beginning of the experiment via skull trephine openings. The central venous and arterial pressures were measured through polyethylene catheters threaded into the femoral vein and artery.

d. Results and conclusions: Sernylan had long lasting effects on the brain, cardiovascular and respiratory systems which might contra-indicate its use in physiological or pathophysiological studies.

[a] Parke, Davis Co., Detroit, Michigan

Sernylan produced abnormal brain wave activity, fall in heart rate and arterial and central venous pressures, a respiratory acidosis and a mild hypoxemia. These effects could alter baseline events occurring in a pathophysiological experiment.

Our work indicates that M. mulatta, given tranquilizing levels of Sernylan will go through stages of sleep as demonstrated by the presence of slow delta waves, sleep spindles, and K-complexes. Additional intramuscular injection of Sernylan, which in effect elevates the blood concentration of the drug, produced a multitude of high voltage peaks persisting for several hours following the last injection. These spikes may represent a mild convulsive activity or they may be artifacts. It should be noted that EEG activity similar to that observed in grand mal seizures can be elicited by injections of large or repeated doses of Sernylan.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122 Anesthesia and pulmonary complications of combat injury

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PROJECT 3A062110A822
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	72 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
71 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	3A062110A822	00	120
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE (Procede with security Classification Code)

(U) Metabolic Response to Disease and Injury (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 PHYSIOLOGY 003500 CLINICAL MEDICINE 002300 BIOCHEMISTRY

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	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 CURRENT		
	72	20	365
	73	20	365

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide 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 General Hospital.

25. (U) 71 07 - 72 06. Investigations of alterations in metabolic-hormone-energy-fuel relationships induced by trauma, immobilization, infection, and drugs were conducted. Studies of the hypercalcemia and hypercalciuria which occurs with immobilization and fractures have revealed that serum parathyroid hormone does not increase, but that it is still detectable. It may play a permissive role. Urinary cyclic three prime five prime adenosine monophosate falls at bed rest supporting the conclusion that parathyroid hormone is not the etiologic factor. Anxiety, hyperventilation, soldiers heart and neurocirculatory asthenia can alter lactate metabolism during stressful events. Lactate infusion in five dogs indicate that both serum pH and lactate alter the ionized calcium. Ionic calcium plays a major role in cell membrane permeability and nerve conduction, and it may be through this mechanism that symptoms develop. Treatment of five acromegalics with large doses of medroxyprogesterone and thorazine gave unimpressive results, either clinically or biochemically. Preliminary cell culture studies evaluating the development of dependence and tolerance to morphine have only demonstrated the phenomena of dependence. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 June 72.

*Available to contractors upon originator's approval.

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1124-b

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators:
Principal: COL Jerry M. Earll
Associates: Marcus Schaaf, M.D., Maj Leonard Wartofsky, MC; MAJ William J. Howard, MC; MAJ Richard C. Dimond, MC; MAJ James C. Low, 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 approach 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 General Hospital 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. Thyroid Metabolism.

Earlier thyroxine turnover studies in malaria patients have been completed and will be published in J. Clin. Invest., August 1972. These altered thyroid hormone kinetics are being pursued further with a highly sensitive radioimmunoassay for thyroid stimulating hormones (TSH). Thyrotropin releasing hormone (TRH) is being evaluated in a Phase II study with Abbott Laboratories. These studies have been completed in over 60 individuals and are extremely useful in defining the nature of disturbed pituitary thyroid function.

2. Calcium Metabolism.

Refinements of earlier immobilization and fracture studies continue to support observations of pathologically elevated ionized calcium while parathyroid hormone does not rise and cyclic 3′ 5′ AMP tends to fall. This work is being reported at the June 1972 Ford Bone Symposium and will be published in detail in proceedings of the meeting.

The role of ionic calcium is being evaluated in the disease complexes of "neurocirculatory asthenia", "soldiers heart", and anxiety. Preliminary work suggests abnormalities of lactate metabolism are involved. The mechanical aids and stresses of high altitude pilots and mountain troops place them at risks of hypoxia, hyperventilation and anxiety. Hyperventilation which is common in adverse environments causes respiratory alkalosis which decreases blood ionic calcium as well as increasing lactate. Symptoms associated with hyperventilation and anxiety are similar to those of hypocalcemia. The physiologically active component of serum calcium, the ionic fraction, is pH dependent. Measurements *in vitro* by an Orion (R) ion-selective electrode showed a decrease of 0.05 mg/100 ml of ionic calcium per 10 mg/100 ml rise in lactate. Following the procedure of Pitts, *et al.*[1], we infused Sodium L (+) lactate or NaCl in dextrose solutions in randomized order into seven awake dogs. Blood pH, pCO_2, HCO_3, ionic and total calcium were measured prior to, during and after each infusion. Peak blood levels of 96 mg/100 ml lactate obtained at the end of infusion was associated with a mean drop of 0.86 $\pm$ 0.06 (S.E.) mg/100 ml of ionic calcium ($p < 0.005$) compared with a decrease of 0.13 $\pm$ 0.07 mg/100 ml during infusion of NaCl in dextrose. Alkalosis (H^+ decreased 8 nM) due to bicarbonate generation during lactate infusion accounts for 0.2 mg/100 ml of the total decrease. The *in vivo* results confirm the *in vitro* finding that lactate complexed calcium. This allowed lactate to make a significant contribution to the lowering of ionized calcium. Serum ionic calcium in normal subjects vary in a narrow range of 4.8 to 5.28 mg/100 ml ($\pm$ 2 S.D. = 0.8 mg/100 ml). Ionic calcium plays a major role in maintaining normal cell permeability, conduction, and physiology. Any condition in which an increase in serum lactate and respiratory alkalosis co-exist may drop ionic calcium to levels which may produce symptoms and impair performance.

Studies are being completed to determine if measurements of cyclic 3′ 5′ AMP, parathormone, calcium and ionic calcium during suppression and stimulation of parathyroids will permit more accurate diagnosis of disorders of parathyroid glands. EDTA infusions are administered to patients to lower serum calcium. This lowering normally stimulates the parathyroids to secrete parathormone which subsequently increases cyclic 3′ 5′ AMP in the urine. Calcium infusions are given to suppress parathormone and cyclic 3′ 5′ AMP should decrease in the urine. A total of 15 patients have been studied on this protocol - 4 surgically proven cases of hyperparathyroidism, 3 suspected but not yet operated on, and 8 patients with idiopathic hypercalciuria. Although the data is still tentative and incomplete, we have found that the patients with idiopathic hypercalciuria respond normally to calcium suppression and EDTA stimulation, while the patients with hyperparathyroidism show at least some response to these agents. Even those patients with

definitive parathyroid adenoma seem to respond, but at a higher level of blood calcium. We postulate that their calcium regulating mechanism is set at a higher level. We plan to study other patients as they become available.

3. Polypeptide Hormone Metabolism.

Recent reports of treatment of acromegaly with medroxyprogesterone acetate (MPA) have been uniformly favorable. Treatment of five active acromegalic patients with MPA failed to confirm the favorable responses reported by others. The patients were studied prior to and after one week (5 patients), three months (3 patients), and six months (2 patients) of treatment. Basal growth hormone levels and responses to glucose, arginine, and insulin were studied during each period. Daily fluctuations in basal growth hormone levels were observed in all patients before and during therapy. No significant change in mean basal growth hormone levels occurred in any patient. Glucose tolerance did not improve in any patient and significantly worsened in three patients. No objective improvement of physical features was noted in any patient. Our findings suggest that (1) in an individual acromegalic, basal growth hormone levels are very variable; therefore, interpretation of response to MPA based on single measurements should be made with caution; (2) MPA therapy did not significantly change mean basal growth hormone levels in any patient at any time; (3) MPA therapy may be associated with deterioration of glucose tolerance; (4) the optimistic reports of the efficacy of MPA treatment should be viewed with reservation.

An even more recent report suggested medical treatment of acromegaly with chlorpromazine. Five acromegalic patients were treated with chlorpromazine (CPA), 100 mg/day, for 1 to 3 months. Growth hormone (GH) was measured in an average of four basal blood samples, obtained on different days, and during an oral glucose tolerance test in each patient prior to and after 1 and 3 months of therapy. Each patient's specimens were measured in duplicate within the same radioimmunoassay.

Five patients failed to show a significant decrease in their mean basal GH after one month of treatment. Their pre- and post-treatment mean values in ng/ml were: 9.2-9.8, 21-25.3, 25-24, 9.8-9.1, 18-179. Patient 6 decreased from 11.1-7.8 after one month. No further significant reduction in his mean basal GH occurred after three months of treatment, and his GH has never suppressed to 5 ng/ml following a glucose load. Four of these patients treated previously with medroxyprogesterone acetate for 4 to 6 months failed to respond to that agent as well. Patient 7 responded to CPZ with a significant decrease in his mean basal GH. His pre-treatment value of 94 ng/ml fell to 56 ng/ml after one month without any signs of clinical improvement. In

addition to patient 6, patients 7 and 2 were treated for three months without further decrease in their mean basal GH.

It is concluded that the long term therapeutic efficacy of CPZ in acromegaly has yet to be demonstrated. Our preliminary data suggest that a one month treatment period may suffice to predict the responsiveness to CPZ in an individual patient.

4. Steroid Metabolism.

The urinary excretion of a 17-hydroxycorticosteroid, pregnanetiol, 17-ketosteroid, 11-deoxy-17-ketosteroid, and testosterone were measured in normal men and men with endocrine disorders before and during four days of chorionic gonadotropin administration. Testosterone excretion at least doubled in all normal men and showed the greatest change. Androsterone and etiocholanolone increased to at least 150% of the control. Patients with hypopituitarism and primary testicular disease could not be consistently differentiated on the basis of their response to gonadotropin. Patients with delayed puberty and hypogonadotropic hypogonadism have variable responses. In patients with acromegaly, baseline steroid excretion and response to gonadotropin did not differ from normal men. This work was published in the Archives of Internal Medicine, October 1971.

Plasma 17-OH corticosteroid procedures are too numerous to elaborate. However, the basic quality of most procedures reside in the final technique used to measure the isolated steroid. These techniques may employ such analytical tools as colorimetry, fluorometry, gas liquid chromatography, thin-layer chromatography, column chromatography, isotope dilution, radioimmunoassay and competitive protein-binding procedures.

The purpose of this investigation was two-fold. One to determine if any correlation existed between several different procedures; second, to evaluate the relative sensitivity and reliability of these procedures. The sensitivity and reliability problem is necessary in order that the methods used can be scaled down to handle small aliquots of plasma. This requirement has become necessary due to the great demand of blood from patients for a variety of studies.

The several procedures evaluated were: (1) The Porter-Silber procedure as outlined by Peterson, et al.[2]. This has been a routine procedure in their laboratories for years. This procedure requires 5 ml of plasma, and drug therapy discontinued during study period. It will measure all 17-OH corticosteroids. (2) The Clark-Rubin fluorometric procedure[3] requires 1.0 ml of plasma and may be used during limited drug therapy. However, this procedure is handled in such a way as to measure only

cortisol in plasma. (3) The double-isotope derivative procedure as outlined originally by Kliman, et al.[4], is a commercially available procedure. This is a very sensitive procedure and it is used to measure a wide variety of plasma steroids. For each analysis 1.0 ml of plasma is required. This procedure is too costly for our laboratories. (4) A competitive protein-binding procedure as outlined by Murphy, et al.[5], is a very sensitive and reliable procedure for measuring a wide variety of plasma steroids. This is a new addition to our laboratories and it requires only 0.1 ml of plasma for a single analysis. This procedure is not influenced by drug therapy and analyses can be made during a wide variety of drug treatment. In addition, a large number of samples can be processed during a single run.

In conclusion these procedures have all be thoroughly investigated and correlation data obtained. They have all been subjected to examination during drug studies and results are now being tabulated for publication.

5. Intermediary Metabolism and Drug Studies.

Preliminary cell culture studies involving the development of dependence and tolerance to morphine have only demonstrated the phenomena of dependence. Several lines of cell culture are now being evaluated and studies of the addicted monkeys' fibroblasts have begun. Earlier studies in this literature indicates that urinary 3′ 5′ AMP was low in depressed patients and fluctuated with mood. Preliminary studies of acute ingestion of alcohol fail to reveal changes in urinary cyclic 3′ 5′ AMP.

Project 3A062110A822 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 OA 6445	72 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. GRP'N INSTR'N	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A822	00	121
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of Enteric Disease (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
59 05	CONT	DA	C. IN HOUSE

17. CONTRACT/GRANT N/A
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 72	4	115
FISCAL YEAR CURRENT 73	4	115

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME: DA
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, and the identification and characterization of agents involved in "travelers diarrhea".

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

25 (U) 71 07-72 06 A new E. coli hybrid expressing the somatic determinants of S. flexneri 2a has been employed as a vaccine in volunteers. Dosages of 10^{10} cells in volunteers have produced no illness. For technical report see Walter Reed Army Institute Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* Available to contractors upon originator's approval.

DD, FORM 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 3A062110A822, 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 diseases is studied to elucidate mechanisms by which enteric pathogens produce symptoms. By understanding the steps involved in provoking disease, improved procedures for prevention and treatment of diarrheal diseases will become evident.

Progress.

1. By employing an integrated immunological, cytological and genetic approach to study pathogenesis of bacillary dysentery by Shigella flexneri, studies from this department have previously established that an essential feature for establishing disease is the penetration of epithelial cells of the colonic mucosa by the pathogen and its subsequent multiplication. During the past year we have addressed ourselves to the question of O-antigen specificity and its effect on the invasiveness of pathogens. The similarity of chromosomal position for antigen genes of E. coli and S. flexneri (near the histidine operon, his), has enabled us to construct by intergeneric hybridization techniques, S. flexneri derivatives which express either the E. coli O-25 or O-8 somatic antigen rather than their native parental serotype. Table 1 is a summary of the serological properties of his^+ S. flexneri hybrids obtained from matings with O-25 E. coli Hfr W3703 and O-8 E. coli Hfr 59.

Their slide agglutination properties were tested in E. coli monospecific factor 25 or 8 serum, unadsorbed S. flexneri 2a serum, S. flexneri monospecific type II serum and S. flexneri Y serum containing agglutinins for group factors, 3,4. With hybrids derived from matings with the O-25 E. coli W3703 donor, three agglutinin classes could be scored. Class C, in which cells agglutinated only in S. flexneri antisera (type II and Y) represents his^+ hybrids which did not inherit E. coli determinants, this serological pattern being typical of the parental recipient strains. Among those hybrids which inherited antigen 25, two serological patterns were distinguished. Class A hybrids agglutinated strongly in factor 25 serum and in the S. flexneri

type 2 serum but were unreactive in the group factor S. flexneri Y serum. It appears therefore that these hybrids had replaced their native group antigens with the E. coli O-25 antigen in the process of recombinant formation. The type specific II antigen, whose gene(s) map near the pro locus (distal to the his region) remain unaltered and are still expressed in such hybrids. With Class B hybrids, agglutination was detected in all three sera, although it was noticeably weak in the S. flexneri group factor serum. These results suggest that class B hybrids may be diploid for the his chromosomal region, conserving their native S. flexneri group factors in addition to inheriting antigen 25 from E. coli. With His^+ hybrids derived from the antigen 8 donor, E. coli Hfr 59, only two distinct agglutinin classes were evident (Table 1, bottom). Class A hybrids agglutinated only in factor 8 serum, being unreactive in any of the S. flexneri antisera. Class B hybrids behaved serologically as typical S. flexneri 2a.

Virulence of S. flexneri hybrids with E. coli antigenic characteristics. The Sereney test for keratoconjunctivitis was employed in initial screenings to assess the virulence of S. flexneri hybrids expressing E. coli antigens. This test determines the ability of organisms to penetrate epithelial cells of the cornea, causing keratoconjunctivitis. A provocation of keratoconjunctivitis by S. flexneri in turn reflects the ability of the organism to invade bowel epithelium, a necessary attribute for S. flexneri virulence. Both E. coli Hfr 59 and Hfr W3703 failed to evoke keratoconjunctivitis and hence were considered avirulent. When the 88 His^+, antigen 8 positive hybrids derived from a mating with Hfr 59 (Table 1), class A, bottom) were so screened, none caused keratoconjunctivitis thus indicating that they were avirulent. Subsequent testing of these hybrids for sensitivity to "rough-specific" bacteriophages revealed that 77 of 88 were lysed by at least one of them. Thus, the avirulence of a high proportion of the O-8 hybrids could be due to their rough state. Nevertheless, the remaining 11 hybrids, which exhibited no sensitivity to the "rougn-specific" phages and agglutinated strongly in O-8 antisera, still were avirulent.

Sereney tests on S. flexneri hybrids which inherited E. coli antigen-25 revealed that some of these indeed retained the ability to penetrate epithelial cells. Screening tests were limited to class A hybrids (Table 1, W3703 donor) since this type of hybrid had apparently replaced S. flexneri group factors with the O-25 donor antigen. Six of 44 such hybrids provoked a positive Sereney test and when tested with the rough-specific phages, scored as smooth. As in the case of the O-8 S. flexneri strains, many, but not all, of the avirulent O-25 hybrids appeared to be rough, showing sensitivity to at least one of the rough-specific phages employed.

Upon reisolation of organisms from diseased eyes, the virulent cells were serologically indistinguishable from those employed in the

initial challenge, being agglutinated only by E. coli O-25 and S. flexneri type II antisera. One such strain, O-25 hybrids 547-1-7 was chosen for further study of virulent properties.

The virulence of O-25 hybrid strain 542-1-7. Both the parent S. flexneri 2a strain 17-14-18 and the O-25 hybrid 542-1-7 were fed to groups of starved-opiated guinea pigs at a dose of about 1×10^8 cells. Animals were sacrificed 24 hours later and their intestines compared histologically to assess the extent of bowel damage.

Sections of the small intestine from guinea pigs fed the parent his- S. flexneri 2a strain 17-14-18 showed marked alterations of the mucosal architecture. These included; villous clubbing with elongation and dilation of the glandular crypts; decreased numbers of goblet cells; increased focal sloughing of the epithelium; congestion and increased cellularity of the lamina propria. The large intestine was characterized by diffusely scattered areas of altered mucosal architecture. There was an increase in the mucosal thickness. The epithelial lining of the crypts showed nuclear hyperchromatism, increased numbers of mitoses and decreased numbers of goblet cells. The surface epithelium was focally uncerated with an associated acute inflammatory exudate. Occasional epithelial crypt abscesses and microabscesses in the mucosal lymphoid aggregates were observed. The lamina propria was congested and diffusely scattered collections of polymorphonuclear neturophils were present. Sections from both the small and large intestines of guinea pigs fed the hybrid S. flexneri O-25 strain 542-1-7 showed similar and essentially indistinguishable morphological alterations. Control animals, fed either an avirulent strain or broth alone, showed normal small and large intestinal mucosal architecture without evidence of acute inflammatory reaction.

Additional examination of diseased tissue with the fluorescent antibody technique, using labeled antisera against E. coli O-25 or S. flexneri 2a revealed that the degree of invasion of mucosal epithelial cells by the O-25 hybrid approximated that of the virulent S. flexneri parent 17-14-18.

Our finding that S. flexneri hybrids with E. coli O-8 factor (chemically divergent to S. flexneri group factor) are avirulent while those with the chemically related E. coli O-25 factor can conserve virulence thus indicates that the O-repeat unit composition of surface lipopolysaccharides can be altered within limits without significant alteration to virulence.

2. Another aspect of our program on diarrheal disease has been concerned with the disease caused by toxin producing strains of Shigella dysenteriae, interest in this organism having been increased due to the recent epidemic of Shiga dysentery in Central America. We

have been involved during the last year in assessing the relative importance of mucosal invasion versus enterotoxin production in the pathogenesis of disease. We have studied in various experimental animal models the disease provoking capacity of S. dysenteriae 1 and some mutant derivatives. Specifically, the disease provoking capacities of (a) a wild-type strain capable of both penetrating the intestinal mucosa and producing toxin; (b) a naturally occurring mutant unable to invade, but capable of elaborating high yields of toxin; (c) a mutant strain capable only of cell penetration having lost its toxigenicity and (d) a mutant strain unable to penetrate or elaborate toxin, were compared.

Comparative studies on toxigenic S. dysenteriae 1 employing penetrating and non-penetrating derivatives. Initial experiments were conducted with S. dysenteriae 1 strain 3818. This strain is similar in behavior to a strain of S. flexneri 2a previously studied, in that two colonial types are observed, an opaque (O) and a translucent (T) form. As with S. flexneri 2a, the T colonial form of strain 3818 was able to penetrate epithelial cells as evidenced by an ability to evoke keratoconjunctivitis in the guinea pig; (Sereney test) the O-form lacked this property.

Since S. dysenteriae 1 elaborates toxin, its possible involvement in the penetration step of pathogenesis had to be assessed. Thus, comparative titrations for toxin production by the O and T forms of strain 3818 were performed to determine whether the O form produced toxin and if so, whether the amount elaborated was significantly lower than its T form parent. Two-fold serial dilutions of sterile filtrates from shaken overnight broth culture of both the O or T form were tested for their capacity to kill monolayers of HeLa cells. The preparation form a T-colony form of strain 3818 killed HeLa cells at a dilution of 1:800 while that from the O-colony form had a cytopathogenic effect at a dilution of 1:1600. The finding of comparable levels of toxin in both both the O (non-penetrating) and T (penetrating) form of strain 3818 suggests no significant involvement of toxin in the initial steps of epithelial cell penetration.

Experiments with the T and O derivatives of 3818 were next conducted utilizing the rabbit ileal loop model. Sterile filtrates from shaken overnight broth cultures of both colonial forms contained enterotoxic activity in that both produced positive ileal loops. Likewise when bacterial cells of both the penetrating and non-penetrating derivatives were inoculated, positive loops resulted. However, when fluorescent antibody studies were performed on sections of these positive ileal loops, a dramatic difference in the distribution of organisms was observed. Organisms of the T-form (Sereney positive) strains were observed within epithelial cells of intestinal mucosa as well

as in the bowel lumen, while the cells from the O-form (Sereney negative) derivative were present only in the lumen. Thus, it is apparent that despite production of toxin both the O and T form, only the T-form had the ability to penetrate the intestinal epithelial cell.

The toxigenic O and T forms of strain 3818 were next fed to groups of Rhesus monkeys in dosage levels of 5 x 10^{10} cells. The results of this study (summarized in Table 2) were unequivocal, in that 9 of 15 animals fed the penetrating strain (T form) exhibited evidence of disease; 4 of these animals died with acute dysentery while the remaining 5 diseased monkeys suffered severe diarrhea. There was no evidence of paralysis in any of the animals. None of the monkeys which were fed the toxigenic but non-penetrating strain 3818-O showed any signs of illness. Three additional monkeys suffering from dysentery following an infection with strain 3818-T were sacrificed for pathological examination. At necropsy, gross abnormalities of all three were confined to the colon. The small intestine appeared normal. The colonic contents consisted of a mucopurulent of muco-hemorrhagic exudate which covered the mucosa. The colonic wall was edematous and thickened; the mucosa was injected, granular, unevenly swollen and markedly edematous, with patchy hemorrhage. Histologically, the colonic mucosa was involved by acute colitis, which was indistinguishable from that caused by Shigella flexneri infections previously reported. From these findings it is evident that toxin production alone is not responsible for the capacity of S. dysenteriae to cause clinical disease in monkeys.

Comparative studies on penetrating S. dysenteriae 1, employing toxigenic and non-toxigenic derivatives. As is the case with S. flexneri and S. sonnei, S. dysenteriae 1 must penetrate the intestinal mucosa to cause disease. Nevertheless, neuro-enterotoxin production is an important characteristic of this organism and we next attempted to assess its role in the pathogenesis of the disease. Our approach was first to obtain a mutant strain which did not produce toxin but which could invade the bowel wall and then to obtain a double mutant which lacked both of these characteristics. Initially, attempts were made to isolate a toxin negative mutant from cell populations of S. dysenteriae 1 strain 3818-T which had been exposed to mutagenic agents (N-methyl-N'nitro-N-nitrosoguanidine, ethidium bromide or treatment with UV). Sterile filtrates from over 700 such clones were tested for lack of ability to kill HeLa cell monolayers and none were found.

Other studies in this laboratory, however, led to the fortuitous observation that 10 to 25 percent of chlorate resistant mutants of several S. dysenteriae 1 strains lost the ability to produce toxin. At the present time, the precise chlorate resistance locus involved and the mechanism by which a proportion of these clones become toxin

negative is unknown. Nevertheless, we decided to employ this approach to recover toxin negative derivatives of botn strain 3818-T and strain 3818-O. A toxin negative, penetrating strain (725-78), derived from 3818-T, and a toxin negative non-penetrating strain (735-19), derived from 3818-O, were chosen for further study. Crude toxin extracts, prepared by the procedure of van Heynigan, were made from comparable amounts of cells of the parent and mutant strains. Extracts from the mutant strains failed to kill HeLa cells and failed to cause a positive ileal loop in the rabbit. On the other hand, the preparations from the parent strains had more than 600,000 tissue culture lethal doses per mg Kjeldahl nitrogen and did evoke a positive ileal loop.

Since both mutant strains failed to produce detectable toxin, it was of interest to compare their behavior with that of parent strains (3818-T and 3818-O) when bacteria rather than cell-free material were employed in both the rabbit ileal loop and starved opiated guinea pig models. In addition to positive ileal loop responses with the toxin producing 3818-T and 3818-O, it became immediately evident that the penetrating non-toxin mutant 725-78 was capable of eliciting a positive loop. There remained the distinct possibility, however, that this non-toxigenic strain had reverted in the animals and produced toxin. To detect such revertants, 5 to 20 clones were isolated from each positive loop, and were tested for toxin production using the HeLa cell cytotoxicity test. No such revertant clones were found among these isolates from loops infected with the non-toxigenic strain. These results thus indicate that in vitro production of toxin is not an attribute which S dysenteriae 1 must possess to cause fluid loss in this model. In contrast to strain 725-78, the non-penetrating, toxin negative derivative 735-19 consistently failed to produce a positive rabbit ileal loop. Samples of intestine removed from rabbits and starved guinea pigs infected with either strain 3818-T, 3818-O, 725-78 or 735-19 were examined for the distribution of organisms and for pathological alterations in the intestinal mucosa using the fluorescent antibody technique and routine histologic procedures. The degree of mucosal alteration in each experimental group varied from animal to animal, but the distribution and localization of organisms and the evolution of mucosal lesions showed a uniform pattern. Similar results were found in both the rabbit loop and the small intestine of guinea pigs, although the changes in the latter were more extensive than those of the former. Both penetrating strains (3818-T and 725-78), and structural changes in the mucosa of the rabbit ileal loop and of the small and large intestine of guinea pigs; the degree of inflammation and mucosal alterations caused by the toxigenic penetrating strain 3818-T was more severe than that caused by the non-toxigenic, penetrating strain 725-72. The non-penetrating, toxin positive 3818-O remained localized in the bowel lumen, evoking minimal or no inflammatory response and no mucosal alterations. The intestinal mucosa of animals infected with the non-toxigenic, non-

penetrating strain 735-19, showed neither inflammation nor structural changes and therefore was indistinguishable from a non-infected control.

Because the non-toxigenic penetrating derivative 725-78 appeared fully virulent in the rabbit ileal loop and starved guinea pig models, we tested its disease evoking capacity in Rhesus monkeys. Six monkeys were fed strain 725-78 at a dose of 5×10^{10} cells and examined daily for illness. Three of the 6 monkeys developed typical bacillary dysentery. A total of 80 clones of 725-78 isolated from the disease monkeys were screened for toxin production to exclude the possibility of reversion to toxin production within the host. No such revertants were recovered.

The results of these studies on several animal models (rabbit ileal loop, starved guinea pig, monkey) employing an immunological and cytological approach indicate that the disease caused by a non-toxigenic penetrating hybrid is not distinguishable from that caused by the original toxigenic penetrating parent strain. Strains which have lost both of these properties are unable to cause disease and those which form toxin but do not penetrate bowel epithelium can not cause a pathogenic infection. Although these findings do not necessarily exclude a function for toxin in the pathogenesis of shiga dysentery, they emphasize that a far more important property for causing natural disease is the ability of the pathogen to penetrate and grow in the bowel mucosa.

TABLE I

Serological properties of *his*$^+$ *S. flexneri* hybrids obtained from matings with *E. coli* Hfr strains W3703 and Hfr 59

Donor Strain	No. *His*$^+$ Hybrids Tested	Agglutinin Class	*E. coli* O-25	*E. coli* O-8	*S. flexneri* type 2	*S. flexneri* Y (3,4)	No. each class
W3703	223	A	+	nt*	+	-	144
		B	+	nt	+	+	23
		C	-	nt	+	+	56
Hfr 59	247	A	nt	+	-	-	88
		B	nt	-	+	+	159

* nt: Not tested

TABLE 2

Studies in Animal Models with S. dysenteriae 1 Strain 3818T and Its Mutant Derivatives

Strain	Invasion	Toxin Production	Sereney* Test	Rabbit Ileal* Loop	Invasion of* Guinea Pig Intestine	Clinical Disease* in Monkeys
3818T	+	+	4/4**	6/6	4/4	9/15
3818O	-	+	0/4	4/4	0/4	0/15
725-78	+	-	4/4	7/8	4/4	3/6
735-19	-	-	0/4	0/4	0/4	Not done

1141

* See text for description of test

** No. positive / Total tested

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 121, Pathogenesis of enteric diseases

Literature Cited.

Publications.

1. Powell, Don W., Gerald R. Plotkin, Ronald M. Maenza, Leif I. Solberg, Don H. Catlin and Samuel B. Formal. 1971. Experimental diarrhea. I. Intestinal water and electrolyte transport in rat salmonella enterocolitis. Gastroenterology 60: 1053-1064.

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6. DuPont, H.L., R.B. Hornick, M.J. Snyder, J. P. Libonati, S. B. Formal, and E. J. Gangarosa. 1972. Immunity in Shigellosis. I. Response of man to attenuated strains of Shigella. J. Inf. Dis., 125: 5-11.

7. DuPont, H.L., R. B. Hornick, M.J. Snyder, J.P. Libonati, S. B. Formal, and E. J. Gangarosa. Immunity in Shigellosis. II. Protection induced by oral live vaccine or primary infection. J. Inf. Dis., 125: 12-16, 1972.

8. Skermam, F.J., S.B. Formal and Stanley Falkow. 1972. Plasmid-associated enterotoxin production in a strain of Escherichia coli isolated from humans. Inf. and Imm., 5: 622-624.

9. Gianella, R.A., S. B. Formal and G. Dammin. 1972. Secretory morphologic and invasive characteristics of Salmonella typhimurium. Gastroenterology 62: 752. (Abstract).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6436	72 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. ORD'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. MU /CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (f)			

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 72	4	125
	CURRENT 73	4	125

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Microbial Genetics; (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) 71 07 72 06. We have investigated the molecular nature of the transmissible, extrachromosomal fertility factor, P, of Vibrio cholerae. We found the P factor to be a supercoiled, circular DNA molecule with a molecular weight of 80 million daltons, and a guanine and cytosine (G plus C) base composition of 40 percent, which is significantly different from the 46 percent G plus C of the V. cholerae chromosomal DNA. In our continuing studies of Salmonella typhosa hybridization with Escherichia coli donors, we determined that the length of the E. coli chromosomal segments incorporated by recombination in S. typhosa is most frequently between 1 and 2 minutes. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* Available in correspondence upon validator's approval

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial Genetics and taxonomy

Investigators.

Principal: Louis S. Baron, Ph. D.

Associate: John A. Wohlhieter, Ph. D.; Edward M. Johnson, Ph. D.; Isaac R. Ryman; Charles A. Life; Janet R. Lazere, B. S.; SP5 Joseph A. Alexeichik, A. B.; SP4 William G. Craig, Jr.; PVT Richard Synenki, M. S.; and Archana Datta, Ph. D.

Description.

1. Escherichia coli Hfr donors bearing negative chromosomal markers were employed in matings with Salmonella typhosa recipients to establish the length of the E. coli chromosomal segments incorporated by recombination in the S. typhosa hybrids.

2. Salmonella typhosa hybrids which contain the receptor for phage λ and a region required for λ replication acquired from Escherichia coli K-12 become sensitive to this coliphage.

3. The fertility factor, P, of Vibrio cholerae which exists in cells as a supercoiled circular DNA molecule has been isolated and characterized.

Progress.

1. Recombination of Escherichia coli chromosomal segments in Salmonella typhosa.

As a genetic recipient in the cross with an Escherichia coli K-12 Hfr strain, Salmonella typhosa is capable of recombining transferred K-12 deoxyribonucleic acid (DNA) with homologous regions of its own chromosome. Frequently, however, the E. coli DNA persists, unrecombined, in S. typhosa as an addition to the Salmonella genome, thus creating a partially diploid hybrid. When an inherited E. coli gene determines a positive phenotypic character for which the corresponding Salmonella allele is a negative determinant, its diploid nature may be discovered by its loss, i. e., segregation, from the S. typhosa hybrid. However, the absence of detectable segregation, although suggestive of the haploid state of the E. coli gene, does not establish it.

To circumvent the problem of distinguishing haploid S. typhosa recombinants from stable (rarely segregating), partially diploid hybrids, we recently examined phenotypic expression of negative E. coli donor markers (for which the corresponding recipient alleles were positive) to indicate the haploid state of those E. coli genes in S. typhosa hybrids. We found that haploid inheritance of negative E. coli characters (as unselected markers) in S. typhosa hybrids occurred to a significant extent only when those characters were closely linked to the positive markers used for selection, indicating that recombination involves relatively short segments of the donor chromosome (see Annual Report, WRAIR, 1971). To determine the lengths of these E. coli DNA segments undergoing recombination in S. typhosa, we have, in the present study, concentrated on a single positive selective marker, rhamnose utilization (rha^+), and have examined the unselected inheritance of several closely linked negative donor genes.

Two negative allele-bearing E. coli Hfr strains were employed. E. coli Hfr WR2017 has the negative markers $metB^-$, $argH^-$, and $thiA^-$ situated within 2 min of the selective marker rha^+. Its transfer order is O-thiA-argH-metB-rha. The negative markers are thus transferred proximal (with respect to the transfer origin) to the rha^+ selective marker. E. coli Hfr WR2018 has the same transfer order as WR2017, and bears the negative marker $metE^-$, which is situated 1.5 min distal (with respect to the transfer origin) to the rha^+ marker; it also has the negative $argH^-$ marker. Matings between these Hfr strains, and the S. typhosa recipient strain, WR4200, were accomplished on a minimal agar selective medium, in which rhamnose (utilized by both of the donor strains but not by the S. typhosa recipient) served as the carbon source. In addition to the cysteine and tryptophan required by S. typhosa WR4200, methionine, arginine, and thiamine were included in the selective medium to allow recovery of those hybrids in which recombination of the negative donor alleles $metB^-$, $metE^-$, $argH^-$, or $thiA^-$ might occur. Counter-selection of the E. coli donor parent, in each instance, was accomplished by the omission of histidine, which both of the Hfr strains require for growth.

The inheritance pattern of 100 hybrids of S. typhosa WR4200, selected for receipt of the rha^+ marker E. coli Hfr WR2017 was as follows:

Hybrid Class	No.
rha^+	75
rha^+ $metB^-$ $argH^-$	18
rha^+ $metB^-$ $argH^-$ $thiA^-$	5
rha^+ $metB^-$	2

The negative donor allele, *metB*$^-$, most closely linked to the *rha*$^+$ marker at 0.8 min, was expressed by 25 hybrids, and 23 of these also expressed the next closest negative marker at 1.2 min, *argH*$^-$. However, only five hybrids expressed, in addition to the donor *metB*$^-$ and *argH*$^-$ markers, the least closely (1.8 min) linked *thiA*$^-$ marker. The haploid inheritance of *E. coli* DNA in this cross thus appears to fall off rather sharply at a distance of 1.2 to 1.8 min from the selected marker.

Recombinational inheritance of an *E. coli* marker located distal to the *rha*$^+$ selective marker was examined by mating *S. typhosa* WR4200 with *E. coli* Hfr WR2018. This Hfr transferred the negative *metE*$^-$ marker, which is located 1.5 min distal to *rha*, as well as the proximally located negative marker, *argH*$^-$. The inheritance pattern of the hybrids obtained from this cross was as follows:

Hybrid Class	No.
rha$^+$	61
rha$^+$ *argH*$^-$	26
rha$^+$ *metE*$^-$	13

Of the 100 *rha*$^+$ *S. typhosa* hybrids examined, 13 expressed the negative *metE*$^-$ allele. In addition, 26 other *rha*$^+$ selected hybrids from this mating expressed the donor *argH*$^-$ marker, which compares with the 23% haploid inheritance observed for this allele in the previous cross with WR2017. Significantly, no hybrid from this cross was found to express both negative donor markers, which would represent a minimum map distance of 2.7 min.

The results of these analyses indicate that the segment length of *E. coli* DNA most frequently incorporated by recombination in *S. typhosa* hybrids is between 1 and 2 min. Considering that only 5% of the *S. typhosa* hybrids derived from *E. coli* WR2017 were observed to incorporate as much as 1.8 min of donor chromosome, and that none of the WR2018-derived hybrids were seen to incorporate as much as 2.7 min, it is likely that recombination in this mating system normally does not involve DNA segments much longer than 2 min.

None of the *S. typhosa* hybrids derived from either of these matings which expressed a negative donor marker were observed to lose the selected *rha*$^+$ marker by segregation. This stability of the selected positive marker whenever closely linked negative donor markers are expressed in the hybrid was observed also in previous studies, and it is probable that in those instances, the selected marker has been incorporated by recombination along with the neighboring negative marker. On the other hand, all but 16 of the 61 hybrids obtained with *E. coli* WR2018 which expressed only the *rha*$^+$ marker were observed to be unstable (diploid) with

respect to this character. If we assume that most of those 16 *rha*$^+$ hybrids from which no segregants were detected are recombinants (which seems quite probable), and if we add in the 39% observed recombinational inheritance (26 *rha*$^+$ *argH*$^-$, 13 *rha*$^+$ *metE*$^-$) in this cross, it would appear that the overall incidence of recombination among *rha*$^+$ selected hybrids is in the neighborhood of 50%. In view of our previous finding of 52% recombination among *S. typhosa* hybrids selected for receipt of the *E. coli ara*$^+$ marker (Annual Report, WRAIR, 1971), it seems reasonable to conclude that roughly half of the *S. typhosa* hybrids obtained from this mating system will be recombinant with respect to the marker (and closely linked genes) for which they are selected.

2. Sensitivity to wild-type λ in *E. coli-S. typhosa* diploid hybrids.

The isolation of partial diploid hybrids from a cross between *E. coli* Hfr WR2004 as donor and *S. typhosa* Hfr WR4000 as recipient provided a means for establishing the continuous nature of the K-12 diploid genes within such hybrids. Thus, continuity of the K-12 genetic material could be demonstrated by transfer of the *E. coli* genes carried by the *S. typhosa* Hfr diploid hybrids to suitable F$^-$ K-12 recipients. The expected gradient of transfer frequencies, inheritance of unselected donor characters, and marker entry times, as determined by interrupted matings, were observed using these *S. typhosa* Hfr diploid hybrids as donors of K-12 genes.

Three relatively stable partial diploid hybrids containing continuous K-12 segments, which had previously been obtained from a cross between WR2004 and WR4000, were examined for sensitivity to wild-type λ. Two of these *S. typhosa* hybrid strains, WR4270 and WR4271 had been shown to contain and transfer an *E. coli* diploid segment which included the selective markers *pro*, *thr*, *ara*, *leu*, *arg*, *xyl*, and *fuc*, but not the *his* locus. The third *S. typhosa* hybrid, WR4272 possesses an *E. coli* genetic segment shown to be genetically complete through the *strA* locus, but not including the *fuc*$^+$ marker. All three of these *S. typhosa* hybrid strains were found to be sensitive to wild-type λ using the spot test procedure. To confirm the spot test results, these three *S. typhosa* hybrid strains were also examined in the standard TB agar overlay procedure for the assay of λ, with *E. coli* WR2000 employed as the control indicator strain for plaque formation. All three *S. typhosa* hybrids plated λ with an efficiency of plating (eop) of 1. The appearance of the plaques formed on the hybrids, however, was considerably different from those observed on the K-12 indicator strain WR2000. The plaques are smaller and less distinct on the *S. typhosa* hybrids than on *E. coli*; cloudy diffuse areas in the center of these faint plaques are poorly defined as compared to typical λ plaques.

The finding of sensitivity of these S. typhosa hybrids to wild-type λ is in distinct contrast to our previous results with S. typhosa hybrid WR4255, a strain which does not permit plaque formation by wild-type λ. WR4255 is lysed only by sx mutant of λ, or by phage hybrids between λ and phage 21 which, in addition to the immunity of phage 21, have obtained the N gene of this phage in place of the N gene of λ.

We also examined 24 newly isolated hybrids from a cross between WR2004 and WR4000 and found them to be uniformly sensitive to wild-type λ. Among these were strains similar to WR4272 which possess a physically continuous diploid E. coli chromosomal segment from proA through the strA locus.

Lysogeny of λ in S. typhosa hybrids. Although S. typhosa hybrid WR4255 is unable to replicate λ normally, phage production can be detected following transduction of the gal^+ genes by HFT lysates composed of λ and λdg. However, the expected lysogenic response seen with λ lysogens of K-12, wherein phage release is inherent in all clones, is not observed with the gal^+ transductants of WR4255. Rather, phage production by the gal^+ transductants of WR4255 is an unstable trait which can be permanently lost upon repeated cloning of the transductants. Despite the loss of phage producing ability by the transductants of WR4255, immunity to superinfection remains as a stable trait even after segregation of both phage producing ability and the λdg element. These results in conjunction with the non-inducibility of λ and λdg in transductants of WR4255 indicated that at least the immunity genes and perhaps the entire λ prophage was being maintained in this hybrid, but was no longer being replicated to form free phage as occurs in typical K-12 lysogens. The fact that S. typhosa WR4272 displays cloudy plaques when λ is plated on it was taken as presumptive evidence that λ lysogens could be isolated in this strain. Accordingly, the cloudy centers of a number of plaques were picked and purified on MEA agar. As previously reported with other similar E. coli-S. typhosa partial diploid strains, the colony type is seen as a small dense form which will occasionally segregate a translucent larger colony resembling the parent strain WR4000. Also correlated with colony type in the S. typhosa diploid is the presence of K-12 type I pili which can be detected by a slide agglutination test with antiserum prepared against E. coli K-12. Dense appearing colonies of WR4272 are diploid and uniformly carry the K-12 region extending from proA...leu...ara...thr...rm...pil...malB...rha...tna...xyl...mal...strA, whereas, the translucent segregant clones generally have lost all of these markers. To insure maintenance of the diploid K-12 genes in hybrids needed for further study, only the dense colonies which appeared after streaking from plaque centers were further purified by restreaking on MEA agar plates. These dense clones were then

tested for λ phage release and immunity to superinfection to establish the lysogenic state. Most of the dense colonies tested were immune to λ and free phage could be demonstrated in the supernatant fluid. Those dense colonies which did not release phage remained sensitive to lysis by λ and thus were considered non-lysogenic for λ.

A number of λ lysogenic derivatives of the S. typhosa diploid hybrid WR4272 were examined for conservation of the ability to release phage by replica-plating colonies onto overlays of the E. coli K-12 λ indicator strain WR2000. Over 4000 dense colonies tested in this manner showed that phage was released by all colonies, whereas in a similar experiment with heterogenotes of WR4255, loss of phage production was detected at frequencies of 50% or more of the colonies tested. In addition, a λ lysogenic strain of WR4272, designated WR4290, was subjected to thirty consecutive single dense colony clonings. At the end of this period, 400 individual dense colonies were tested for phage release as above and all were found to be still capable of producing λ. These results indicated that the λ lysogenic diploid S. typhosa clones of WR4290 were similar to K-12 in being able to maintain the normal lysogenic relationship with regard to phage release in contrast to the frequent loss of this ability by hybrid WR4255.

Quiescent nature of prophage λ in haploid segregants from S. typhosa diploids. To follow the fate of lysogenized λ after segregation of the entire K-12 diploid segment, a single translucent haploid segregant was picked from each of 100 individual dense WR4290 λ lysogenic colonies isolated on individual MEA plates. Each of the translucent colonies was shown on further genetic analysis to have lost all of the detectable markers of the E. coli diploid segment, and upon testing failed now to show phage release. We considered the possibility that loss of the K-12 segment might involve removal of K-12 genes affecting the vegetative replication ability of λ. The phage could still persist, however, in the prophage state in these segregants but would be unable to form free phage. If this were the case, the replacement of the diploid piece should result in the exposure of λ lysogeny evidenced by concomitant immunity to λ and phage release by all clones. To test this possibility, a translucent haploid segregant unable to produce phage was selected from the λ lysogenic diploid hybrid WR4290. This strain, WR4291, being negative for all testable K-12 markers, thus expressed a typical haploid phenotype. A mating was then performed using a λ sensitive E. coli Hfr, strain WR2009, to replace the requisite K-12 region (from pro^+ through the str marker) in WR4291. Hybrids of this mating were selected for acquisition of the diploid strains containing K-12 genes from pro^+ through str. This class of recombinants was previously shown to contain the necessary K-12 region for λ replication. After purification and

genetic analysis, 24 of the suitable diploid hybrid class were tested for release of λ and were all found to produce the phage again; they were also immune to superinfection by λ but sensitive to λ*imm*434 and λ*vir*. As expected, the parental translucent segregant WR4291 was unable to produce λ under the same conditions; its immunity pattern cannot be tested since it lacks the λ receptor and is, therefore, insensitive to lambdoid phages. These findings thus support the view that the diploid segment contains gene(s) necessary for vegetative replication of λ which, if lost by segregation, prevent the production of free phage in lysogenic cultures.

Preparation of low frequency transduction (LFT) and high frequency transduction (HFT) lysates of λ in S. typhosa hybrids. Occasionally, large diploid hybrids such as WR4272 will segregate derivatives which have lost a portion of their K-12 segment, but still maintain part of the previously inherited K-12 genes with a high degree of stability. A segregant of this type from WR4272 was encountered which conserved the K-12...*rha*...*tna*...*xyl*... region, having lost the *lac* and *fuc* markers. This strain, WR4272, proved to be sensitive to λ, and because it possesses the native *gal* allele of its S. *typhosa* parent, was studied to determine whether LFT and HFT lysates of λ carrying the *gal* operon of S. *typhosa* could be produced.

After S. *typhosa* hybrid WR4273 was lysogenized with λ*tl* to yield WR4272, a Gal^- mutation was introduced into WR4274 by mutagenesis with NTG to provide a recipient strain, WR4275 for the transduction experiment. The next step in the procedure was the production of a λ lysate by heat induction of the λ*tl* lysogenic, Gal^+ strain WR4274. This lysate, assumed to be an LFT, was found to contain approximately 5×10^9 pfu/ml when assayed on the λ sensitive S. *typhosa* hybrid WR4273. Transduction experiments were performed with this lysate at an moi of 5, employing WR4275 as the recipient for transduction of the S. *typhosa gal* operon. After a 72 hr incubation period, Gal^+ colonies which appeared on the MacConkey galactose agar plates were subjected to repeated purification. Most of these transductants were found to behave as unstable Gal^-/Gal^+ heterogenotes. HFT lysates were prepared by heat induction of these heterogenotes, and were shown to contain approximately 5×10^8 pfu/ml after assay on the WR4273 host.

Transduction of S. typhosa gal operon by HFT lysates. An HFT lysate from an S. *typhosa* hybrid heterogenote was compared with an HFT from an E. *coli* K-12 heterogenote to determine Gal^+ transduction frequencies. The λ lysogenic E. *coli* strain, WR3061, λ lysogenic S. *typhosa* hybrid WR4272 and λ insensitive S. *typhosa* hybrid WR4255 were employed as recipients. The results show that the HFT lysate from the S. *typhosa* hybrid heterogenote produced

a thousand-fold lower frequency of Gal$^+$ transductions to E. coli and to the S. typhosa WR4255 hybrid than is observed with the WR4275 recipient. With the E. coli HFT lysate, however, the differences in transduction frequencies among the three recipients were minor in extent. The similarity in transduction frequencies between E. coli WR3061 and S. typhosa hybrid WR4255 has been previously noted and is thought to reflect the presence of the K-12 restriction-modification (hsp) region which is located between the ara and pil markers known to be carried by WR4255. These results suggest that when λdg is propagated in S. typhosa it is subject to restriction by E. coli K-12 as well as by S. typhosa hybrid WR4255 which contains the restriction-modification locus of K-12. S. typhosa hybrid WR4275, which does not possess this K-12 region, and by inference, S. typhosa itself, do not restrict λdg propagated in K-12. To confirm the result exhibited by the presence of the K-12 restriction-modification locus, the WR4275 hybrid was back-crossed with E. coli Hfr WR2002 and an Ara$^+$ hybrid was obtained which was found to contain the K-12 pil hsp ara region. This strain, WR4276, was now able to restrict λdg previously propagated in heterogenotes of S. typhosa hybrid WR4275 to the same extent observed with the E. coli K-12 or WR4255 recipient.

Localization of K-12 chromosomal region allowing lytic replication of λ. The finding that haploid segregant WR4291 lacks a K-12 chromosomal region essential for the vegetative replication of λ suggested that a similar deficiency might be responsible for the observed behavior of WR4255. Our next experiments were, therefore, designed to ascertain the location of that region of the K-12 chromosome which would permit lytic replication of λ in the S. typhosa WR4255 hybrid. Since WR4255 already possesses the λ receptor as evidenced by its being transducible for the K-12 gal operon by λdg, we were able to employ it as the recipient in mating experiments with appropriate K-12 donors.

The fact that WR4255 does not have the ..rha..tna..xyl.. region of the K-12 chromosome present in hybrids such as WR4272 which plaque λ indicated an obvious K-12 region for transfer to WR4255. For this purpose, a K-12 Hfr strain, WR2015 having the transfer orientation O..metA..arg..rha..tna..xyl.. was employed as donor with WR4255 in a cross with selection for Rha$^+$ and Xyl$^+$ recombinants. After purification, genetic analysis of both the Rha$^+$ and Xyl$^+$ hybrids established them to be unstably diploid for the ..rha..tna..xyl.. region of the K-12 chromosome. Although these markers were rapidly segregated, it was possible to demonstrate by the spot test that when hybrids possessed this K-12 region, they were susceptivle to lysis by wild-type λ whereas the WR4255 parent could be lysed only by λsx. In characterizing this group of hybrids, we found that though most segregatns lost the entire ..rha..tna..xyl.. segment and became insensitive to λ, some segregants lost only the ..rha.. portion retaining the ..tna..xyl.. region and λ

sensitivity. This result thus eliminated the O..metA..arg..rha.. segment of WR2015 from contention as a region providing genes essential for λ replication to WR4255.

We next made use of two E. coli Hfr strains, WR2010 and WR2021 which transfer the ..tna..xyl.. segment of the chromosome from opposite directions. Hfr WR2010 has the tranfer orientation O..tna..xyl..malA..strA.., its point of origin being approximately at min 73 of the K-12 chromosome, while Hfr WR2021 has its point of origin at min 60 with a transfer orientation of O..strA..malA.. xyl..tna.. Approriate matings with these Hfr strains would provide limits for the chromosomal region containing the gene(s) necessary for λ replication in WR4255. Xyl^+ hybrids of WR4255, obtained using both of the above K-12 Hfr donors, were found to show λ sensitivity. On the other hand, Rha^+ hybrids from a cross with the K-12 F'14 donor, WR2016 which carries the ..arg..metB..rha..ilv.. region, on acquiring this episome, remain insensitive to λ. These results indicate that the necessary genetic region responsible for λ replication resides within the K-12 chromosomal segment which extends from the origin of Hfr WR2010 near min 73 to the origin of Hfr WR2020 at min 60.

The region required for λ replication was refined somewhat by employing a K-12 F-merogenote strain which we isolated as a partial deletion. The F-merogenote strain, WR2030, deleted from the ..mal..strA.. region, was characterized as having retained the ..pyrE..mtl..xyl.. segment of the K-12 chromosome (approximately min 72 to min 66). This episome could be transferred at high frequency to WR4255 by selection for Xyl^+ recombinants. Such Xyl^+ hybrids became sensitive to λ, reverting to insensitivity when the episome is lost on segregation.

Restoration of thermoinducibility to λtldg in S. typhosa hybrid transductant WR4255 (λtldg). We have previously reported on a transductant of S. typhosa hybrid WR4255 inferred to be carrying the defective phage λtldg. In the Gal^+ state, WR4255 (λtldg) is immune to λ as determined by its insensitivity to λsx while exhibiting sensitivity to λimm434sx. Gal^- isolates behaved as typical prophage segregants because they become sensitive to λsx indicating that immunity to λ is lost in association with loss of the gal genes (2). Despite this behavior, λtldg is not inducible in the WR4255 (λtldg) transductant by procedures which result in the lysis of similar K-12 transductants carrying λtldg. We, therefore, considered the possibility that the non-inducibility of λtldg in WR4255 (λtldg) could also be due to the absence of the appropriate K-12 λ replication region. Consequently, the F-merogenote strain WR2030 was mated with the WR4255 (λtldg) transductant for transfer of the λ replication associated ..mtl..xyl.. region and Xyl^+

hybrids were then tested for temperature inducibility of the λtldg element. The results of such an induction experiment show that restoration of thermoinducibility is conferred on λtldg when the WR4255 (λtldg) transductant contains the F..mtl..xyl.. episome. These results make it apparent that the λ replication gene(s) needed for vegetative λ replication after infection are also essential for successful induction of λtldg in the WR4255 lysogen.

Acquisition of sensitivity to λ derivatives by NG induced mutants of S. typhosa hybrid WR4255. We were interested in determining whether it was possible to isolate mutants of the λ insensitive S. typhosa hybrid WR4255 which would be susceptible to wild-type λ and its derivatives. We, therefore, subjected WR4255 to NG treatment and plated the treated cells on MEA agar plates. Individual colonies which appeared were purified and then grown in TB medium for use as indicator strains for phage sensitivity. The NG treated strains were initially screened for sensitivity by plating approximately 500 pfu of λvir (as determined on E. coli K-12 strain WR2000) on them. Out of about 300 colonies examined, in this manner, three mutants were detected on which plaques were produced by λvir. None of the three mutants plating λvir were susceptible to wild-type λ, though one of the mutants was now susceptible to λimm434, in addition to λvir.

3. Isolation and characterization of supercoiled circular DNA molecules from V. cholerae.

The P factor is a fertility factor that is transmitted between V. cholerae cells at high frequency and occasionally transfers some chromosomal genes along with it to a recipient cell.

We examined the V. cholerae strain V58P$^+$ for the presence of supercoiled circular molecules that could be associated with the P factor. To detect the presence of a supercoiled circular component, labeled cells were gently lysed and centrifuged to equilibrium in an ethidium bromide-cesium chloride (EtBr-CsCl) density gradient. In such a gradient, supercoiled circular DNA bands as a separate component distinct from the rest of the chromosomal DNA so that the circular DNA can be isolated when the gradient is fractioned. The DNA from V. cholerae V58P$^+$ did contain two such components, one the cellular or chromosomal DNA and the other smaller component containing supercoiled circular DNA. The ration of the counts in the two components indicated that about 3% of the total DNA from V58P$^+$ was in the supercoiled circular form. When active donor cultures of V58P$^+$ were used this procedure always resulted in the detection of a supercoiled circular component containing 2-4% of the total DNA. As a control, the V. cholerae V58P$^-$, which is isogenic to V58P$^+$ except that it lacks the P factor, was processed in the same way and run at the same time. This P$^-$ strain contained a small

amount of DNA in the supercoiled circular form. The amount of supercoiled circular DNA was always small and was observed occasionally. When this circular component was detected in V58P$^-$, it was never more than 1/10 of the circular component which was always present in V58P$^+$ donors.

The P factor can be transferred to a number of other V. cholerae strains including V63P$^-$ and GS-1-65. These strains were then examined for the presence of supercoiled circular material by the dye-buoyant density method. A comparison was made between these strains both with and without the P factor. The results were similar to those observed for V58P$^+$ and V58P$^-$. In addition, when the P factor was transferred from V63P$^+$ to V58P$^-$, the newly isolated V58P$^+$ donor strain consistently showed supercoiled circular DNA fractions smaller in amount to that present in the original V58P$^+$ strains.

The supercoiled circular DNA isolated from the P$^+$ and P$^-$ strains was then subjected to sucrose density gradient centrifugation to determine the molecular weight of the different circular components. Supercoiled circular DNA that has been isolated by the dye-buoyant density method may show two peaks when centrifuged in a sucrose gradient. The faster moving peak is the intact supercoiled circular DNA and the slower fraction represents the corresponding open circular or linear DNA that results from strand breakage. Using the ^{14}C labeled λ DNA as a reference, it is possible to calculate the sedimentation coefficient of the two components. In the P$^+$ supercoiled circular preparation, the faster moving one corresponding to the supercoiled circular form of the molecule had a sedimentation coefficient of 84s. The molecular weight of the supercoiled circular DNA from V58P$^+$, GS-1-65P$^+$, and V63P$^+$ was calculated to be 80 million daltons using the relationship between the sedimentation coefficient and molecular weight. The sedimentation coefficient of the slower moving one was 54s and in the range expected for open circular DNA of 80 million daltons.

The sedimentation velocity in sucrose of the circular DNA isolated from the P$^-$ strain was also measured. The circular DNA from the P$^-$ strains did not contain any 84s material, but contained two components, one 58s and the other 43s. The 58s peak is the supercoiled circular DNA that has a molecular weight of 40 million daltons. The other component is the open circular or linear form of the same molecular weight.

An examination of the supercoiled circular DNA isolated from the V58P$^+$ cells in the electron microscope showed large circular molecules and in addition, a few smaller circular molecules. A number of both kinds of molecules were photographed and measured. See Table 1. The large molecules had a molecular weight of 80 million daltons which agree well with the molecular weight

TABLE 1

ELECTRON MICROSCOPY OF CIRCULAR DNA

Origin of Circular DNA	No. of Molecules Measured		Average Length (μ)	Molecular Weight (x 10^6 daltons)
V. cholerae V58P$^+$	a (large)	11	41.09 ± 1.78	78.5 ± 3.4
	b (small)	9	19.20 ± 1.97	36.7 ± 3.6
V. cholerae V58P$^-$		17	20.21 ± 1.83	38.6 ± 3.5

calculated from the sedimentation coefficient. While no smaller molecules were detected by sucrose gradient centrifugation, the presence of smaller molecules in P^+ cells is not unexpected since similar size molecules were observed in the P^- strains and the P^+ and P^- strains are isogenic except for the presence of P.

An attempt was made to determine the G + C composition of the P^+ circular DNA. The ^3H labeled P^+ circular DNA that was isolated by the dye-buoyant density method was combined with unlabeled V. cholerae DNA extracted from $V58P^+$ and centrifuged to equilibrium in a CsCl density gradient. The density gradient was fractionated and the unlabeled V. cholerae DNA was located by optical absorbance at 260 nm. The ^3H labeled P^+ was detected by radioactive counts. The majority of the P^+ circular DNA was less dense than the V. cholerae chromosomal DNA, it was difficult to calculate the G + C composition from such an experiment.

In order to calculate the G + C composition of DNA using the Marmur relationship between density and G + C composition, the DNA has to be run in an analytical ultracentrifuge. $V58P^+$ DNA was fractionated in a preparative CsCl density gradient, and the lighter fractions were pooled and run in the analytical ultracentrifuge. In addition to the V. cholerae chromosomal DNA, another lighter DNA component was observed. This result confirms the previous experiment which showed the labeled circular P^+ DNA had a lighter density than V. cholerae DNA. It was possible to calculate that the G + C composition of the P^+ DNA was 42% G + C, which is significantly less than the average 48% G + C composition of the V. cholerae chromosomal DNA.

No separate component was observed when the $V58P^-$ DNA was fractionated and examined in the same fashion in a CsCl density gradient in the analytical ultracentrifuge. In addition, the labeled supercoiled circular DNA isolated from the $V58P^-$ strain banded with the V. cholerae chromosomal DNA when the two were mixed and centrifuged together. Thus, the P^- supercoiled circular DNA isolated from the various V. cholerae strains has the same average G + C composition as the V cholerae chromosomal DNA and differs from the G + C composition of the P^+ DNA.

Summary and Conclusions.

1. Escherichia coli Hfr donors bearing various negative chromosomal markers located within 2 min of the rha locus were employed in matings with a Salmonella typhosa recipient. S. typhosa hybrids selected for receipt of the donor rha^+ marker examined for the expression of these negative markers as indicators of genetic

recombination. The E. coli markers metB⁻ and argH⁻, situated within 1.2 min of the selected rha⁺ marker, were inherited by recombination along with the selected marker by 23 to 26% of the hybrids examined. The E. coli metE⁻ marker, located 1.5 min from rha⁺, was inherited by recombination in 13% of the E. coli thiA⁻ determinant occurred in only 5%. Linked recombinational inheritance was not detected for E. coli markers separated by 2.7 min. These findings indicate that the segment length of E. coli DNA most frequently incorporated by recombination in S. typhosa hybrids is between 1 and 2 min.

2. Hybrids between Escherichia coli K-12 and Salmonella typhosa which conserved a continuous K-12 chromosomal diploid segment extending from pro through the strA locus were sensitive to plaque formation by wild-type λ. These partially diploid S. typhosa hybrids could be lysogenized with λ and subsequently induced to produce infectious phage particles. When the K-12 genes were segregated from a lysogenic S. typhosa hybrid, phage productive ability was no longer detectable due to loss of a genetic region necessary for vegetative replication of λ. However, λ prophage was shown to persist in a quiescent state in the S. typhosa hybrid segregant with phage productive ability being reactivated after replacement of the essential K-12 λ replication region. Low frequency transduction (LFT) and high frequency transduction (HFT) lysates containing the gal⁺ genes of S. typhosa were prepared by induction of λ lysogenic S. typhosa hybrids indicating that the attλ site is chromosomally located in S. typhosa in close proximity to the gal locus as in E. coli K-12. After propagation in S. typhosa hybrids, λ was subject to restriction by E. coli K-12 recipients thus establishing that λ DNA is modified by S. typhosa. Hybrids of S. typhosa, however, did not restrict λ grown previously on E. coli K-12. The K-12 genetic region required for λ phage production in S. typhosa was located within min 66 to min 72 on the genetic map of the E. coli chromosome. Transfer of an F-merogenote encompassing the 66 to 72 min E. coli chromosomal region to λ insensitive S. typhosa hybrids enabled them to replicate wild-type λ. The λ insensitive S. typhosa hybrid, WR4255, which blocks λ replication, can be mutagenized to yield mutant strains sensitive to λvir and λimm434. These WR4255 mutants remained insensitive to plaque formation by wild-type λ.

3. We have shown that there are supercoiled circular DNA molecules present in the V. cholerae strains with the P⁺ fertility factor. These P⁺ circular DNA molecules have a molecular weight of 80 million daltons and an average G + C composition of 42% which is substantially different from the average G + C composition of the V. cholerae chromosomal DNA which is 48%. In addition, we found that all of the V. cholerae strains tested had some smaller supercoiled

circular DNA molecules present. These DNA molecules had a molecular weight of about 40 million daltons and had an average G + C composition the same as the V. cholerae chromosomal DNA. The function of these smaller molecules are not known, but they might be due to some kind of lysogenic phage present in the cell. While no recipient was found for the P^+ fertility factor that lacked some kind of a supercoiled circular component, there was no difficulty in distinguishing the P^+ DNA in the P^- cells because of the difference in molecular weight and G + C composition.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial Genetics and Taxonomy

Literature Cited.

References.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6446	72 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
710701	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	3A062110A822	00	123
b. CONTRIBUTING				
c. [redacted]	CDOG 114(F)			

11. TITLE (Provide with Security Classification Code)

(U) Histopathologic Manifestations of Diarrheal Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 26 00 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
b. NUMBER: Not Applicable
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM. AMT.
EXPIRATION

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR (PRECEDING) 72	6	100
FISCAL YEAR (CURRENT) 73	5	150

19a. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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NAME: DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U)Diarrhea; (U)Intestine; (U)Electron Microscopy; (U)Infection; (U)Toxin

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

23. (U) This work unit is mainly concerned with studies on the mechanisms of diarrheal diseases of infectious origin with the greatest emphasis on ultrastructure. These studies are considered essential parameters for a comprehensive and scientifically based treatment of military personnel with diarrheal diseases and enteric infections.

24. (U) Utilizing various morphologic techniques including light and electron microscopy, histochemistry and cytochemistry, autoradiography, immunotracer method, histologic and ultrastructural alterations of the gastrointestinal tract in diarrheal diseases and those of other organs are being studiod in experimental animals and human subjects.

25. (U) 71 07-7206 A review paper, Penetration of the intestinal epithelium by various microbes, has been published. The study of the epithelial penetration has been extended into an adenovirus and Ent. histolytica. Studies on the effects of cholera and shigella enterotoxin upon the gut mucosa are nearly complete. Cholera infections in man are currently being studied which provides ultrastructural evidences of hypersecretion by epithelial cells and alterations of capillary endothelial cells. The interaction between epithelial cells and anaerobic spirochetes by scanning and transmission electron microscopy are covered by two papers currently in press. A paper, Duodenal ulceration in fasting guinea pigs, has been submitted for publication. Collaborative studies with other Divisions on diseases of the kidney, heart, have been completed and are in preparation for publication.
For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 71 - 30 June 72.

26. (Applicable to contractor's summary)

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Diarrheal Disease

Investigators.
Principal: Akio Takeuchi, M. D.
Associates: Maj. Jack Zeller; Maj. Joseph Ballo; Maj. Raymond Nagle; Maj. John Mooney; Helen R. Jervis, Dr. Nat. Sc.

Problem

To clarify the mechanisms of diarrheal diseases with emphasis on those of infectious origin which have military importance.

Background

This department has, since its inception, been concerned with studies of diarrheal diseases. Those mediated by bacterial enterotoxins and those caused by invasion of enteric pathogens have been continuously studied. During FY 1972, under the new Division Director and Department Chief, the department has strengthened its collaborative and supportive activities with various in-house research projects in WRAIR, WRGH and AFIP.

Approach to the Problem

Utilizing various morphologic techniques including light and electron microscopy, histo- and cytochemistry, autoradiography, fluorescent antibody technique and immuno-tracer method, this department studies histo- and ultrastructural manifestations of the bowel in diarrheal diseases and those of other organs in experimental animals and human subjects.

Results and Discussion

1. Studies on diarrheal diseases.

In most enteric infections, penetration of the gut epithelial barrier is the first step in establishing lesions. Yet, the study of this first step had long been neglected. This department has continuously studied the mechanism of this first step by various organisms including the shigella, salmonella, coccidia, intestinal spirochetes and Giardia, the summary of which has been published in two review papers (Takeuchi 1971) (Takeuchi 1972). The study of this first step has extended into other types of organisms which now include adenovirus (Takeuchi and Hashimoto 1972) and Entamoeba histolytica (Takeuchi and Phillips 1972).

Injection of the cholera enterotoxin into the small bowel wall in rabbits is not associated with fluid accumulation in contrast to that usually seen with intraluminal installation which further provides evidence that cholera toxin effects primarily the small gut epithelium then stimulates fluid exsorption into the gut lumen. The results are currently in preparation for publication (Sheahan and Sprinz 1971).

Collaborative studies on cholera vibrio infections in man have been currently under investigation with SEATO Laboratory, National Institute of Allergy and Infectious Diseases and Peter Bent Brigham Hospital have been progressing well. Our electron microscope studies showed ultrastructural evidences of hypersecretion by the intestinal epithelial cells and of alterations of capillary endothelial cells in the lamina propria.

Studies of the pathogenesis of amoebic lesions in the gut have been reinvestigated by experimentally producing lesions in the cecum of guinea pigs with trophozoites of Ent. histolytica in collaboration with the National Institutes of Health. In this model, we have demonstrated for the first time that at the ultrastructural levels, Ent. histolytica penetrates the intact epithelial barrier from the lumen without eliciting "lytic changes" at the site of penetration (Takeuchi and Phillips 1972). The work further clarifies that "lysis of tissue" and ulcer formation, the hallmark of clinical amoebic dysentery, are the result of secondary effects from disruption of neutrophils, accelerated epithelial cell turnover and altered microcirculation in the lamina propria after penetration.

Spiral-shaped organisms occupy a significant proportion of the normal enteric flora of mammals including man. Little is known about these organisms because these fastidious anerobes have thus far been impossible to culture and isolate (Takeuchi 1972). We extended studies on these organisms in our monkey model (Takeuchi and Zeller 1972) (Zeller and Takeuchi 1972). Using transmission and scanning electron microscopes, we were able to identify the morphologic character of these organisms in their natural environment which provides the baseline for further clarification of the role of these organisms in man.

Studies on acute duodenal ulcerations in fasting guinea pigs have been completed and presented at the FASEB meeting (Jervis et al 1972). A paper entitled "Acute duodenal ulcerations due to fasting in the guinea, delineation of the experimental model" has been submitted for publication in Archives of Pathology.

2. Collaborative studies with other divisions within WRAIR and WRGH.

(a) Experimental Pathology in nephrology. Collaborative studies on experimentally produced nephropathy has been expanded with

the Division of Medicine (Nagle et al 1972). An acute obstructive nephropathy experimentally produced in rabbits were studied by a multidisciplinary approach. Glomerular filtration rate, osmolar clearance, free-water reabsorption and glucose reabsorption were determined for the obstructed and contralateral kidney. All of these determinations demonstrated significant decreased in the obstructed kidney and were well-correlated with ultrastructural and histochemical studies. A part of this joint investigation was presented in the annual meeting of the American Association of Pathologists and Bacteriologists (Nagle et al 1972 [a]). A complete paper on this project has been submitted for publication in Laboratory Investigation.

(b) In conjunction with Renal Transplant Service, WRGH, histopathological and histochemical studies on nephrectomized kidneys and renal biopsies are currently under investigation.

(c) In collaboration with the Division of Medicine, the effect of reestablishment of blood flow to areas of acutely ischemic myocardium was investigated in dogs, by physiologic and morphologic parameters. Biopsies obtained following two hours of ischemia were noncontractile and elicited injury potentials to an epicardial recording electrode. Ultrastructure examination of such biopsies demonstrated distinct mitochondrial changes, deranged nuclear chromatin, disrupted sarcoplasmic reticulum and disorganized myofibrillar structure. Upon reestablishment of blood flow, all of these changes except the damage to the filaments were substantially repaired at the end of 24 hours after reflow was initiated. This information provided an important baseline for recent clinical efforts in surgically reestablishing coronary blood flow to the acutely ischemic heart. A portion of this study has been presented and this successfully joint study will be completed soon (Ballo and Snow 1972).

(d) In collaboration with the Division of Communicable Diseases and Immunology, a series of experiments are in progress which consist of in vitro studies on the phagocytic role of mouse macrophages utilizing synthetic liposomes, with known quantities of antigen as phagocytic material. The objective is to better understand the precise role of complement and antibody in phagocytosis.

(e) This is a light and electron microscope study of the composition of microaggregates that form in blood under normal clinical storage conditions. Before storage there are no aggregates. In the first few days of storage microaggregates are of variable size and composition form. Most of the smaller aggregates resemble reversible aggregates, as induced by adenosine diphosphate. Larger aggregates resemble irreversible aggregates, as induced by low con-

centrations of thrombin and collagen. The nuclei of degenerate neutrophils which appear on the third to fourth day of storage evert from the cell interior, adhere to platelets and augment the size of platelet aggregates. Fibrin and red blood cells do not participate in the formation of aggregates, although earlier studies by other authors, using the light microscope, indicated otherwise. All types of aggregates persist throughout the course of storage.

This study has determined the composition of the variety of types of microaggregates that form in stored blood. Many of these microaggregates are irreversible and thus should be called microthrombi. The appearance, in vivo, of these microthrombi in human beings will be the objective of another study.

With the use of Coulter Counter Model T, Major Solis, Division of Surgery, WRAIR, has investigated the size and number of these microaggregates in stored blood and his results complement our findings.

Conclusions and Recommendations

While enteric infections share a common basic pattern of morphologic responses in the host, the nature of microbes and the types of host-microbe interactions in the enteric epithelial barrier result in significant differences in the subsequent pathologic mechanisms. Electron microscopic observations have greatly contributed to the clarification of these mechanisms. However, further studies are required to define the extent and nature of this mechanism.

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Diarrheal Diseases

Literature Cited.

References:

1. Sprinz, H.: Pathogenesis of intestinal infections. Arch. Path. 87: 556, 1969.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6485	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SUM'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	125
b. CONTRIBUTING				
c. [illegible]	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Hematology of Nutritional Deficiencies of Military Importance (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 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 72	2	45
FISCAL YEAR CURRENT 73	2	45

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
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NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Diet; (U) Intestine; (U) Iron; (U) Protein; (U) Hemoglobin; (U) Vitamin B-12

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

23. (U) The nutritional anemias are correctable diseases that are commonplace in geographic areas of military importance. These diseases markedly reduce the capability of affected populations to perform work or sustain a military effort and remain self-supporting.

24. (U) Establishment of standards and standard methods for detection and quantification of these diseases. Studies of the nutrient content of various foodstuffs and the availability of these nutrients for absorption from these foodstuffs in normal subjects and in populations where nutritional deficiencies and chronic infections are commonplace.

25. (U) 71 07 - 72 06 Trypsin, an exocrine excretion of the pancreas was shown to be essential for the absorption of dietary vitamin B-12 in both experimental animals and in certain patients with pancreatic insufficiency and a megaloblastic anemia. In collaboration with WHO and ICSH standard methods for the measurement of serum iron concentration and for standardizing these methods were developed. Several factors preventing the standardization of measurements of the total iron-binding capacity in serum specimens were identified. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 71 - 30 Jun 72.

*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 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 125, Hematology of Nutritional Deficiencies of Military Importance

Investigators.
Principal: COL Marcel E. Conrad, MC
Associate: MAJ Phillip P. Toskes, MC; Harold L. Williams

Description.

Standard methods for the measurement of serum iron concentration and the serum transferrin concentration were investigated in collaboration with the International Committee for Standardization in Hematology and World Health Organization. Studies of the problems involved in the measurement of iron-binding capacity in serum were continued. Intestinal factors affecting the absorption of iron and vitamin B_{12} were investigated.

Results.

During the last five years, this laboratory has made and distributed serum specimens to nine international laboratories participating in a collaborative endeavor to establish standards and standard referee methods for the measurement of iron and iron-binding protein in serum specimens. A simple method that permits the reproducible measurement of iron in serum specimens in the various collaborating laboratories was developed and reported.

In our laboratory the standard magnesium carbonate method for measurement of the total iron-binding capacity and unsaturated iron-binding capacity was studied to disclose causes of error and to improve techniques. Failure to regulate the pH of reaction mixtures at all stages of the procedure and inadequate amounts of protein in the specimen each caused a significant error in measurements of both the total and unsaturated iron-binding capacity. An exchange of unbound radioiron in the iron-saturating solution with transferrin-bound iron caused an increase in unsaturated iron-binding capacity. The use of undiluted serum specimens, regulation of the pH of iron-saturating solutions, and the addition of sodium barbital buffer to serum before the addition of iron-saturating solution seemed to improve these measurements. More recently we found that the iron-binding capacity

is significantly increased in aged and lyophilized serum specimens and that this error is accentuated if the lyophilized specimen is stored at room temperature, the serum iron concentration of the specimen is elevated and is more abnormal with certain batches of magnesium carbonate than others. These latter variables will be proposed for group study within the next few months based upon our observations. It is believed that the factors mentioned make other serum proteins--and most particularly albumin--a protein that binds iron. This possibility is currently being investigated.

Iron deficiency is a worldwide problem and may mask the coexistence of folate and vitamin B_{12} deficiency. In many publications iron deficiency has been associated with a deficiency of serum folate. Thus experimental studies in animals were initiated in which some animals were fed an iron-deficient diet and others received an iron-replete diet. Both diets contained adequate amounts of folate and vitamin B_{12}. Following 12 weeks of experiment, the animals maintained on an iron-deficient diet were not only iron-deficient but also had a significantly low serum folate level. Although lactase values in the gut of affected rats were diminished, maltase, sucrase, B_{12} absorption, fecal fat excretion, C^{14} transport, and the absorption of C^{14} glucose were normal. The histology of the gut and quantification of both aerobic and anaerobic intestinal bacteria were normal. Thus folate metabolism can be linked to iron absorption and this abnormality requires additional investigation.

Vitamin B_{12} malabsorption was found in patients who had intrinsic factor and evidence of pancreatic insufficiency. These patients had diminished serum levels of vitamin B_{12} and a megaloblastic anemia. Gastric secretions contained both free acid and intrinsic factor. There was poor absorption of vitamin B_{12} when administered alone or with intrinsic factor. The addition of either pancreatic extract or trypsin to an oral dose of vitamin B_{12} and intrinsic factor normalized absorption. This was not observed when pancreatic extract was added to vitamin B_{12} in the absence of intrinsic factor. An animal model of this condition was made by partially pancreatectomizing rats. Studies in these animals showed that the pancreatic factor was soluble, heat labile, acid stable, and non-dialyzable. It passed through an Amicon PM 30 membrane and had a molecular weight greater than 5000 on Sephadex columns. Enzymatic studies showed it had the characteristics of trypsin. Studies were performed in rats to determine if the pancreatic factor was necessary to allow gastric intrinsic factor or the B_{12} ileal receptor site to function optimally. Gastric intrinsic factor obtained from pancreatectomized rats with

B_{12} malabsorption was shown to stimulate B_{12} uptake in control rat ileal sacs. Further, when ileal sacs were prepared in pancreatectomized rats and control rats and exposed to gastric intrinsic factor from control rats, B_{12} absorption was similar in both groups of animals. Thus the mechanism of action of the pancreatic factor appears not to be mediated by an effect upon gastric intrinsic factor or the ileal epithelial cell receptor, but by an effect upon an as yet poorly described intraluminal phase of vitamin B_{12} absorption.

Although potassium deficiency has been associated with decreased intestinal motility, little is known about the effects of the depletion of this cation on intestinal function and integrity. Thus rats were fed a low potassium diet for one month. These animals exhibited decreased growth rate and had proteinuria. The serum potassium concentration and muscle potassium concentration were decreased with an increase in the sodium concentration in both organs. Grossly, the proximal and distal small intestine appeared dilated in potassium-deficient animals. In vivo measurements of transmural potential difference in the jejunum and ileum was less negative than in control animals. Iron absorption was increased in the potassium-deficient animals. However, this was believed to be caused by the diminished intestinal motility because measurements of iron absorption from isolated intestinal loops were similar in both normal and potassium-deficient animals. There was no difference in stool fat excretion or intestinal disaccharide levels between the two groups. Histologic sections demonstrated the characteristic changes of potassium deficiency in the kidneys and heart, but the intestine appeared normal by light microscopy.

Conclusion and Recommendations.

An acceptable and reproducible method was developed and studied for the measurement of iron in serum specimens that has been recommended for use as a referee method by WHO and ICSH. A similar method for measuring iron-binding protein is needed. A number of factors leading to technical error in the performance of measurement of the iron-binding capacity of serum were identified. Studies were made of the effect of potassium depletion upon the gut, the relationship of folate and iron absorption, and of the pancreas and vitamin B_{12} absorption.

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 125, Hematology of Nutritional Deficiencies of Military Importance

Literature Cited.

Publications:

1. Conrad, M.E.: Iron storage diseases. In: Current Diagnosis. Ed. Conn, H.F., W.B. Saunders, Philadelphia, 1971, p. 673.

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PROJECT 3A062110A823
MILITARY PSYCHIATRY

Task 00
Military Psychiatry

1173-a

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6470	72 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
71 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	6211OA	3A062110A823	00	030
b. CONTRIBUTING				
c. CONTRIBUTING	CD OG 114(F)			

11. TITLE (Precede with Security Classification Code)

(U) Military Psychiatry (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002500 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: Not Applicable 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 72	8	220
FISCAL YEAR CURRENT 73	8	200

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) Stress Performance; (U) Human Volunteer; (U) Military Adjustment; (U) Psychiatric Treatment; (U) Endocrine Response; (U) Aggressive 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 psychologic, sociologic, organizational and physiologic factors which predispose the soldier to perform ineffectively or develop psychiatric, psychosomatic disease or drug addiction, and to develop appropriate preventive and treatment techniques.

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

25. (U) 71 07 - 72 06 A study was completed investigating racial perceptions by soldiers at various posts. Studies in readjustment patterns and difficulties of CONUS troops in combat units who have served in Vietnam were completed. Data has been collected in a descriptive study of poly-drug users in the Army and comparisons of two groups of Vietnam addicts are being made according to a number of variables. Data concerning the establishment of a drug treatment program has been obtained, and the study currently is being completed. A pilot study investigating the aspects of staff functioning in service delivery at the Mental Hygiene Consultation Service at Fort Meade has been completed. Project design for a retrospective and prospective study of command social and personal determinants, and the prevalence and spread of opiate and other drug use has been submitted for CONARC approval. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*Available to contractors upon originator's approval.

DD FORM 1498 1 MAR 68 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 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Investigators.
Principal: COL Harry C. Holloway, MC
Associate: LTC James T. Grosshans, MC; MAJ Donald R. Bardill, MSC; MAJ Jonathan F. Borus, MC; MAJ Edgar P. Nace, MC; CPT Larry H. Ingraham, MSC; and David H. Marlowe, Ph.D.

I. INTRODUCTION

Within the Department of Psychiatry increasing emphasis has been placed upon studies of military mental health principles and the delivery of services with the major focus being on drug abuse problems. Data was gained from field studies of the reentry and adjustment process of Vietnam veterans, racial perceptions, the development of a drug treatment program, delivery of mental health services, and drug-using active duty personnel.

II. ADJUSTMENT PROCESS OF VIETNAM VETERANS AND RACIAL PERCEPTIONS

MAJ Borus is completing his work on the reentry and readjustment process of Vietnam veterans. He has completed three papers based on interview studies of Vietnam returnees at Fort Meade, defining the common adjustment issues facing these veterans, differentiating successful from less successfully coping returnees, and proposing a military intervention program to facilitate healthy readjustment. As chairman of a Special Session of the American Psychiatric Association, MAJ Borus presented these findings at the 1972 Annual Scientific Meeting in Dallas. Statistical analysis of the prospective veteran adjustment study of 1000 men at Fort Meade is now in progress.

Work with the Racial Perceptions Inventory (RPI) was also continued in conjunction with CPT Byron G. Fiman of Computer Support to Military Psychiatry (COMPSY), CPT M. Duncan Stanton of Walter Reed General Hospital (WRGH), and SP5 Albert F. Dowd. An initial paper by these investigators on the RPI was published in the May 1972 American Journal of Psychiatry. Major use of the RPI at Fort Benning, U.S. Army Hawaii (USARHAW), and Walter Reed Army Medical Center (WRAMC) has provided a data base of 1384 respondents. Three reliable factors have been delineated in the RPI items, and the data is currently being compliled in papers dealing with development of the instrument and racial perceptions

of Vietnam. A revised streamlined edition of the RPI has been produced, and an administration manual has been composed to allow independent utilization of the RPI by interested Army posts. Contracting for further development and study of the instrument is to be coordinated with the Office of Deputy Chief of Staff for Personnel (ODCSPER) and the Office of the Chief of Research and Development (OCRD). Findings from the RPI were presented by MAJ Borus at the Army Medical Department conference in Denver, Colo. and the Psychology in the Air Force Symposium of the Air Force Academy.

III. DELIVERY OF MENTAL HEALTH SERVICES

During the early part of the year, field studies relating to the delivery of mental health services continued to progress on a limited basis. As a prelude to the originally projected larger study of the delivery of mental health services by an Army Mental Hygiene Consultation Service (MHCS), MAJ Bardill initiated a pilot study of service delivery at the MHCS at Fort Meade, Maryland. In the beginning, plans were to study aspects of clinic staff functioning in service delivery, but a preliminary examination of staff activities revealed that the social work/psychology specialists, because of overwhelming numerical strength, are the staff sub-group having the largest amount of patient contact. Given the amount of staff time spent and the patient contact by the specialists, study attention was directed to methods of examining the nature of specialists' patient activities in terms of form of interview, individual group, etc. and activities related to the training and supervision of the specialist. MAJ Bardill and SP5 Griffey used and compared two data gathering instruments. Preliminary impressions are that for accurate descriptive type data, a daily-log technique seems feasible for use in an Army MHCS, and indications are that data is more reliable and valid than data obtained from a questionnaire.

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6456	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A823	00	031
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(F)			

11. TITLE (Precede with Security Classification Code): (U) Military Performance and Stress; Factors Leading to Decrements of Performance and Disease. (09)

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

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 72	5	150
FISCAL YEAR CURRENT 73	3	165

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20. GENERAL USE: Foreign Intelligence not Considered

21. KEYWORDS (Precede each with Security Classification Code): (U) Electrophysiology; (U) Biorhythms; (U) Psychophysiology; (U) Operant Conditioning; (U) Stress; (U) Performance; (U) Human Volunteer

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. 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) 71 07 - 72 06 Progress includes the isolation and characterization of physiological and behavioral power spectra under conditions of extended (48 hr) sleep deprivation. A study of perceived stress, methods of coping and performance in Officer Candidate School has been completed. Follow-up study of performance in the field is in progress. Work on brief changes in stress-related autonomic functions specifically related to different types of information processing is continuing. The relationship between information processing, brief autonomic changes and obesity is being delineated. Instrumentation for acquiring and processing autonomic signals is under development with a special emphasis on subject safety. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 71 - 30 JUN 72.

* Available to contractors upon collaborator'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 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 031 Military performance and stress: Factors leading to decrements of performance and disease

Investigators.

Principal: Frederick W. Hegge, Ph.D.

Associate: MAJ Malcolm G. Robinson, MC; CPT John R. Jennings, MSC; CPT William C. Orr, MC; Guy C. Sheatz, Ph.D.; Ann. B. Barnet, M.D.; Robert M. Chapman, Ph.D.; Harold Lawson, B.A.; Stanley Hall, M.A.

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 psychological processes and physiological activity are made. Within this approach, applicable techniques range from those of clinical medicine to those of mathematics and statistics. Specific areas of research include: 1) vigilance performance decrement associated with behavioral and physiological spectra under conditions of extended sleep deprivation (48 hrs); 2) longitudinal study of endocrine reactions and stress in Officer Candidate School; 3) autonomic correlates of information processing; and 4) statistical problems associated with biological measures.

Progress

1. Vigilance performance decrement associated with behavioral and physiological spectra under conditions of extended sleep deprivation.

A study of visual signal detection and heart rate was conducted with the goal of extending previous work that utilized a maximum of twenty-four hours of sleep deprivation. Due to the rigorous vigilance task no subject reached the planned limit of forty-eight hours without sleep, however, all went beyond thirty-six hours.

When the data of the present study were subjected to a more rigorous and sophisticated spectral analysis than was previously available, our earlier report of increases in low frequency components of the spectra could not be confirmed. The new analysis involves preliminary treatment on the data in a manner that reduces the generation of artifact when the Fast-Fourier Transform (FFT) is computed. While commonly employed in the construction of hardware spectrum analyzers, end-point tapering and the Hanning filter have not been used in software spectral analyses. Information concerning the activity of a particular frequency

in this case a 90-minute ultradian rhythm, is extracted during the technique of complex demodulation. This procedure permits the continuous extraction of both amplitude and phase information that is unobtainable from the conventional spectral analyses.

Preliminary results of the present study indicate that there is a strong tendency for maximal reported fatigue to be assoicated with a maximal amplitude of the 90-minute ultradian rhythm observed in both heart rate and vigilance performance. Further, there is a direct relationship between the amplitude of the 90-minute rhythm and detection error variability. Errors in vigilance performance may come in 90-minute cycles whose amplitude increases with increasing fatigue. In previous work (24 hours deprivation) we have found a circadian variation in the amplitude of the 90-minute rhythm. Under conditions of longer deprivation, this variation in the 90-minute rhythm is disrupted. Further, circadian trends normally observed in the raw data virtually disappear.

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

The study of Officer Candidates at Fort Benning provided two advantages: 1) OCS was clearly a highly stressful, but controlled, situation which should produce biological stress reactions comparable to other stress situations; 2) the continuing nature of OCS made it important to assess the predisposing factors to the extreme biological and psychological stress reactions experienced by many officer candidates. The study was conducted in three phases: 1) acute stress phase during the third week of OCS, 2) baseline stress phase during the 23rd week - a period of minimal stress, and 3) follow-up phase after the candidate had been at their PCS six months. A battery of endocrine measures, interviews, and tests were administered during both phases 1 and 2. The follow-up phase consisted of a telephone interview assessing the former candidate's duty performance. The data collection for this study has been completed, however, the data analysis is only nearing completion.

One striking result has already been assessed -- the levels of plasma testosterone (male sexual hormone) are dramatically reduced during the early, stressful period of OCS. This is true of almost every man irrespective of performance in OCS. Expected relations between endocrine measures, performance, and personality did not emerge over the company studied. Men who were in active leadership roles did show a positive correlation between level of 17-hydrocorticosterone (a stress-related hormone) and level of command (squad leader vs. company commander). Psychiatric interview assessments showed a moderate (+.4) correlation with final rank in the OCS class. Turning to the follow-up phase, final OCS rank was not related to performance on active duty. In fact, none of the variables measured showed a strong relation to active duty performance. Variables significantly correlating with

ratings of later performance were a set of scores assessing time of puberty, a small number of personality measures, and level of urine 17-hydrocorticosterone.

A number of avenues are being explored prior to a final interpretation of these data. The variables and intercorrelations between variables seem markedly affected by platoon membership and by relative standing in OCS. For example, the three platoons showed significantly different amount of plasma testosterone suppression. With regard to relative standings, among men not doing well in OCS psychological, performance, and endocrine scores are intercorrelated. This is not the case for men in the upper half of the OCS class. It is hoped that some of these complex relationships will identify the sort of man who is particularly likely to overreact to the stress of OCS. The current data seem to indicate that the stress of OCS may not be highly related to other types of stress such as that found in initial duty assignments.

3. Autonomic correlates of information processing.

The reactions of the autonomic nervous system have been related classically to stress. Recent evidence (Lacey, 1967) has indicated, however, that relatively non-stressful information processing may cause autonomic reactions. These non-stress reactions may either exacerbate or lessen classical autonomic stress reactions. Of equal importance, certain autonomic reactions may facilitate information processing. Thus, change in autonomic function due to stress, may serve to impair information processing by interfering with the autonomic aspects of this cognitive functioning.

Preliminary data suggested that three types of function -- input, storage, and manipulation -- were associated with distinctive autonomic reactions. Futhermore, these differences were influenced by constitutional factors -- specifically, overweight persons reacted differently than underweight persons (See Jennings & Orr, 1970, Schachter, 1970). A factorial experiment was designed to look jointly at the reactions due to the tasks and due to the constitutional factor of obesity. In order to set the cardiac changes within a biological and psychological framework, measures were taken of muscle activity, skin conductance, respiration, life history, and personality. This experiment has been completed, however, only the heart rate data have been analyzed. These results showed significant differences between the tasks with those requiring a combination of memory and manipulation producing the largest cardiac speeding. Difficulty level of the tasks influenced general level of heart rate but did not alter the relationship between tasks. Relatively speaking, memory requirement appeared to produce speeding in obese subjects, while manipulation requirements produced speeding in underweight subjects. A complete interpretation must be contigent, however, upon a further analysis of the heart rate and the analysis of the remaining variables.

Upon the completion of this analysis, experiments are planned to induce biologically inappropriate cardiac reactions via stress or operant techniques and then to observe the effect upon information processing efficiency.

4. Statistical Problems Associated with Biological Measures.

The measurement of biological data characteristically takes place over time within an individual. This produces a series of dependent data points, which are not readily dealt with by classical statistical techniques. In addition, certain traditional statistical problems, such as the nature of the distribution of a variable, arise continually within an ongoing research project.

Two problems dealing with cardiac function are currently under study. First, external events will elicit a brief and complex variation in heart rate. Part of this variation is due, in all probability, to biological dependency and part due to the reaction to the external event. Techniques such as factor analysis are being investigated in hopes of separating such components of the cardiac evoked response. The second problem is the problem of the distribution of heart rate as a variable. In particular, heart rate can be expressed as a rate per unit time as a temporal period between beats, i.e. a rate of 60 beats per minute, is equivalent to a heart period of one second. In a sample of five subjects, it is clear that the statisticaly distribution of cardiac activity are different depending upon the unit of measurement chosen. The quantification and analysis of these differences is currently going on.

5. Cortical Evoked Potentials During Sensory and Cognitive Performance.

Using averaging techniques to isolate cortical reactions following brief stimuli, the influence of different sensory and cognitive processes have been studied. Two studies have concentrated on demonstrating that subjective shifts in perception will produce changes in cortical evoked potentials. In one study subjects were presented with brief flashes of constant wavelength and intensity. Subject perceived these flashes as differing in hue in spite of their objective identity. Preliminary analyses indicate that shift in perceived hue was related to a shift in evoked potential.

Previous research demonstrated that manipulations making numbers relevant and irrelevant to a task caused concomitant evoked potential shifts. Current research is investigating the possibility of obtaining shifts in evoked potentials relating to shifts in the meaning of words and nonsense syllables. Sematic differential and sematic conditioning techniques are being employed. Additional exploratory work is underway on the ability of subjects to control the brain wave, alpha, under a variety of conditions.

6. Sensory Evoked EEG Responses, Sleep and Perceptual-Cognitive Development of Children.

The purpose of the research is to examine and evaluate electrophysiological measures which are related to the developing sensory, perceptual and cognitive processes of children. The general method is that of recording of computer averaged EEG responses to visual and auditory stimuli in normal children and in children with abnormalities of sensory and mental development. EEG sleep patterns are also being studied. The relationship between sensory evoked potentials, electroencephalographic, behavioral and biochemical parameters of development are being examined. The applications of evoked response recording to clinical diagnosis, i.e. in patients with sensory, mental and perceptual abnormalities, are being investigated and evaluated.

Auditory and visual evoked responses have been collected from normal children at birth and up to three years of age. Comparison groups of Down's syndrome infants have been studied. The results demonstrate the habituation of the auditory evoked potential in normal infants (6 to 12 months), but not in neonates or in infants with Down's syndrome. The 5-HTP-treated Down's syndrome infants also showed abnormalities in their EEG sleep patterns and sensory-evoked potentials. The abnormal EEG bursts of the 5 HTP treated children were not found in untreated Down's syndrome children. The detailed analyses of the sensory evoked potentials of normal children has shown regular increases and decreases in certain components with age. The regularity of these changes has allowed application of evoked potential techniques to the assessment of hearing loss in infants.

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Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 031 Military performance and stress: Factors leading to decrements of performance and disease

Literature Cited.

References:

1. Schachter, S. Some extraordinary facts about obese humans and rats. American Psychologist 26: 129-144, 1971.

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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 0B 6485	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISPN INSTN	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A062110A823	00	032
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Drug Abuse in Military Personnel (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002300 Biochemistry; 012900 Physiology; 013400 Psychology; 003500 Clinical Medicine; 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
1 July 71	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 72	20	600
	CURRENT 73	100	3000

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ASSOCIATE INVESTIGATORS
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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Drug Abuse; (U) Epidemiology; (U) Biochemistry; (U) Treatment/Rehabilitation; (U) Prevention; (U) Toxicology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede EACH with Security Classification Code.)

23. (U) To develop and assess screening techniques for military settings; to determine the extent, prevalence, and incidence of drug abuse by soldiers, to devise methodologies for the identification, treatment and prevention of drug abuse by soldiers, and to assess the effects of drug usage upon military performance.

24. (U) All systems purported to be useful for screening are evaluated in the laboratory and, if indicated, tested in the field; developmental changes in these systems are made. Studies assessing drug usage are conducted in military populations; intervention programs are evaluated, using epidemiological, social, and psychological methods; effects of drugs upon performance investigated, and the laboratory assessment of the toxicological, biochemical and psychological basis of illicit drug use are examined.

25. (U) 71 07 - 72 06 The following activities have been pursued: 1) Phase I of the Drug Abuse counteroffensive was planned, organized, and executed in RVN; 2) The intercomparison of screening and confirmatory urine tests has been systematically evaluated. Drugs that cause false positive reactions in the testing system are being identified. Collaborative efforts with the NIMH Drug Addiction Center have been established and a sensitivity and reliability study completed. Technical evaluations on fluorescence assay, new FRAT test reagents, and the radioimmunoassay for opiates are in progress. A survey of drug problems was carried out in PACOM and an appropriate staff document completed, as well as operational, preliminary profile of the Vietnam drug abuser. An investigation was completed to evaluate the motivational effects of Vietnam DEROS screening and detoxification upon heroin users. Material concerning drug users' desire for treatment and rehabilitation upon return to CONUS has been disseminated to command. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 June 72.

*Available to contractors upon submitter's approval

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Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 032 Drug abuse in military personnel

Investigators.

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DESCRIPTION

This work unit was initiated in the last year in response to operational and research requirements imposed upon AMEDS as a result of an increase in illicit drug use by soldiers. During the first quarter of the Fiscal Year 1972, the major emphasis of work performed under this work unit was to provide maximal operational support to presidentially initiated drug abuse counteroffensives. As the operational requirements were rapidly assumed by the standard military health care and command system of the U. S. Army, the work carried out under this unit shifted its emphasis to research and development tasks associated with the perfection of biochemical, epidemiological, and behavioral methodology required to adequately describe the dynamics and causes of the spread of drug abuse in a military population. The fundamental goals of this work were to document the nature of the problem, to develop and improve methods for maintaining surveillance on drug abuse, and to generate appropriate behavioral, physiologic, and epidemiologic information so that current programs of primary and secondary prevention may be improved or replaced by more effective methods.

PROGRESS

1. BIOCHEMICAL METHODOLOGY FOR DETECTION OF DRUGS OF ABUSE.

Extensive work has been done concerning the evaluation of biochemical and biophysical methods for detecting drugs in body fluids and urine. The ultimate goal of this work is the devel-

opment of an optimum system of chemical procedures that will combine maximal sensitivity for detection with complete specificity for verfication of the compounds of interest.

a. Planning, development, and execution of large mass screening in RVN. The USAMRDC and the WRAIR assumed the responsibility for planning, developing and placing centralized laboratories in the Republic of Vietnam (RVN) in order to accomplish Phase I of the presidentially directed drug abuse counteroffensive. At the time, state of the art evaluations indicated that thin layer chromatography (TLC) was available for screening and gas liquid chromatography (GLC) could be used for confirmation. A third technology, the free radical assay technique (FRAT), although developmental, was placed in RVN as a screening method for opiates. A plan was developed to place two laboratories capable of analyzing up to 3000 urine samples per twenty-four-hour day for opiates, amphetamines, barbiturates and other miscellaneous drugs. Both laboratories were operational by 7 July 1971.

Several points are to be emphasized:

(1) The effort represented the first system to mass screen urine for drugs.

(2) A reliable, practical method of urine collection had to be established. A chemical finding was valueless unless it could be reliably referred to a named individual.

(3) The only way any laboratory screening for drugs can effectively operate is by continuous technical surveillance over methodology.

(4) Internal quality control and intra-laboratory comparisons must be made on a regular basis.

(5) All systems currently in use are imperfect systems and require considerable research and development effort.

(6) Drug screening laboratories of the type established are costly and present many logistical problems. Out of this effort came a testing system, FRAT (opiates) and TLC (barbiturates, amphetamines, etc.) for screening and GLC for confirmation. This produced a level of standardization of methodology heretofore not achieved.

(7) The major impact of the system that evolved from this effort was to provide a means to identify drugs in a urine sample from known individuals. Biochemical methodology cannot diagnose "drug abuse." It can provide critical information to a military physician who, on the basis of a thorough evaluation of a man, may conclude that the soldier in question is inappropriately using drugs.

b. Development of quality control procedures. Quality control is a must in the urinalysis laboratory if performance is to be of the highest order. The following facts have been established relative to quality control:

(1) Physiological urine is the best substance for quality control testing.

(2) Single drugs per sample are most appropriate for quality control samples which will be used to evaluate and detect technologic and systems failure.

(3) A variety of drug concentrations above and below the established level of sensitivity is desirable.

(4) The quality control specimens should be submitted to the laboratory for analysis on a double-blind basis.

(5) A quality control specimen entered in the system on the basis of one control specimen per twenty-five urines is adequate.

(6) A separate, independent quality control laboratory must be established in order to maximize credibility.

c. Evaluation of technologies that have evolved over the reporting period. During the past year, the following technologies for urine assay have evolved:

(1) Radioimmunoassay (RIA). This technology is based on the same principle as the FRAT test, but employs a radioactive label indicates an increase in sensitivity to the nanogram level (50-75 nanograms) of morphine. Being an antigen-antibody complex, it is anticipated that specificity and cross reactivity with chemical structures similar to morphine would be of the same order of magnitude as the FRAT. Since the RIA requires a scintillation counter, the cost per test will be high. The radioimmunoassay will be evaluated in a time motion study to better assess its potential as a tool in operational drug screening programs.

(2) Fluorescence. The automated fluorescence assay system as sold by Technicon Instruments has been evaluated. The system is valid to a level of 0.2 micrograms/ml of morphine and seems to be free of the problems of false positives. The system can, in experienced hands, perform 60 assays per hour, a speed too slow for mass screening operations. In addition, the system is costly.

(3) Hemoagglutination Inhibition. The hemoagglutination inhibition test for morphine is simple and requires little or no expensive equipment. The test has the same order of sensitivity as the radioimmunoassay test and probably has the same cross reactivity as radioimmunoassay and FRAT. This procedure will be initially field tested in July 1972.

(4) New Free Radical Assay Technique Reagents for Other Drugs. Several antibody preparations for amphetamines, barbiturates, methadone and cocaine have been evaluated in collaboration with the SYVA Corporation which makes and sells the opiate reagent. Sensitivity levels of these preparations have been of the same magnitude as the opiate reagent (0.5 micrograms per ml.). There appears to be a cross reactivity with a large number of common pharmaceutical preparations so that specificity of methods using these reagents may be low. At present, these reagents have not been released for marketing. The new FRAT reagents will be further evaluated in the July time motion and sensitivity study.

(5) Electron Spin Resonance Technique Employing the Use of Gels. In current drug mass screening efforts, the cost per opiate assay is 0.50 dollars. The antigen antibody complex for opiates is subject to interaction with numbers of structurally similar molecules. This produces large numbers of false positives; specificity is insured by use of GLC. In an effort to increase specificity of the antigen-antibody step and reduce cost per test, the use of stereoselective gel is being investigated. Employing the technology as outlined by Beckett and Anderson (1960), a stereoselective gel has been successfully prepared. This gel has approximately 10^{14} selective binding sites for morphine per milligram. Titration of the gel with spin labeled morphine indicates that at 0.08 micrograms of spin labeled morphine per mg gel all binding sites are filled. Using this preparation, morphine can be measured over the range from 0.2 microgram to 50.0 micrograms. Specificity studies are in progress. The ease of preparation and lower cost make the gel system a potential replacement for the antigen-antibody system. The only limiting factor for extension of similar gel systems to other drugs appears to be successful spin labeling of the compound of interest.

(6) Overview of Evaluation of New Technologies. Each of the above technologies is being evaluated in terms of its application for mass screening. It must be pointed out that unless a technology significantly enhances sensitivity or specificity, reduces logistical burden, and decreases cost significantly, it has little chance of replacing the current testing procedure.

d. Studies of methodology sensitivity. The urinalysis test system currently in use Army-wide consists of FRAT for opiates and TLC for all other drugs as screening tests followed by confirmation for all positives by GLC. Continuous evaluation of all technologies has been carried out to determine levels of sensitivity.

The FRAT system has a cut-off sensitivity of 0.4 microgram opiate per ml. This has been firmly established by the development of a logit transformation of raw FRAT data concomitant with regression analysis. The procedures have been written in fortran for computer usage and adapted in the programmable calculator. In addition, the above procedures allow for the determination of instrument error as well as reagent failure.

The determination of pseudomorphine by fluorescence has an absolute sensitivity level of 0.1 microgram of morphine. Application of the statistical procedures outlined above indicates that the fluorescent assay is linear over the range tested.

An extensive study of the nonionic XAD2 resin with respect to its absorption and elution characteristics has been completed. Specific conditions have been defined for optimum absorption and elution. The use of this resin is being extended to other drugs. Changes have been made in amphetamine and barbiturate extraction procedures to improve methodology.

2. EVALUATION OF PACOM DRUG ABUSE TREATMENT AND REHABILITATION PROGRAMS.

At the request of CINCPAC and COMUS MACV, a survey of drug abuse treatment and rehabilitation programs was carried out at selected sites throughout PACOM with an emphasis upon Army, Navy, and Air Force programs in Vietnam. Critical to accomplishing this survey were the formulation of its goals by the MACV Surgeon, BG Robert Bernstein, and the personal support which he gave this work in Vietnam.

This comprehensive survey initiated in April 1971 of U. S. Armed Forces drug treatment programs in Vietnam, Thailand, Okinawa, and Korea was formulated into a preliminary report of findings submitted to CINCPAC on 1 September 1971 titled "Evaluation of PACOM Drug Abuse Treatment and Rehabilitation Programs." This report concentrated on documenting the nature of the drug abuse problem in all three military services in PACOM, evaluating the adequacy of the response by line and medical programs, and the development of a plan for an adequate treatment program. This work was carried out by COL Harry C. Holloway, MC, Director, Division of Neuropsychiatry, Walter Reed Army Institute of Research, LTC James M. Timmens, MSC, USA (USARV), CDR G. P. Fitzgibbons, USN, Drug Abuse Control Officer Staff CINCPAC, and SP4 J. Keith Ostien, CINCPAC. Valuable consultation was provided by COL Stewart L. Baker, Jr., MC, Consultant in Psychiatry and Neurology, OTSG, USA. Some of the findings reported by this group were that:

a. Drug Abuse in the U. S. military forces represented a significant threat to combat readiness.

b. Drug abuse problems in PACOM consisted of two distinct categories:

(1) Endemic drug abuse of several categories of drugs including alcohol, sedative, stimulants, various forms of cannabis products, and opiates which varied in prevalence and incidence on a local basis, but, in general, reflected the patterns of drug misuse seen in demographically similar civilian populations.

(2) The epidemic of heroin use and addiction in a segment of the U. S. forces in the Republic of Vietnam.

c. Meaningful, preventive, treatment, and rehabilitation programs required chemical surveillance of the at-risk military population to ensure the earliest possible identification of drug abuse. Sensitive and reliable chemical surveillance of drug program participants and staff appeared to be mandatory to assure a drug free treatment environment.

d. Treatment and rehabilitation required that there be adequate staffing by professionally trained personnel and that civilian expertise should be called upon to assure that desired skill levels are achieved in the professional military staffs.

The staff report suggested the basic outline of an integrated program of primary prevention and treatment.

3. PLAN FOR EVALUATION OF DRUG TREATMENT PROGRAMS.

In August 1972, at the request of the Office of The Surgeon General, three teams, each consisting of two professionals representing both medical and behavioral scientific disciplines, were assembled to collect data to form a basis for developing an appropriate methodology for monitoring and evaluating drug programs. In addition to WRAIR staff from the Divisions of Neuropsychiatry and Preventive Medicine, Major Eric Nelson, MC, of the Department of Psychiatry and Neurology, Letterman General Hospital, and LTC Edward Maillet of Brooke Army Medical Center participated on the teams. These teams visited three CONUS post programs for one week and collected data concerning the input and output of the programs, the relation of the drug program to ther post institutions, and the therapeutic approach being implemented. Critical assistance was provided by CONARC Surgeon and the participating MEDDAC Commanders. The data and study groups' recommendations were presented to the Director, WRAIR, and made available to representatives of the Consultant in Environmental Health of OTSG. Using the resultant data base as background, representatives of the Office of the Director of WRAIR, Division of Biometrics, WRAIR, Division of Preventive Medicine,

WRAIR, Environmental Health, OTSG, and the Division of Neuropsychiatry, WRAIR, met with the Armed Forces Epidemiology Board (AFEB) Ad Hoc Committee on Drug Abuse, composed of Dr. Vincent Dole, Dr. Robert Dupont, and Dr. Colin McLeod. Dr. Dole and Dr. Dupont made available information concerning the methods utilized in their extensive civilian programs to record individual patient data. The methods used to formulate this data for Automatic Data Processing (ADP) were investigated and compatible data forms for recording information on individual military users were developed. As a result, the data form designed provided basic demographic, medical information and follow-up information on participants in military drug treatment programs which was compatible with the data to be collected on a nation-wide population drug program under the direction of The President's Special Action Office for Drug Abuse Prevention (SAODAP).

a. In November 1971, a proposed approach was submitted to the U. S. Army Medical Research and Development Command in the form of an annex to the Alcohol and Drug Abuse Prevention and Control Plan (ADAPCP). The general recommendations emphasized were:

(1) Collection of systematic patient-oriented data should be instituted.

(2) Any data collection system must identify and record all individuals entering a program and confidentiality of the data collected is essential to establish the credibility of the rehabilitation program.

(3) That goals against which achievement of treatment programs should be evaluated should be the same as the goals of ADAPCP (i.e., a drug-free functioning military member on active duty or of an appropriate transfer to Veteran's Administration or other civilian care for a discharged member requiring further rehabilitation).

(4) Any data collection system must provide useful local feedback, be as simple as possible, be self-correcting, be amenable to interfacing with civilian data collection systems, and have a central coordinating and control manager.

(5) Redundant reporting through several channels on the same drug-using individual ought to be replaced by a single reporting system for all data concerning an individual. Such a system would not preclude other reporting systems for actions of an agency (e.g., CID, JAG, PM, etc.).

(6) Concurrent with the initiation of an individual data reporting system, a method for reporting objective descriptions of medical rehabilitation systems should be initiated to report staffing patterns, costs, modes of rehabilitations, and interface arrangement with other military institutions and civilian community programs.

(7) Three to six months after initiation of the data collection, the information on hand should be arrayed and examined to judge its sensitivity and reliability in providing comparative information about operative programs.

b. These recommendations were accompanied by a plan for their implementation which outlined:

(1) Data collection format for initial and follow-up individual data.
(2) Assumption of responsibility for individual and programmatic data collection by The Surgeon General,
(3) Personnel requirements,
(4) An approach to rehabilitation program description.

This document was then utilized by OTSG in the formulation of a plan for evaluation of the U. S. Army Drug Treatment Programs. Following appropriate modification, the data collection format recommended became operational as an activity of the Directorate for Environmental Health of OTSG supported by Individual Patient Data System (IPDS).

4. COLLABORATIVE STUDIES OF THE MILITARY DRUG ABUSER IN USARV.

Clinically observed disturbances in patterns of sleeping, feeding, and social interaction in drug users while illicitly using drugs and during the early weeks and months following withdrawal suggests that research on the relationships between drug usage and behavioral and rhythmic physiological phenomena will provide critical information about the addictive process and factors which contribute to subsequent relapse. The measures applied range from those of clinical observation through continuous electrophysiologic monitoring of bodily functions. The hypotheses being addressed are: (1) drug usage results in abnormalities of ultradian and circadian cyclicity, e.g., sleep, respiration, cardiac, gastrointestinal, and behavioral performance cycles; (2) the rhythm abnormalities will be seen as changes in phase, amplitude and the relationship of one rhythm to another; (3) rhythm abnormalities will extend through time and will be altered as the physiological state of the addict is altered (for example, by alternating drug usage patterns); (4) confirmation of these hypothesis would strongly suggest that purposeful modifications of these rhythms in the ex-addict will enhance the effectiveness of some therapeutic measures.

Between 12 April 1972 and 18 June 1972 a five-man research team from Neuropsychiatry Division, WRAIR, plus physicians, technicians, enlisted medics, monitors, nurses, social workers, and support personnel assigned to this study by USHSGV, USARV, completed a

data collection phase of a comprehensive study of heroin use, addiction and withdrawal at the 24th Evacuation Hospital, Long Binh, RVN. Two research wards were utilized: one for initial intensive physiological monitoring of early abstinences and a second rehabilitation ward facility which provided the opportunity for behavioral, biochemical, and neuroendocrine follow-up.

More than 100 soldiers were initially screened in their current military unit. All prospective study participants were evaluated by the research team for a specific history of heroin abuse, absence of other drug abuse, and willingness to enter a research unit which was officially recognized as part of the USARV Drug Amnesty Program. Ten users were selected who had begun heroin use in Vietnam, were not using other drugs and had taken their heroin dose via the respiratory tract on a daily basis for at least one month. Ten others were studied who had used to various extents, some of whom had used the intravenous route of administration. Five drug-free individuals were selected to serve as concurrent controls assigned individually to each group of patients investigated.

Briefly, the organization of these investigations consisted of five to seven days of around-the-clock clinical evaluation, physiological monitoring, support of the withdrawing addict (specifically withholding all medications) and collection of biological specimens. Subsequent to release from this period of intensive monitoring, patients moved to the follow-up ward for continuation of selected investigational procedures in a "normalized rehabilitative" environment (less rigidly controlled in terms of activity restriction and exclusion of periodic stimuli).

Data currently to be analyzed from the WRAIR field research investigations of heroin abuse in Vietnam will include the following measurements:

a. On-line continuous monitoring of multiple physiological parameters by four electrode radiotelemetry:

(1) EEG (electroencephalogram)
(2) EKG (electrocardiogram)
(3) EGG (electrogastrogram)
(4) RPG (rheopneumogram)
(5) EMG (electromyogram)
(6) EOG (electroculogram)

b. Hematologic, biochemical, and endocrine parameters (serum sampling 3 times per day):

(1) Hematocrit
(2) Leucocyte counts and differentials
(3) Sedimentation rate
(4) Erythrocyte and leucocyte morphology
(5) Platelet evaluation
(6) Electrolytes, calcium, phosphorus, BUN
(7) Liver function tests (bilirubin, SGOT, LDH, alkaline phosphatese
(8) Serum lipids and lipoproteins
(9) Glucose
(10) Immunoglobulins
(11) Serum proteins
(12) Australian antigen
(13) Evaluation of the hypothalamic-pituitary-adrenal-gonadals axis
(14) Radioimmunoassay for morphine and morphine antibodies

c. Urine Parameters

(1) Volume and timing (per specimen, 24-hour)
(2) Specific gravity
(3) Qualitative urinalysis
(4) Creatinine
(5) Neuroendocrine battery
(6) Morphine radioimmunoassay and FRAT
(7) Amphetamines and barbiturates

d. Performance Variables

(1) Grip strength
(2) Motor coordination and steadiness
(3) Reaction time

e. Clinical evaluations

(1) Vital signs (temperature, pulse, respiration, blood pressure
(2) Pupillometry (Polaroid photographs)
(3) Symptom classification and quantification and observation of withdrawal signs

f. Social, psychological, behavioral and medical parameters

(1) Comprehensive drug history and demographic information
(2) Medical history and complete physical examination
(3) MMPI administration
(4) Time series questionnaire (a retrospective report of diurnal behavior patterns)

(5) Activity and status log (a comprehensive minute-to-minute account of each subject's objective behavior, sleep-wakefulness, eating, drinking, restlessness, cigarette consumption, position, and symptomatology).

INTERIM FINDINGS

a. Withdrawal (abstinence syndrome) in short-term Vietnam addicts is less severe than has been classically described in civilian "street" addicts, most dramatically so among patients with nonparenteral routes of heroin administration.

b. A pattern of heavy intermittent use exists among some Army heroin users in Vietnam. These patients exhibit no overt evidence of abstinence syndrome (clinical or physiological) when heroin is withdrawn.

c. Rhythm disturbances in sleep-wakefulness cycles and "life style" are significant concomitants of heroin use and withdrawal in Vietnam. The extent, ramifications, and clinical significance of these changes remain to be determined in subsequent data analysis from the completed studies.

d. The drug using population studies were unique in that:

(1) Environmental and social concomitants of drug use in USARV were unique,

(2) A considerable portion of the population studied had only been using heroin for a short time,

(3) The heroin being used prior to withdrawal was relatively pure,

(4) A segment of the population insufflated the drug as their only route of administration

OTHER STUDIES IN PROGRESS

Additional collaborative studies with LTC Norman Ream, USARV and Dr. John A. McCutcheon (formerly MAJ, USARV) are in progress on larger samples to evaluate hormonal patterns during a 14-day period following withdrawal from chronic heroin use in Vietnam. Daily 24-hour urine samples were obtained and multiple blood samples for circadian rhythm studies were drawn at regular intervals during the withdrawal period. Clinical symptoms persist during most of the first week, but relatively normal eating, sleeping and activity cycles are usually established during the second week. Extensive clinical and physiological data on these patients are available for comparison with hormonal data. At present, hormonal data are too preliminary to warrant any general conclusions.

LTC Ream, is also collecting data concerning the reflexology of heroin abusers in collaboration with the Department of Medicine,

WRAIR, and providing samples of urine from heroin users and non-users which are being used to evaluate the sensitivity of the radio-immunoassay determination of opiates. It is anticipated that Colonel Ream will return to WRAIR during FY 1973 to analyze considerable psychologic, sociologic, and medical data concerning heroin abusers in Vietnam.

5. STUDIES OF MILITARY DRUG ABUSE AND TREATMENT IN CONUS.

Study of the Development of Drug Counselling Service. From September through December 1971 LTC James Grosshans, MC, and SP4 Terry Zuelke carried out a study of the development of drug abuse counselling and rehabilitation counselling at Ft. George G. Meade, Maryland. The major goal of this organizational study was to describe the administrative and technical problems encountered in the initiation of a new institution on a garrison post and document the process by which these problems were resolved. Data collection was accomplished by intensive day-to-day naturalistic observation and open-ended structured interviews during a period extending from August to December 1971. Data collection was suspended following the discharge of SP4 Zuelke from the service. The resultant narrative data were reduced to a more manageable chronologic format during January through May. Preliminary findings indicate that many of the problems encountered by this program resulted from inter-institutional conflict and the loose egalitarian structure of the counselling program. During the period of observation, it became clear that the drug urine detection program being utilized to monitor the patient population was not detecting all instances of manifest and self-confessed drug use. The obvious lack of staff experience in working with drug abusers has resulted in a more vigorous in-service training program. In part, the early problems which beset this program have been corrected by the recruitment of a more experienced civilian staff, and increased emphasis on intra-program discipline. Further data analysis is in process.

Captain Larry Ingraham, MSC, in the Department of Psychiatry, Division of Neuropsychiatry, a social psychologist, has worked on two projects related to drug abuse in the Army. The first project involved the construction of a paper and pencil drug education inventory. The purposes of the inventory were three: (1) to provide a service to unit commanders that would facilitate entry into their units for intensive research, (2) to assess the drug education needs of Army units, and (3) to secure basic epidemiologic data concerning drug use in Army units. The inventory was pretested, revised, and readied for mass administration, but the project was discontinued when research attention shifted from drug problems in CONUS to those in Vietnam. The second research project was entitled, "A Preliminary

Assessment of the Army Drug Abuse Control and Treatment Program from the Perspective of the Patient." The respondents in this research were Army enlisted men who had been detected as heroin users by means of a urine test at the time they were to leave Vietnam. The major objectives of the research were three: (1) to provide a description of the detection/detoxification/rehabilitation program of the Army from the experimental perspective of the patients, (2) to assess the responsiveness of the detected users toward Army and VA sponsored treatment/rehabilitation opportunities, and (3) to suggest hypotheses and strategies for further research. The major results were that the returnees did not define themselves as addicts and emphatically denied both the need and desire for further treatment/rehabilitation opportunities. The results further indicated that heroin use in Vietnam is best viewed in terms of the social structure that encouraged and maintained usage rather than in terms of personality, demographic, or pathological characteristics of individual users. A preliminary report and fact sheet were provided to the CONARC Surgeon and a brief paper summarizing the findings of this study was presented to The Third Annual Psychology in the Air Force Symposium held at the Air Force Academy in April. An extended presentation and discussion of the results have been prepared for publication.

Major Edgar P. Nace, MC, Division of Neuropsychiatry, a psychiatrist, has completed a study of drug-using, active-duty personnel. Drug-taking patterns in their pre-service civilian life and in their military careers and the relationships between drug-taking behaviors and other behavioral variables were investigated. A semi-structured interview was constructed for use with drug-abusing patients who presented for help at mental hygiene facilities, and 101 such patients as well as 44 non-drug-using mental hygiene patients were interviewed and administered standardized, non-projective psychological tests. This work was carried out at Fort Meade, Maryland; Fort Carson, Colorado; Fort Knox, Kentucky; and Fort Hood, Texas. The findings from Colonel Holloway's study team in USARV, and data collected by Major Nace in CONUS Led to a paper (mimeo) entitled "Profile of the Military Drug Addict" and was presented to Army World-Wide Drug Abuse Conference sponsored by the Chief of Staff of the U. S. Army in September 1971 and at the symposium on "Current Trends in Army Medical Department Behavioral Science," Fitzsimons General Hospital, Denver, Colorado, 8 November 1971. The data on hand are currently being more extensively analyzed.

Preliminary field work undertaken in Southeast Asia and in CONUS has resulted in the formulation of several hypotheses concerning the critical importance of social reinforcers and organizational circumstance in affecting the spread of drug abuse in the military group. In order to garner data which would allow the testing of

these hypotheses and development of more effective programs of primary prevention, a study of the social networks of drug users has been designed and forwarded to CONARC through the Medical Research and Development Command. It is anticipated that this study, which combines the technologies of urinary screening, contact epidemiology, and other techniques of clinical psychiatry and social science, will be initiated in the next fiscal year.

SUMMARY AND CONCLUSIONS

During the reporting period, laboratories for the mass screening of urines for drug content were developed and placed in RVN. A screening and confirmatory system evolved that has become an Army-wide standard. A quality control program for laboratories supporting the Army Drug Abuse Counteroffensive was initiated in the Division of Biochemistry, WRAIR, and transferred to the Armed Forces Institute of Pathology for further operational implementation. A variety of new technologies for drug detection have been evaluated. Sensitivity criteria on each method have been established.

Surveys and intensive studies of drug users and the military programs which serve them, have been initiated and have formed the basis of several staff reports which have:

a. Recommended and detailed programs of treatment and prevention for PACOM.

b. Formulated a plan and instruments for a data-gathering system concerning individual drug users which is compatible with similar civilian systems.

c. Provided a preliminary plan for evaluation of drug rehabilitation programs.

d. Made initial progress toward providing data concerning factors which determine a soldier to be at-risk for non-alcoholic drug abuse.

e. Provided preliminary descriptive and social data about soldiers detected to have used opiates in Vietnam and particularly information about their self-image and wish for treatment.

f. Completed data collection in several studies of military drug users.

g. Intensively studied the physiology and behavior of a population of Vietnam heroin users following their last insufflation of heroin and during withdrawal.

Perhaps the most important development accomplished under this work unit was the development of the technology and conceptual basis for unit drug screening procedures which permit the early detection of opiate, barbiturate, and amphetamine use prior to the development of addiction or chronic dependency on these drugs.

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 032, Drug Abuse in Military Personnel

Literature Cited.

References:

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2. Holloway, H. C.: Perspectives in Neuropsychiatry at the Walter Reed Army Institute of Research: An Overview. Current Trends in Army Medical Service Psychology, Fitzsimons General Hospital, Denver, Colorado, 14-18 December 1970.

3. Holloway, H. C.: Profile of the Military Drug Addict. Proceedings of Army World-Wide Drug Abuse Conference, Fort McNair, MDW, 27-29 September 1971, (mimeo).

PROJECT 3A062110A824
IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00
Ionizing Radiation Injury, Prevention and Treatment

1202a

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6471	72 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
71 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	621110A	3A062110A824	00	055
b. CONTRIBUTING				
c. CONTRIBUTING	1212B (21)			

11. TITLE (Previous 10 Security Classification Code)

(U) Chemical Protection Against Irradiation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

014000 Radio and Radiation

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)
PRECEDING FISCAL YEAR 72	6	150
CURRENT FISCAL YEAR 73	6	150

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Rothe, William E., COL

NAME:

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 addition, chronic toxicity studies, dose reduction factor studies and drug antagonism studies are being carried out.

25. (U) 71 07 - 72 06 The synthesis effort has been concentrated on aminoalkylaminoalkylphosphorothioates, new sulfur blocking groups, cycloalkyl aminothiols, and purification studies on the phosphorothioates. Screening in mice has continued as well as oral studies on WR - 3689 and WR - 2721 in monkeys. Preclinical pharmacology studies were completed for WR - 2823 and WR - 2721 in support of supplements to the respective claims for investigational exemptions for these new drugs. Phase I human tolerance studies were completed up to the 5 g. per day level for WR - 2721. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971 - 30 June 1972.

* Available to contractors upon established 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 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 055, Chemical protection against irradiation

Investigators.
Principal: Thomas R. Sweeney, Ph. D.
Associates: COL William E. Rothe, VC; CPT Robert W. Caldwell, MSC; LTC David E. Davidson, VC; LTC Gale E. Demaree, MSC; Miss Marie Grenan; Melvin H. Heiffer, Ph. D.; Daniel L. Klayman, Ph. D.; Robert S. Rozman, Ph. D.; 1LT Thomas S. Griffin, OrdC; 2LT Thomas S. Woods, CmlC; 1LT James H. Smith, MSC; 1LT Rolla R. Rich, MSC; LTC Kenneth E. Kinnamon, VC; June A. Schafer; Albert Einheber, Ph. D.

Progress

I. General

Progress on the development of prophylactic drugs to counter the effects of ionizing radiation on exposed troops, or personnel, has been made at a somewhat diminished pace, compared to previous years, because of certain operational difficulties that were beyond control and because of a drop in the rate of introduction of new compounds into the pipeline which resulted from a decreased synthesis program. Although a number of excellent compounds have emerged, compounds which will be discussed later, the well known fundamental problems associated with antiradiation drug development viz., species variability, toxicity, and the variability in activity with route of administration for various classes of compounds continue to make difficult extrapolation of mouse data and generalizations in structure/activity for the large animals.

Phase I studies in the clinic have been completed on WR-2721. Two other compounds WR-2823 and WR-2529 are awaiting start of clinical studies.

Chemistry

II. Chemistry-Contract Synthesis Program

Two chemical synthesis contracts were terminated during FY-72 leaving

only three as of the end of the fiscal year. The preparations laboratory continues to be active.

During FY-72 there were 82 target antiradiation compounds submitted by the synthesis contractors. The preparations laboratory resynthesized 10 compounds on a large scale.

The fragility as well as the synthetic and purification difficulties associated with the important class of compounds, the aminoalkylamino-phosphorothioates, made necessary a careful study of the purification of certain of the compounds in this class. This study was successful and a general approach to the purification of the class of compounds is now in hand. Any given compound will require its own slight variation in the general approach, but this should present no problem. Direct determinations of phosphorothioate and water in this class of compounds would be extremely useful analytical procedures and it is planned to attempt to develop these methods.

Synthesis efforts during the last year have emphasized 1) new sulfur covering functions, 2) compounds related to WR-2721 in which new N-functions have been introduced in place of the amino function and 3) no-nitrogen polysulfides. The latter type of compound is particularly interesting from the theoretical standpoint as well as from the practical because of its unexpected and potent antiradiation activity. A breakthrough in synthesis was achieved when a compound was synthesized having a thiopyrophosphate group as the sulfur-covering function. This method of blocking will have to be applied to several compounds before its value as an antiradiation agent can be assessed. Because of the expiration of the contract devoted to synthetic work in the alpha amidinium thiols and related compounds, work in this area has ceased. Some of this work will be picked up by a still active contract.

Two patent applications have been filed, one on S-substituted thiosulfuric acid derivatives, SN-016240, and one on organic disulfides, SN-874677.

III. Chemistry-Organic Chemistry Section

Studies of synthetic methods used to produce more effective antiradiation agents have led to the discovery of a number of new and useful chemical phenomena.

During the past year, the investigation of the reaction of hydrogen sulfide with aminoalkanethiosulfuric acids has been completed. It was learned that where the amino group is primary or secondary, the resultant

product is the corresponding disulfide as the thiosulfuric acid salt [$(RS-)_2 \cdot H_2S_3O_3$]. This constitutes an excellent method and one of choice for the conversion of organic thiosulfates directly to disulfides. Where the amino group is teriary, the outcome of the reaction is less predictable, for di-, tri-, and tetrasulfides have resulted.

The very unusual compound derived from 2-diheptylaminoethanethiosulfuric acid and hydrogen sulfide, namely, bis (2-diheptylaminoethyl) tetrasulfide $6H_2S$ complex,

$$(C_7H_{15})_2NCH_2CH_2SSSSCH_2CH_2N(C_7H_{15})_2 \cdot 3H_2S \cdot 3H_2S$$

has evoked much curiosity. This is the first amine which has been reported to form a stable complex with more than one equivalent of hydrogen sulfide at room temperature. Some limited studies are underway to determine the structural requirements of this type of compound. What has been learned thus far is that: (1) if the heptyl groups are replaced by methyl groups, the complex does not form; (2) the dioctyl congener is stable, forming what appears to be a complex with eight molecules of hydrogen sulfide; (3) ordinary tertiary amines do not form stable complexes with hydrogen sulfide; and (4) bis (2-octylaminoethyl) disulfide also forms a stable complex with H_2S. These complexes do not react with lead acetate solution unless a "triggering" agent, such as chloroform, is added to immediately release the hydrogen sulfide.

The reaction of organic thiosulfates with sodium borohydride has been investigated, for it was hoped that through the use of this reagent, a smooth conversion to the thiol could be achieved. The reaction has been performed in the traditional solvents--water, methanol and ethanol--as well as in tetrahydrofuran. Surprisingly, great difficulty has been experienced in obtaining thiols in hydroxylic solvents. There is evidence that indicates that the thiosulfates are converted to disulfides but that scission of the S-S bond by borohydride occurs very slowly. In tetrahydrofuran, borane complexes of 2-mercaptoethylamine and bis (2-aminoethyl) disulfide are obtained.

Another study in progress also involves organic thiosulfates and is concerned with their reaction with thiobenzoate anion. A number of potentially active antiradiation agents are emerging from this work.

The reaction of chalcogens with sodium borohydride in protic solvents has been examined. Sulfur and tellurium were found to be less reactive than selenium both in water and in ethanol.

The synthesis of the thiosulfate derivative of quinine has been achieved. Work is now underway to convert this new compound to the corresponding disulfide and thiol. Also, a sulfur derivative of thiamine is now being synthesized.

Much effort has been expended on the study of the stability and purity of several phosphoroate-containing antiradiation agents. A method has been devised to enable the detection and quantitation of trisodium thiophosphate, a highly toxic contaminant found in some batches of aminoalkaneaminoethylphosphorothioates synthesized on contract.

Patents Granted

(1) D. L. Klayman and W. F. Gilmore, U.S. Pat. 3,595,899 (1971); 2-(Phenylalkylamino) ethanethiosulfuric Acids as Antiradiation Agents.

(2) D. L. Klayman and R. J. Shine, U.S. Pat. 3,597,444 (1971); Method of Synthesizing Selenoureas from Thioureas.

(3) D. L. Klayman and W. F. Gilmore, U.S. Pat. 3,655,715 (1972); Synthesis of N-Substituted 2-Aminoethanethiosulfuric Acids.

IV. Rodent Testing Program

Candidate antiradiation chemicals synthesized under Government contract for the Antiradiation Drug Development Program are evaluated for radioprotective activity in the mouse test system. Female ICR mice, 9-10 weeks of age from the WRAIR colony are used in the test. Each chemical is administered intraperitoneally 15 or 30 minutes before lethal irradiation in the primary screening test. A determination of the dose reduction factor, duration of action, and effectiveness by oral administration is made in secondary mouse tests. Thirty day survival of treated mice after exposure to 975 rads of Cobalt-60 gamma radiation (LD_{90} 30 days) is used to evaluate protective activity in the mouse tests. Prior to radiation screening all compounds are studied for their toxicologic and pharmacologic properties.

The following table summarizes the results of screening for the period July 1, 1971 to January 20, 1972. In the intraperitoneal tests, compounds were administered 15 or 30 minutes before irradiation. In the oral tests they were administered 15, 30 or 60 minutes before irradiation.

		Per Cent Survival			
Compounds Tested	Route	50-100% Active	35-49% Mod. Active	20-34% Fair	0-19% Poor
144	IP	54	5	27	58
140	Oral	32	4	10	94

During this testing period some interesting new protective compounds were uncovered. Dr. Lamar Field, on the basis of his previous active organic disulfide (WR 76,873), submitted a highly potent and interesting trisulfide (WR 168,643) which in the rodent test system produced one of the highest protective indices of any compound thus far screened. It is an unusual radioprotectant since it contains no nitrogen function. Its structure is:

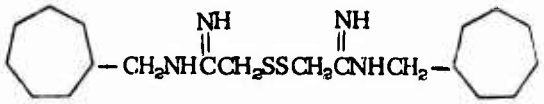
$$NaO_3S(CH_2)_4SSS(CH_2)_4SO_3Na$$

This agent, like WR-76,843, disproportionates *in vivo* and its protective activity is apparently associated with a product of the disproportionation. In the IP test, WR-168,643 affords good protective activity at 1/25th of its LD_{50} dose. Orally, it is effective at 1/6 of its LD_{50}.

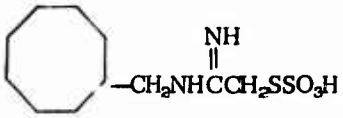
Another interesting and highly active series of protective compounds is the amidine series, synthesized by Ludwig Bauer and by Parke Davis. Amidines with various substituents on nitrogen and sulfur are still among the most important structural types with antiradiation activity in mice. Cycloalkylalkyl and adamantylalkyl groups show the greatest activity as nitrogen substituents. The best sulfur covering groups are the thiol, the phosphorothioate, and the disulfide.

The most effective N-substituted amidines orally and parenterally are the cycloheptyl methyl and cyclooctyl methyl compounds. The disulfide of the cycloheptyl methyl (WR 151331) offered the best activity by both routes of administration. In the cyclooctyl methyl series, the Bunte salt (WR 166829) produced the best protection intraperitoneally, while the disulfide (WR 166764) was more effective orally. Of the 3 sulfur covering functions studied, the disulfides were the most toxic.

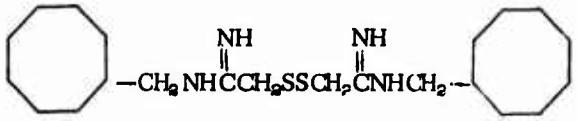
WR 151331:



WR 166829:

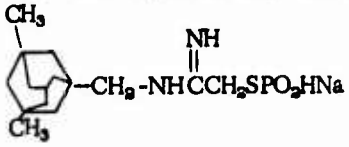


WR 166764:

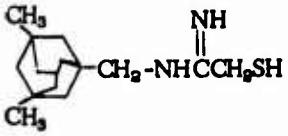


When the substituent on nitrogen was adamantyl methyl, there was further enhancement of activity. The best in this series of amidines was WR 159243 (3,5-dimethyl adamantane methyl) tested as the phosphorothioate. This compound was active intraperitoneally at 1/30th of the LD_{50} dose and orally at 1/4 of the LD_{50}. Its thiol form (157078) was surprisingly better orally, protecting mice at 1/7 of its LD_{50}, but in the intraperitoneal test it was less effective than the phosphorothioate.

WR 159243:



WR 157078:



Another interesting compound showing good activity in the mouse test is a guanidino substituted 2-aminoethanethiol tested as the phosphorothioate (176240). It was effective intraperitoneally at 1/10 of its LD_{50} dose, and orally at 1/6 of its LD_{50}.

WR 1. $NH_2C(=NH)-NHCH_2CH_2SPO_3H_2$

A number of analogues of the outstanding radioprotective phosphorothioate WR 2721 were tested, with emphasis on alkyl substituents on the nitrogen or on the alkyl chain. WR 3689, in which a terminal methyl group was added, was superior to WR 2721 in the mouse test. This compound was less toxic than WR 2721, and had superior oral protective activity. The compounds with methyl substituents in the 1 or 2 position of the alkyl chain (WR 156787 and WR 151324 respectively) were very active intraperitoneally, but were not particularly effective orally. Two analogues of WR 3689 with terminal methyl groups (WR 151327 and WR 151326) were tested and had excellent oral and intraperitoneal radioprotective activity. The hydroxyl analogue (WR 151325) was protective only after intraperitoneal administration.

WR 2721: $NH_2CH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$

WR 3689: $CH_3NHCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$

WR 156787: $NH_2CH_2CH(CH_3)CH_2NHCH_2CH_2SPO_3H_2$

WR 151324: $NH_2CH_2CH(CH_3)CH_2NHCH_2CH_2SPO_3H_2$

WR 151325: $NH_2CH_2CH(OH)CH_2NHCH_2CH_2SPO_3H_2$

WR 151327: $CH_3NHCH_2CH_2CH_2NHCH_2CH_2CH_2SPO_3H_2$

WR 151326: $CH_3NHCH_2CH_2CH_2NHCH_2CH_2CH_2SH$

V. Radiation Protection in Dogs and Monkeys

Outstanding radioprotective compounds from the mouse testing program are selected for studies in dogs or monkeys. Before radiation protection studies are initiated, acute toxicity studies are performed and a maximum tolerated drug dose is established. Animals are exposed to lethal whole-body gamma radiation in the exposure room of the Triga Mark IV Nuclear Reactor of the Harry Diamond Ordnance Laboratory Facility. Beagle dogs weighing 9-13 Kg are restrained in lucite boxes and exposed to a total dose of 650 rads ($LD_{99}/30 + 50$ rads). Rhesus monkeys weighing 1.5 - 3.5 Kg are restrained on flat lucite boards and are exposed to 850 rads ($LD_{99}/30 + 50$ rads).

With the reactor operated at 250 KW of power, and with 70 cm of water interposed between the reactor core and the exposure room, the midline air dose rate is 110-120 rads/min of gamma radiation as measured by a beryllium tissue equivalent integrating ionization chamber. Gold foil dosimetry has indicated a neutron contribution which is predominantly thermal, but which contributes less than 2.5 per cent to the total dose.

During the past year the reactor core has been re-loaded, necessitating extensive dosimetric studies to re-establish the configuration of the isodose curves, and to adjust dose rates and cage placement. $LD_{50}/30$ studies for the new configuration are in progress with normal dogs and monkeys, and drug studies will be resumed in the near future.

VI. Liver Perfusion Studies

Certain aminothiols synthesized as radioprotective drugs by the Army Antiradiation Drug Program have been shown to antagonize the biological damage produced in mammals by injection of nitrogen mustard. We indicated in the 1970-71 Annual Reports that this property of aminothiols could be potentially useful in the chemotherapeutic treatment of hepatic tumors. In previous experiments, anesthetized dogs were perfused with 2 mg/kg of nitrogen mustard (Mustargen) directly into the hepatic artery. This dose of mustard was shown to produce lethal hepatic and systemic effects in all dogs. These lethal effects could be prevented when the portal vein was simultaneously perfused with a protective mixture of aminothiols consisting of 100 mg/kg of DL-threo-3-(2-mercaptoethyl) amino-1,2,4-butanetriol (WR 2347) and 400 mg/kg of cysteine hydrochloride (WR 348). Thes experiments suggested that hepatic tumors, which derive their blood supply in man from the hepatic artery in most cases, could be treated by perfusion of much larger doses of nitrogen mustard than were previously possible, provided the hepatic parenchyma and the other tissues of the body were protected by the antagonists.

Unfortunately, dogs were not suitable for studying effectiveness of the perfusion technique in actual cases of hepatic tumor, since tumor induction in this species is difficult. A collaborative arrangement with the National Cancer Institute has been made to study the perfusion technique in Rhesus monkeys with chemically induced primary hepatomas. The surgical procedure has been modified for the monkey, and the appropriate relative doses of aminothiol and mustard are being investigated in normal monkeys prior to initiating experiments in monkeys with tumors.

VII. Immunosuppressive Properties of Radioprotectants

Several radioprotective aminothiols have been tested to evaluate their effects upon the allergic response of Macaca mulatta monkeys sensitized with indermally administered reaginic sera. Although this family of compounds appears to have antiallergic properties, the monkey assay system has produced results which have frequently been erratic. Many modifications and refinements of the test procedure have failed to produce a more uniform response, and this assay system has been abandoned in favor of more complex but more reliable standardized definitive tests for assessing immunosuppressive activity. These are the spleen colony assays described by Till and McCulloch (1) and the infant mouse assay of Simonsen (2). These systems are currently being employed to identify and evaluate the immunosuppressive activity of selected aminothiols.

VIII. Therapy of Radiation Injury by Hematopoietic Tissue Grafting. A Study of Graft Elimination

This work is being accomplished in collaboration with Dr. John E. Nutter of the Armed Forces Radiobiology Research Institute, Bethesda, Maryland. The purpose of the study is to develop a treatment regimen which will permit effective bone marrow therapy of radiation injury.

Deaths caused by the effects of ionizing radiation on the hematopoietic system may be prevented by bone marrow transplantation. However, unless the donor and recipient are identical with respect to histocompatibility antigens, the recipient may subsequently succumb to secondary disease. Any measure that reduces this immunologic reaction increases the chances of successful therapy. Hematopoietic death in mammals, if it occurs, is usually during a "crisis" period between 10 and 15 days after radiation exposure. It has been shown that foreign marrow transplants given after radiation and prior to the crisis period will greatly reduce the mortality from radiation damage. However, due to graft proliferation, the incompatibility between the graft and host eventually results in secondary disease, and death usually follows at some time after the radiation crisis.

An important characteristic of the hematopoietic system that receives little emphasis is its tremendous potential for recovery. No secondary disease would occur if the graft could be eliminated after it had sustained life through the crisis period, and, in addition, the subject would completely recover if his own hematopoietic tissues were regenerating at that time.

It has been shown that unirradiated animals receiving a foreign marrow transplant are not significantly affected by the graft. Presumably this is due to the recipient's fully competent and unsuppressed immune mechanisms which are able to rapidly remove the foreign cells. One way to approximate the normal immunologic state in an irradiated immunosuppressed subject is to inject an antiserum similar to that which would have been produced by the unirradiated recipient, i.e. confer specific passive immunity. It is conceivable that specific antibodies against the transplantation antigens on the bone marrow cells of the graft might eliminate those cells from the host. It is postulated that the administration of graft-specific cytotoxic antibodies to irradiated recipients of allogeneic bone marrow grafts will selectively eliminate these grafts and leave the host's own surviving hematopoietic tissue undamaged. Furthermore, if this is done after endogenous host marrow recovery and before secondary disease is evident, complete recovery should ensue.

Initial experiments are being conducted to establish the relationship between the time after irradiation and the minimum size of the C57Bl/6 marrow graft which will permit CBA mice exposed to a lethal dose of whole-body irradiation to survive the radiation crisis. The incidence and time course of secondary disease in animals surviving the radiation crisis is also being determined.

IX. Pharmacology

The general pharmacology of WR 2721 was studied in anesthetized dogs to further support claims for investigational exemption for this new drug. The results of these studies are reported in the appropriate supplement to the claim. Phase I human tolerance studies were completed. The results of these studies will be reported in the appropriate supplement to the claim.

Patents Granted

Heiffer, M.H. and Jacobus, D.P.: Alpha Adrenergic blocking Agents Pat No 3,629,410

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 0B 6484	72 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. DISB'N INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	D. Change	U	S	3	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110	3A062110A824	00	057
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212B(21)			

11. TITLE (Precede with Security Classification Code)

(U) Biological Effects and Hazards of Microwave Radiation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

01 3400 Psychology;016208 Stress Physiology;005700 Electronic & Electrical Engineering

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

17. CONTRACT/GRANT 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 72	3	200
	CURRENT 73	4	380

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Larsen, MAJ L.E.

NAME: Hawkins, T.D. DA

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 minimum personnel risk from the environment. 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 established and maintained. Initial scientific efforts will use evaluative methods from experimental psychology, electrophysiology and neurochemistry. Exposure parameters will be chosen for relevance to Army radiating equipment and operational requirements.

25. (U) 71 07 - 72 06. During the reporting period effort has been concentrated on dosimetry; behavioral assessment of the effects of power levels up to 150 mW/sq. cm at various wavelengths; initial lethality studies; and replication of experiment involving turnover of brain serotonin. With work stoppage as an end point a definite effect of the wavelength of radiation has been observed with a maximum effect at 1700 MHz. A survey of the literature on microwave cataractogenesis has been conducted and a protocol prepared for study in this area during FY 73. Attempts to replicate the serotonin experiment(cf.contract DADA17-69-C-9144)met only partial success.For technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 72 - 30 Jun 72.

*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 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 057 Biological effects and hazards of microwave radiation

Investigators.
Principal: H. Mark Grove, M.Sc.
Associate: Sandra H. Githens, B.S.; T. Daryl Hawkins, M.A.; Stuart E. Hirsch, MAJ, MC; Lawrence E. Larsen, MAJ, MC; James L. Meyerhoff, MAJ, MC; John F. Schrot, M.A.

FY 72 was the first year for funding of the WRAIR Microwave Research Program. The initial studies, therefore, are in various stages of completion and will be submitted for publication beginning in early FY 73. This Annual Report summarizes the salient points of progress in five general areas.

BEHAVIORAL BIOLOGY STUDIES OF MICROWAVE EFFECTS.

Microwave studies with rectal temperature, lethality, and behavioral performance as dependent variables were initiated. Prior to the microwave work *per se*, several baseline explorations were made with fixed-ratio, variable-ratio, and variable-interval schedules. The goal was to determine if the average female rat would perform reliably on these simple schedules for food pellets if the rat's body weight was maintained at exactly 200 grams irrespective of the initial free-feeding body weight. Consistent performance was maintained on each of the behavioral schedules indicating the feasibility of using rats having standard mass as subjects in a variety of different microwave studies. Use of standard subject serves to eliminate one source of variation and thereby possibly to facilitate determination of the interrelationships between microwave parameters and physiological and behavioral variables.

Preliminary Lethality and Temperature Work.

Initial lethality investigations were designed to gain estimates of the maximum microwave exposure which could be tolerated by 200 g rats. These data were needed in order to establish protective exposure limits for performing rats in which a considerable training investment had been made. At 3000 MHz (10 cm.) and a power density of 250 mW/cm^2 the lethal exposure duration was 4.75 minutes. At this duration only 50 per cent of the animals survived for 24 hours following the exposure. Rectal temperature data were collected before and after the exposure of each rat.

Temperature increases were approximately linearly proportional to the exposure duration. Increases in temperature of 5°C were lethal in most cases. Control exposures with anesthetized animals, however, produced smaller temperature increases suggesting that confinement stress in addition to the microwave exposure was contributing to the temperature increases observed in the normal rats.

Lethality Effects at High Power.

In late spring 1972, studies with the high-power focusing device were initiated. This device can provide a focused beam of microwave power within the range of 0 - 4000 mW/cm^2 with the specific generator available at 10 cm. The data obtained by using this device are quite preliminary. The beam is so narrow that minor changes in the orientation of the rat's head produce markedly different lethality effects. For example, with 3000 MHz independently determined estimates of the lethal power density have ranged from 900 to 1400 mW/cm^2 depending on the individual exposure box employed. Once the initial apparatus problems are worked out, the high-power device should provide a useful tool for examining lethality at very short exposure durations.

Behavioral Performance.

Behavioral studies of the effects of microwaves have systematically examined the variables of power density and frequency. In this series of studies a simple fixed-ratio schedule of food reinforcement was used as the baseline procedure against which to compare microwave effects. On the base schedule each ten responses on a work lever delivered a food pellet to the rat during the fifteen-minute work sessions (FR-10).

Power Studies.

Effects of power density were initially examined using frequencies of 2450 and 3000 MHz (12.24 and 10 cm, respectively). Each individual rat tested showed clear-cut differential reactions to exposures at different power densities. The higher the power density, the sooner the rat stopped performing. For example, 50 mW/cm^2 produced very little disruption during the 15-minute work sessions. A level of 300 mW/cm^2 produced complete stoppage in three to four minutes. Effects for the intermediate powers were intermediate between the divergent effects produced at 50 and 300 mW/cm^2.

Frequency Effects.

Preliminary studies of the effects of different microwave frequencies on the behaving rat provided some very unexpected results. It is commonly assumed that the biological potency of microwaves is frequency independent. Since higher microwave frequencies are more readily absorbed than lower ones, however, one might reasonably expect a stronger behavioral effect at high frequencies than at lower frequencies. However, in an ongoing study we have obtained very strong evidence that the relationship between food-maintained behavior in the rat and microwave frequency is not a simple monotonic one. In the current behavioral study we are examining performance decrements produced by five different power levels at each of four frequencies. An intermediate frequency of 1700 MHz has consistently produced greater performance decrements than both higher and lower frequencies at a given power density. This nonmonotonic relation holds for power denisty levels in the range from 50 to 150 mW/cm^2. These unexpected results have several implications: First, since the rat seems particularly sensitive to some frequencies, different species including man may show similar nonmonotonic sensitivities to the same or perhaps different microwave frequencies. Examination of this possibility will be crucial to a complete evaluation of the biological effects and hazards of electromagnetic radiation.

A second related consideration has to do with the observation that at least with 1700 MHz, reliable work stoppages were produced with a power density of only 50 mW/cm^2. The stoppages occurred on the average with nine minutes exposure. Extrapolating from this finding one could project that work stoppages might be produced at 25 mW/cm^2 if the exposure duration were increased to only 18 to 20 minutes. Therefore, the paradigm is being revised to support a longer session. The extent to which future empirical data confirm such prediction is crucial in that we can seriously expect reliable effects of microwave power densities quite near that of the established safety standard.

CNS ELECTROPHYSIOLOGICAL STUDIES.

Background.

The study of CNS electrophysiological effects of microwave radiation begins with the working hypothesis that the primary pathophysiological mechanism is thermoregulatory inadequacy or dysfunction. Primal to this approach is the problem of brain temperature measurement during microwave exposure. This allows the time course of electrophysiological and thermogegulatory responses to be followed.

The development of thermometric methods for this purpose began more than two years ago with needle encased thermistors (Fenwall K 898). These probes are manifestly inappropriate for use in microwave fields due to the metallic shielding which increases total absorbed power by two orders of magnitude and results in totally unreliable temperature measurements.[1] [2] The only exact method for control of artifact was crosspolarization. This method is unsuitable for our use since our preparations are not restrained.[3]

The year 1970 - 71 was spent developing a glass mounted thermistor which promised to offer great improvement of the needle mounted design. Notable design features include: 1) interchangeable probes with a tolerance of 1% of the resistance value to a standard RT curve; 2) miniaturized dimensions; 3) greatly reduced metal content; 4) use of high thermal impedance materials in mount, mount canal, and lead wires. The associated bridges, recording electronics, baths and general techniques of thermometry were designed, constructed and acquired during this period.

The present phase overlaps with 70 - 71 in that bench testing of the second generation probe began in the summer of 1971.

Progress.

The bench testing program applied 33 mW/cm^2 incident power density to the probe immersed in a saline filled test chamber (controlled for geometry and depth of penetration) inside a section of WR-284 waveguide. The results indicate two types of artifact: A fast artifact (T_c <0.1 sec) coincident with onset of the fields; and a slow artifact (T_c ≈3 - 5 sec). Both of these artifacts are eliminated with decoupling of the lead wires from the field. Incident radiation on the thermistor network without the antenna effects of the the lead wire give a residual error <0.1°F.

Ferrite materials were tested as a possible shielding method with less heating than metal. The were found to be largely ineffective.

Rectification was found to be a minor problem (error ≈0.1°F) and lost with decoupling.

Recommendations and Conclusions.

The results from bench testing of the second generation probe indicate a need for a temperature probe which is decoupled from the field. We developed a tentative design for such a probe employing microcircuitry (1μm lead wire spacing lines 300Å thick by 1μm wide). The design was

refined by the determination of appropriate filter parameters, and its performance expectations were predicted on the basis of antenna theory by R. A. Moore, Ph.D. of the Westinghouse Corporation. Based on those calculations, we believe a 50 dB decoupling is available with a dipole heating of <0.1°C.

A contract was let for the construction of a microcircuit based probe. At this writing, we are awaiting delivery of the engineering prototypes for evaluation. We expect that the same principles may be used to design and build EEG electrodes which may be used with audio and sub-audio amplitude-modulated microwave fields. This idea will be pursued following verification of the performance specifications of the temperature probe.

NEUROCHEMICAL STUDIES.

The effect of chronic exposure to low level microwave energy on brain serotonin (5HT) turnover in rat was examined. Walter Reed rats were exposed to 10 cm radiation at 10 mW/cm^2 for eight hours per day for seven consecutive days. Control rats were placed in the exposure chamber during all exposures but were shielded from the beam.

At the end of each day the exposed animals showed clear signs of stress: piloerection, defecation, etc., in marked contrast to controls. Both groups of 12 rats gained weight over the seven-day period. The mean gain of controls was 48.9 grams, the mean gain of exposed rats was only 31 grams.

Forebrain 5HT turnover was estimated by the method of Tozer. Pargyline, a monoamine oxidase inhibitor, was administered (75 mg/kg) i.p. and rats sacrificed at time 0, 30, and 60 minutes post-injection. 5 hydroxyindoleacetic acid (5HIAA) the metabolite of 5HT, was assayed by the method of Udenfriend. As shown by Tozer, the rate of decline of 5HIAA can be used to calculate 5HT turnover. A plot of $\log_{10}$ 5HIAA concentration vs time yields a straight line, the slope of which is 1/2.3 times the rate constant K_2 of 5HIAA efflux. The product of K_2 and initial (time zero) 5HIAA concentration equals the rate of 5HIAA efflux which is equal to the rate of 5HT synthesis.

The turnover rate in exposed animals was 0.104 µg/g/hr and in controls 0.113 µg/g/hr. Regression lines of $\log_{10}$ 5HIAA vs time were virtually superimposable on each other. It was concluded that microwave exposure in this experiment did not significantly change 5HT turnover. It was noted, however, that initial 5HIAA values were almost always very slightly lower in exposed animals. This

may be due to decreased feeding evidenced indirectly by less weight gain in exposed rats, and could be brought on by heat stress. It has been shown by Wurtman that brain 5HT can be directly influenced by dietary intake. Moreover, we have found a marked correlation--0.8--between body weight and initial 5HIAA values (σ = 0.80) as well as with turnover rates (σ = 0.80) as well as with turnover rates (σ = 0.86).

OCULAR EFFECTS.

During the period July 1971 through June 1972 the study of microwave ocular effects was begun. This study is divided into two major parts: clinical research and laboratory studies.

The clinical research portion consists of the examination of personnel at various military installations whose occupation potentially exposes them to microwave radiation. Ophthalmic examinations are performed on these subjects in addition to control counterparts. Examinations have been done at Ft Huachuca AZ; Ft Monmouth NJ; Ft Bliss TX; WSMR NM; and Tobyhanna Army Depot.

Basic in-house laboratory research has been initiated with a great deal of consideration spent on a review of the existing microwave ocular effects literature. Consultation has taken place with Drs. R. L. Carpenter, and David Donaldson of Boston concerning techniques of microwave exposure and animal examination in addition to photographic documentation of ocular lesions. A veterinary ophthalmologist, Dr. Seth Koch, has been appointed a permanent consultant to WRAIR for this project. Beagles are in the process of being irradiated as part of initial pilot studies. The aim of our studies at this point is to become familiar with the requirements necessary for the production of cataracts in various animal species exposed to microwave radiation.

A protocol is in preparation which will draw upon data from the pilot investigation for an appropriate study to establish whether there exist pathognomonic characteristics of cataracts of microwave origin.

DOSIMETRY.

The question of dosimetry is central to any meaningful research into the biological effects and hazards of nonionizing radiation. Although free fields can be well characterized and are amenable to calculation the presence of irregular perturbing objects renders closed-form calculation impossible and simple measurement, in most cases, impractable.

A workshop/seminar on the subject of dosimetry and measurements was jointly sponsored by WRAIR and Georgia Institute of Technology on 1 - 2 June 1972. This workshop brought together some 50 specialists--biological and physical scientists and engineers--in a review of the state of the art and to discuss potential approaches to solution of critical problems. Fourteen papers were presented. Summaries will be published as a WRAIR technical report during the summer of 1972.

LITERATURE DATA BASE.

As a part of the DoD microwave research project, a program was embarked upon to collect, codify, analyze and enter into a computerized data bank the world literature on the biological effects and hazards of microwave radiation. This task was originally funded by ARPA and managed by the Air Force under contract F33615-70-C-1789 to Data Corporation.

Upon consolidation of the microwave program at WRAIR, monitorship of this task was transferred as well. For the past year the Contractor has been engaged in refining the initial data base design, collecting, analyzing and preparing documents for machine entry. As of 30 June 1972, the file will contain an estimated 800 papers in full-text format. Additionally, an estimated 1700 original documents will have been acquired. It is planned to have 2500 documents in the data base by the end of FY 73. Sufficient entries are expected to support valuable searches by early Fall, 1972. It is anticipated that the base will be fully operational by the end of FY 73 and available to all interested users in this specialized research field. Beyond that, it is planned to keep the file up-to-date on a current awareness basis.

The final report on the contract referenced above will be available in September, 1972 and will fully describe the base, its contents, and its operation.

Project 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 057 Biological effects and hazards of microwave radiation

Literature Cited.

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Publications:

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PROJECT 3A663713D829
MALARIA PROPHYLAXIS

Task 00
Malaria Investigations

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6517	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. SUMM'N INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	106
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (F)			

11. TITLE (Precede with Security Classification Code)

(U) Antigenic Fractionation, Serology of Malaria

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: 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. OTHER (In thousands)
FISCAL YEAR PRECEDING 72	3	100
FISCAL YEAR CURRENT 73	3	100

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P. DA

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Plasmodium; (U) Immunity; (U) Erythrophagocytosis; (U) Autoimmunity; (U) Diagnosis

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

23(U) To isolate, purify and characterize various antigens from malarial parasites in order to improve diagnostic specificity and to induce immunoprophylaxis or immunosuppression in military personnel.

24(U) Separate parasite proteins by physical and chemical means. Determine the presence and activity of metabolic antigens in the plasma of acutely infected animals and human patients. Analyze the fractionated proteins by both classical and new methods.

25(U) 71 07 - 72 06 The antigenic components detected in the indirect hemagglutination test and the soluble antigen fluorescent antibody test were isolated from erythrocytes of owl monkeys infected with Plasmodium falciparum. These components were solubilized using either repetitive freeze-thaw or disruption in a French pressue cell to lyse washed erythrocytes. The components were insoluble in ammonium sulphate concentrations of greater than 50% at 0 C. Gel filtration studies showed that the molecular weight of all the reactive material was greater than the upper exclusion limit of Sephadex G-200. Sepharose 6B chromatography indicated that the reactive components lie in or near the void volume eluted from this gel. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 June 72.

* Applicable to contractors upon authorization'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 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 106, Antigenic Fractionation, Serology of Malaria

Investigators.

Principal: E. H. Sadun, Sc. D., Lib. Doc.

Associate: LTC C. L. Diggs, MC; CPT R. O. McAlister, MSC

Previous studies of the antigens of Plasmodium falciparum have used lysates obtained from infected chimpanzee blood demonstrating relatively lower parasitemias. The high parasitemias which occur during the course of this infection in owl monkeys coupled with the greater availability of these animals, present a unique opportunity to obtain large quantities of parasite material. The properties of antigens obtainable in this host-parasite system were therefore studied. The following report details the results of initial investigations into the purification and properties of the serologically reactive components obtained from P. falciparum-infected Aotus erythrocytes.

A. Erythrocyte isolation and lysis

The Camp. strain of P. falciparum was utilized for these studies. Aotus monkeys were housed and cared for as previously reported. An appropriate dose of parasites obtained from either fresh blood from a donor animal or from a frozen stabilate was administered intravenously. Infections were monitored daily using Giemsa-stained films of peripheral blood. When more than 20% of the erythrocytes were parasitized, the animals were euthanized by femoral vein and cardiac puncture; blood was collected in heparinized syringes.

The heparinized plasma was collected following centrifugation at 600-800 X g for 15 min at 2-5 C. The erythrocytes were resuspended with 50 ml cold 0.15 M NaCl and centrifuged as above for 10 min. After washing 4 consecutive times under identical conditions, the cells were centrifuged for 15 min and the packed cell volume measured. The cells were then reconstituted in 4 volumes of PBS (0.01M Na_2HPO_4-NaH_2PO_4, pH 7.5 - 0.15 M NaCl). Erythrocytes were lysed either by repetitive freeze-thaw (Wellde et al., 1969) or by use of a French pressure cell at 1000 lbs/in^2. After lysis, erythrocyte counts were determined using a hemacytometer. The disrupted material following lysis was centrifuged at 15,00 -23,000 at

1-2 C. The supernatant fluid was carefully removed from the pellet containing erythrocyte membrane fragments and free parasites, and stored at -70 C for subsequent studies. The pellet was suspended in an equal volume of PBS. Thin films were subsequently prepared and stained with Giemsa to ascertain obvious morphological effects of the lytic process on the parasites.

B. Fractionation and purification

All ammonium sulfate fractionations were carried out at 0 C. Gel filtration studies were performed at room temperature (22-26 C) using dextran and agarose gels (Sephadex T.M. and Sepharose T.M. respectively).

The gels were equilibrated in 0.1 M Tris (hydroxymethyl) amino methane-HCl, pH 8.0-.022% (w/v) NaN_3 ("Tris") or in this buffer containing 0.15 M NaCl ("Tris-saline"). Fractions were collected with a portable fraction collector (Buchler Instruments) set for drop counting. Protein analyses were conducted using the method of Lowry et al. on washed TCA precipitates, employing crystalline bovine serum albumin as standard, or alternatively by absorbance at 280 nm, using as $E^{1\%}_{1cm}$ the value of 14.6, a published value for human ∂-globulin (McDuffie and Kabat, 1956). Absorbances were measured with a Zeiss PMQII Spectrophotometer equipped with quartz cells of 1 cm light path. Volume reduction of antigenic fractions, when necessary, were carried out in cellulose dialysis tubing of pore size 48 Å avg, under reduced pressure.

C. Assay of antigenic activity

All antigenic preparations were tested in duplicate against pools of normal and hyperimmune human sera. The normal sera were obtained from either healthy individuals who had undergone physical examinations, or from donated plasma obtained from the blood bank at Walter Reed General Hospital. The hyperimmune serum pool was obtained from a blood bank located in a highly endemic area in Nigeria.

The soluble antigen fluorescent antibody (SAFA) test was performed with the following modifications: All sera (normal and immune) were tested at a single dilution of 1:5 unless otherwise specified. The fluorometer dial was set to zero by interrupting the light path with an opaque barrier. Results of dial readings for both normal and immune sera were then recorded, and the difference in fluorescence dial reading (FDR) between the two was used as an expression of serologic activity. Normal

serum pool FDR values varied from 9.0 - 14.5 units, depending on antigen concentrations used.

For testing of antigenic fractions, the standard 47 mm Millipore T.M. filters (pore size 0.45μ) were cut into 6.5-7 mm discs. To each of these discs was applied 0.010 ml of the antigen solution to be tested. After a five minute absorp ion period, the discs were dried at 37 C for 30 min.

The indirect hemagglutination test (IHA) was performed according to the published method with the following exceptions: The sheep cells used for the sensitization step were preserved by glutaraldehyde fixation and stored at 4 C as a 30% suspension (v/v) in distilled H_2O. All sera were absorbed with an equal volume of this 30% suspension for 10 min. The normal rabbit serum used in the diluent was obtained from a large pool obtained from a commercial source. Hemagglutination patterns were developed at room temperature, and serum titers were expressed as the reciprocal of the highest serum dilution giving complete agglutination. Optimal antigen concentrations for use in sensitizing the preserved erythrocytes was determined for each antigen preparation. The use of concentrations above that found to be optimal resulted in nonspecific agglutination in the presenee of both immune and normal sera. All antigen fractions were tested in duplicate versus the paired hyper-immune and normal serum pools; the initial serum dilution utilized was in all cases 1:20. The test was carried out using the Microtiter T.M. apparatus and "U" type plates.

A. Isolation of antigenic material

The freeze-thaw method of lysis as a means for initial isolation of antigen was investigated due to the success reported in previous studies. Erythrocyte counts made on the lysate prior to centrifugation revealed the degree of erythrocyte lysis to be in most cases 99% or more; however, Giemsa-stained preparations revealed the pellet to contain many intact parasites which appeared unaffected by the lytic process. By contrast, it was found that 1000 lbs/in^2 using the French pressure cell gave not only 100% lysis of the erythrocyte, but resulted in disruption of a number of parasites as well, when comparisons were made between the morphological appearance of pellets obtained after aliquots of suspended erythrocytes were lysed by each method. A small amount of additional protein and antigenic activity measurable with the IHA test was recovered in the French pressure cell-lysed preparation. These differences were deemed significant, since both preparations showed identical fractionation properties following $(NH_4)_2\ SO_4$ treatment.

Protein measurements on lysates were performed on TCA precipitates using the Lowry method. It was considered appropriate, however, to use a spectrophotometric assay following $(NH_4)_2\ SO_4$ precipitation and subsequent dialysis.

B. Antigen reactivity and $(NH_4)_2\ SO_4$ fractionation

Initial studies using 10 monkeys were conducted to determine the relative reactivity of various antigen preparations from different animals. The potential use of $(NH_4)_2\ SO_4$ fractionation to remove contaminating host hemoglobin was also investigated.

It was empirically determined that most of the host hemoglobin was soluble in 62% (3.34 M) $(NH_4)_2\ SO_4$ at 0 C. An aliquot of each lysate preparation was subjected to $(NH_4)_2\ SO_4$ precipitation at this concentration at 0 C for 30 min, followed by centrifugation at 11,000 X g for 15 min. The red supernatants were decanted and the pellets washed 3 times with 70% (3.75 M) $(NH_4)_2\ SO_4$ with intermittent centrifugation and finally redissolved in a minimal quantity of PBS. Following dialysis against a large volume of PBS, the dissolved precipitates were adjusted back to the original volume and were tested for SAFA reactivity, along with the original lysate and the dialyzed $(NH_4)_2\ SO_4$ supernatants. The precipitate fractions were assayed for their protein content. It was found that the antigens detectable in all preparations were insoluble at this salt concentration. The pellets recovered following salt precipitation varied in color from pale brown to deep red; the amount of contaminating hemoglobin visually seen appeared to be less of a problem when using small initial volumes of lysate for the precipitation. In all cases, neglibible activity could be detected in the supernatant following precipitation, even when pressure dialysis was utilized to return the supernatant fluid volume back to that of the original lysate.

For further investigation of the properties, the antigen in the presence of $(NH_4)_2\ SO_4$, experiments were designed to elucidate antigen solubility over a wide range of salt concentrations.

A preparation of lysate obtained from an animal which had a terminal parasitemia of 84% was divided into 1 ml aliquots. Each aliquot initially contained 16.5 mg protein/ml. Saturated $(NH_4)_2\ SO_4$ was then added to a final concentration of from 20%-75% M at 0 C at 5% intervals. Following centrifugation and dialysis, the paired antigen precipitates at each point were redissolved in 1 ml of PBS unless the pellet size required the use of a larger volume. All antigen precipitates including the

paired unfractionated control samples were tested for reactivity in the SAFA test and in the IHA test against the pooled immune and normal sera. Following testing, protein concentrations were determined and expressed as total protein (mg) in each pellet. Each fraction was used undiluted for soaking discs to be used in the SAFA test; each preparation was diluted 50 fold for sensitization in the IHA test. The results expressed as mean values indicate all antigenic material to be insoluble at salt concentrations above 50% saturation. When this experiment was repeated utilizing a preparation from another animal which had a terminal parasitemia of 83% and a lysate protein concentration of 7.6 mg/ml, similar curves were obtained. Preliminary studies suggest that the antigens are stable frozen at -70 C. Preparations stored in this fashion have shown no detectable loss in serological reactivity after periods of up to five months. The $(NH_4)_2\ SO_4$ precipitated material after dialysis, appears stable for at least several hours at room temperature, and several of these preparations have been repeatedly frozen at -70 C and thawed with no detectable ill effects on measurable activity. No change in reactivity was detected when one aliquot of the antigen was heated at 56 C for 30 min and tested in parallel with an unheated aliquot.

C. Gel filtration studies

To establish the molecular sieve fractionation properties of ammonium sulfate-purified antigen, a pool of lysate taken from three different animals was batch-treated with 62% $(NH_4)_2\ SO_4$ as described above and dissolved in PBS to a final concentration of 5.34 mg/ml. Three ml (16.1 mg protein) of this preparation were applied to a Sephadex G-200 column of dimensions 2.5 C 38 cm. The gel was equilibrated in Tris buffer and the column was pre-calibrated using 3 ml of a 0.2% solution of Blue Dextran 2000 using a flow rate of 2 ml/min. Each 2 ml fraction was read undiluted at 280 nm. Fractions were tested undiluted in the SAFA test, and diluted 1:5 for testing IHA reactivity. A major part of the activity detectable was present in the first protein peak. Curves were drawn through mean values obtained in the serological tests. This experiment was subsequetnly repeated two times using different antigen pools, each purified with ammonium sulfate. In every case, all detectable serological activity showed similar fractionation properties. Following testing, fractions containing activity were pooled and protein concentrations were determined. It was noticed that 2-3 fractions within the initial peak eluting from G-200 contained sufficient turbid material to interfere with absorbance measurements. This material could be removed by centrifugation 20,000 X g with no detectable loss in serological activity.

In all cases, detectable serological activity was localized in a peak containing 17-27% of the protein present in the $(NH_4)_2\ SO_4$ purified material added to the column, regardless of whether the gel was equilibrated in Tris buffer alone or in Tris-buffered saline. SAFA curves were obtained using normal and immune serum diluted 1:5.

Antigenic fractions obtained from G-200 were studied for fractionation properties on agarose as follows: An infected monkey with a terminal parasitemia of 71.5% was exsanguinated and the lysate prepared as described previously. Following precipitation with $(NH_4)_2\ SO_4$, 10.9 ml of antigen were obtained with a protein concentration of 10.7 mg/ml. Five ml of this solution (53 mg) were fractionated on a 2.5 X 87 cm Sephadex G-200 column equilibrated in Tris-saline. After collection and pooling of the active fractions and centrifugation at 20,000 X g - 1 hr, the preparation had a concentration of 370 µg/ml in a total volume of 53 ml. Five ml (1.85 mg) of this preparation were then loaded on Sepharose 6B column of dimensions 2.5 X 38 cm. The gel had been previously equilibrated with Tris-saline and had been calibrated with a solution of Blue dextran 2000 purified with Sepharose 6B to contain only material of a molecular weight above the upper fractionation limit of this gel. A flow rate of 10 ml/hr was maintained and 1 ml fractions were collected. Measurements of absorbance at 280 nm were performed, and each fraction was tested undiluted for SAFA reactivity and at a dilution of 1:5 for IHA response. All antigenic material was present in the initial peak of protein, corresponding to material voided by the gel.

Repetitive freeze-thaw was found to be an effective means for the initial isolation of serological antigens at 23,000 X g. These observations are in agreement with earlier results using lysates obtained from chimpanzee blood. It was additionally determined that disruption effected by gentle lysis with the French pressure cell gave similar antigenic preparations. Lysates prepared using the French cell were of slightly larger volume, due to the more compact nature of the pellet. This effect was judged due to the much smaller erythrocyte membrane fragments present following French cell treatment. The shearing forces present at the orifice of the French cell apparently yield simultaneous fragmentation of the erythrocyte membranes at several points. By contrast, the pellet following centrifugation of the freeze-thaw isolated lysate contained a dense area of free parasites and a few unlysed erythrocytes overlaid with an amorphous stroma layer. This amorphous layer was composed of large erythrocyte "ghosts." Based on

serological activities, it was concluded that either method of antigen isolation was suitable for initial solubilization of reactive components.

The degree of variation observed (Table I) in SAFA activity of preparations obtained from different animals show no obvious relationship to parasitemias observed in the animal at the time of sacrifice. It is possible that the serological activity measured in the SAFA test is produced at some discrete stage in the life cycle of the plasmodium. The somewhat asynchronous nature of this infection is compatible with this hypothesis.

Following the initial studies, the IHA test was incorporated as an additional means of assaying antigenic activity. The additional sensitivity available using this simple test, as well as the relatively small amounts of antigen necessary and the desirability of assaying the reactive material using two independent and widely used tests for malaria antibody, influenced this decision.

The finding that both SAFA and IHA activities were insoluble in concentrations of $(NH_4)_2\ SO_4$ above 50% (2.67 M) at 0 C (Fig. 1) confirmed the use of 62% $(NH_4)_2\ SO_4$ as a valid salt concentration for initial removal of host hemoglobin. SAFA and IHA values of Fig. 1 are not corrected for volume differences in the re-dissolved pellets; these differences became significant only when concentrations of salt (>67%) of sufficient size were used to bring down the host hemoglobin. If the assumption that serologic activity is diluted proportionately with volume changes and the values obtained above $(NH_4)_2\ SO_4$ are corrected for volume differences, the serological values still do not approach those measured at the lower salt concentrations.

The findings reported following Sephadex G-200 molecular sieve chromatography indicate that the reactive elements fractionate in or near the upper exclusion limit of this gel. No obvious differences in molecular size could be detected using pools composed of lysates obtained from different donor animals.

Comparison of the reactivity of the different antigen preparations following $(NH_4)_2\ SO_4$ purification and Sephadex G-200 chromatography indicate similar behavior over a variety of dilutions in both tests. The SAFA reactivity of both preparations was found to be comparable at several dilutions. Similarly, both preparations gave a maximum titer in the IHA test over a wide range of concentrations of antigen. The G-200 purified antigen gave a higher maximum titer with the hyperimmune

pool than did the antigen which had been purified only with $(NH_4)_2\ SO_4$. This finding indicates that one or more of the lower molecular weight components removed by G-200 gel filtration may partially inhibit the sensitization step with the reactive elements of the antigen, probably due to competition for available sites in the surface of the glutaraldehyde-preserved erythrocytes.

The coupling of salt fractionation with molecular sieve chromatography gives rise to material which has been markedly purified with respect to protein concentration. The $(NH_4)_2\ SO_4$ fractionation normally precipitates around 5-10% of the protein, and of that material, 15-20% of the protein normally appears in the void volume region of dextran chromatography. More precise estimates concerning the actual degree of purification await studies designed to elucidate protein content of the antigenic material.

The results of the Sepharose 6B study, if substantiated, indicate that all antigenic reactive in the two tests employed in these preparations is composed of very high molecular weight components. The upper exclusion limit of Sephadex G-200 is approximately 800,000 for globular proteins. The consistent findings of all serological activity in or near the void volume following G-200 gel filtration imply that all antigens detected in the tests described were of a minimum molecular weight of 800,000. Furthermore, the preliminary result observed using material from one animal, if determined to be representative, imply the reactive components to be at or near the upper exclusion limit of Sepharose 6B as well. The upper exclusion limit for Sepharose 6B is approximately 4×10^6 for globular proteins. Regardless of the final precise estimate of molecular weight, it is obvious that the reactive serological antigens obtained in this study are uniformly of at least 800,000 molecular weight.

Investigations designed to elucidate some of the chemical and biological properties of this partially purified material are currently under way. Considering the methods used for lysis and the number of intact trophozoites seen in these preparations following centrifugation, it is presumed that a major part of the reactive material present in these lysates originates either in the erythrocyte cytoplasm outside of the parasite itself, or else comes from stages of the parasite which are sufficiently fragile to be disrupted by the lytic process, with concomitant release of serologically detectable material.

The finding that the reactive material or materials are of such high molecular weight (>800,000) may indicate a spontaneous aggregation of smaller reactive molecules has occurred during the preparative procedure. Studies are currently under way to investigate if disaggregation into smaller molecules without concomitant losses in the chemical and physical properties of this material is possible.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6505	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DOD'N INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	108
b. CONTRIBUTING				
[redacted]	CDOG 114(f)			

11. TITLE (Procede with Security Classification Code)

(U) Biochemical Effects and Mechanism of Action of Chemotherapeutic Agents (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 07	Cont	DA	C. In-House

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING CURRENT		
	72	11	215
	73	13	245

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic; Military)
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ASSOCIATE INVESTIGATORS
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NAME: Icfberg, R. T. Ph.D. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code)

(U) Drug Effects
(U) Plasmodium berghei; (U) Drug Analysis; (U) Mechanisms; (U) Drug Action;

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

23. (U) Chemotherapeutic agents particularly antimalarial drugs will be examined for their metabolic interaction with the biological environment. The mechanism of action of each drug against the malarial parasite will be biochemically defined.

24. (U) Plasmodium species in various animal hosts will be used to test organisms to evaluate intermediary metabolism of the parasite, and the responses of the host to the antimalarial agent under study. Analytical definitions of the chemotherapeutic agent and its distribution in biological tissues are studied. Synthesis of important metabolites will be done.

25. (U) 71 07 - 72 06 Studies on the effects of iron and chloroquine on primates namely the rhesus monkey infected with P. knowlesi and the owl monkey infected with P. falciparum (Malayan Camp strain) have indicated a marked reduction in parasitemia similar to that observed in P. berghei infected mice. Iron had no effect on the inhibitory action in the case of chloroquine resistant P. berghei. In the hamster infected with P. berghei the following significant biochemical changes have been documented: 1) a significant hyperlipidemia involving all major serum lipid fractions; 2) altered thyroid physiology evidenced by a marked increased uptake of I-131; 3) increased red blood cell ATP concomitant with a reduction in 2,3-DPG tissue cells. Diaminodiphenylsulfone has no measurable effect upon serum amino acids. Methodology has been developed to measure WR 33063 and applied to serum from infected humans. Levels of this drug are maximal at 6 hours and diminish to their lowest levels after 24 hours. Methods are being developed for application to other experimental drugs. For technical report, see the Walter Reed Army Institute of Research Annual Progress Reports, 1 July 1971 - 30 June 1972.

(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 3A6637l3D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigation

Work Unit 108 Biochemical effects and mechanisms of action of chemotherapeutic agents.

Investigators.
Principal: COL Charles R. Angel, MSC
Associate: Betty J. Boone, PhD; Edmund S. Copeland, PhD; Seymour Garson, PhD; Robert T. Lofberg, PhD; Benjamin Mehlman, M.S.; Patrick M. L. Siu, PhD.

Description.

The objective of this work unit is to examine the metabolic interaction of chemotherapeutic agents, in particular antimalarial drugs, with the biological environment. Method development is a necessary part of the work unit in order to measure newly developed agents in physiological fluids. Studies undertaken during the reporting period subdivide themselves into 1) biological studies and 2) analytical chemistry methodology development.

Progress.

1. Biological studies

a. Effect of Iron and Chloroquine on the growth and multiplication of the Malaria parasites, P. knowlesi and P. falciparum.

"In vivo" studies with rhesus monkeys infected with P. falciparum (Malayan Camp strain) and the owl monkey infected with P. knowlesi produce inhibition of parasitemia when treated with iron and chloroquine similar to that observed in mice infected with P. berghei. The degree of inhibition by iron and chloroquine was greater in monkeys than in mice. Iron has similar effects in both the chloroquine sensitive and chloroquine resistent strains of P. berghei. Chloroquine, however, had no effect on iron inhibition in chloroquine resistant strains of P. berghei.

b. Exoerythrocytic stages of Plasmodium fallax grown in tissue culture: Changes in extracellular and intracellular free amino acids.

Althoug' prolonged survival and abundant growth of exoerythrocytic malarial forms have been achieved in tissue culture, little is known of their metabolism or metabolic requirements.

Yet, it is generally recognized that such information may serve as the basis for a rational approach to the chemoprophylaxis of malaria.

In the present study, second subcultures of embryonic turkey brain inoculated with exoerythrocytic stages of an avian malarial parasite, P. fallax, were terminated after 48 hours. During this interval, a rise in parasitemia from approximately 50% to 85% was accompanied by a striking reduction in the free amino acid pool of infected cells as compared to that of non-infected cells. The intracellular concentrations of all measurable amino acids, with the exception of lysine, decreased with progressive infection. The most significant reductions were recorded for glutamine (71%) and methionine (56%). Analyses of the cell-free parasites (merozoites) revealed that glutamine and methionine were present in the lowest concentrations, whereas those found in the highest concentrations were glutamic acid, alanine and serine.

Comparative analyses of the media in which parasitized or non-parasitized cells were incubated showed several quantitative differences. The most significant of these was a large reduction in the glutamine content of the infected medium accompanied by a marked elevation in glutamic acid concentration. Reductions in levels of leucine and isoleucine were also noted in the infected medium.

Since the parasites must synthesize protein at a rate commensurate with their rapid increase in size and number, the present data are thought to provide some insight regarding pertinent free amino acid requirements, some of which are comparable to those reported for the erythrocytic stages.

This study undertaken in collaboration with personnel of the Naval Medical Research Institute has been completed and a manuscript prepared for publication.

c. Serum lipids in Plasmodium berghei-infected hamsters.

Concentrations of serum lipids and lipoproteins in P. berghei infected hamsters were markedly altered in the course of infection. Early changes included a significant increase in free fatty acids and a transient reduction in levels of total cholesterol, phospholipids and alpha-lipoproteins. The latter findings probably reflect increased metabolic requirements imposed by activation of the reticuloendothelial system and subsequent stimulation of erythropoietic mechanisms.

A sustained mobilization of free fatty acids from adipose tissue was accompanied by a steady rise in endogenous triglyceride concentrations. This marked the onset of a profound hyperlipidemia during the second week of infection, which ultimately involved all of the serum lipid fractions. A prominent increase in prebeta-lipoproteins, and a significant, although less pronounced, rise in beta-lipoproteins also occurred. Similar findings pertaining to a mixed hyperlipoproteinemia have been described in the nephrotic syndrome. The data indicate that hyperlipidemia in the infected hamsters is primarily a consequence of increased hepatic lipoprotein synthesis; however, a reduced clearance of serum lipids may also be involved.

d. Ancillary observations on duration of infection and immunity in experimental malaria.

Cumulative data on the course of P. berghei infection in different groups of hamsters over prolonged periods revealed that most fatalities occured between 14 and 23 days after inoculation. Variable numbers of survivors generally showed a progressive reduction in parasitemia, a concomitant elevation in hematocrit values and a gradual abatement of overt illness. These animals, with few exceptions, appeared to recover completely from their infections as indicated by negative blood examinations usually beginning in the sixth week after inoculation. However, recent observations involving sub-inoculations into mice have revealed the persistence of latent infections in hamsters up to seven months subsequent to inoculation, whereas complete eradication of the infection was first evident in the eighth month. During these periods of latency and spontaneous cure, it was subsequently found that the hamsters were highly refractory to homologous superinfection. Tentative indications are that acquired immunity to P. berghei in the hamster is not due entirely to premunition.

e. Thyroid function in experimental malaria.

Malarial infections of primates and rodents present a panoply of complications, both functional and morphological, some of which may reflect endocrine involvement. The present study was undertaken to evaluate the impact of P. berghei infection on thyroid physiology in hamsters. Various indices of thyrometabolic status have been included at intervals of 7, 14 and 21 days in the course of the infection. Initial observations based on a comparison of infected and non-infected animals indicate that: 1) total body disappearance of parenterally administered I^{131} was significantly prolonged as the infection progresses (t 1/2 on day 21: 10 hours for controls vs 35 hours for infected;

2) thyroidal uptake of I^{131} was significantly increased at all time periods studied; and 3) the percentage of administered I^{131} in the blood of infected animals was increased, while protein bound I^{131} was decreased, which suggests a reduced binding capacity. A study of I^{131} thyroxine metabolism with special reference to clearance rates and organ distribution is currently in progress. This project is near completion.

f. Red cell organic phosphate levels in experimental malaria.

Tissue anoxia appears to play a prominent role in the pathogenesis of malaria. Although critical changes in local blood circulation are thought to be the essential cause of poor tissue oxygenation, other potential factors have not been adequately explored. In this regard, it is presently known that oxygen transport is markedly influenced by red cell concentrations of 2,3-diphosphoglycerate (DPG) and ATP. It has been demonstrated that the affinity of hemoglobin for oxygen varies inversely with the intracellular levels of these organic phosphates.

The present data indicate that ATP levels in hamster red cells were significantly increased after infection with P. berghei, whereas DPG concentrations were progressively reduced. The latter were lower than those anticipated on the basis of corresponding hemoglobin levels. In part, these findings may reflect a concomitant influx of reticulocytes in which ATP levels are known to be considerably higher and DPG levels lower than those of mature red cells. However, a significant reduction in blood pH, as presently observed in the course of infection, and the consequent activation of DPG phosphatase would also result in lowered DPG levels. Since the higher levels of ATP in infected red cells may balance the lower levels of DPG in terms of oxygen dissociation properties of the cell, the pathologic implications of these findings remain uncertain.

2. Analytical Chemistry Methodology Development

Synthesis of four quinoline methanols having an α (di n butyl amino methyl) side chain and various substitutents in the 6 position has been completed. If the substituent in the 6 position contained an electron withdrawing group, then the compound was biologically active. However, all the active compounds were also phototoxic.

Mechanistic studies on the quinoline methanols to study the phototoxic effect have been initiated by examining the triplet state with ESR spectroscopy. There is strong evidence that

hydrogen atoms are one of the primary photolytic products formed during decay from the triplet state. This work is continuing in collaboration with Dr. George Yang, Food and Drug Administration.

During the reporting period, extensive development has been accomplished to permit determination of experimental malarial drugs in human serum. Gas liquid chromatography has been selected as the method of choice because of its sensitivity and specificity. The first method was developed for WR33063. Compounds of the 33063 type have low volatility necessitating the formation of silyl derivatives. The final procedure entails deproteinization followed by extraction with ethylene dichloride. After removal of the ethylene dichloride and derivatization, the material is separated on an OV-1 column and measured in an electron capture detector. Average recovery from serum was 74 ± 4%.

The results of analysis of serum samples showed the presence of WR33063 maximally after 6 hours with a sharp decline after 24 hours. Individual variations in serum levels were striking.

Summary and Conclusions.

The work unit during the reporting period has been concerned with biological efforts and analytical chemistry. The biological studies have centered around the effect of iron and chloroquine on parasitemia in infected monkeys. The P. berghei infected hamster has been systematically studied. Intracellular and extracellular amino acid pools have been defined in tissue culture employing P. fallax. A method has been developed for WR33063 and applied to human serum samples.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigation

Work Unit 108 Biochemical effects and mechanisms of action of chemotherapeutic agents.

Literature Cited.

Publications:

1. Siu, P. M. L.: Malaria: Effect of Iron and Chloroquine on Erythrocytic Forms of Plasmodium berghei. Proc. Soc. Exp. Biol. Med. 139:799, 1972.

2. McDaniel, H. G. and Siu, P. M. L.: Purification and Characterization of Phosphoenolpyruvate Carboxylase from Plasmodium berghei. J. Bacteriol. 109:385, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6471	72 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 / A. WORK UNIT
71 07 01	D. Change	U	U	NA	NL	YES / NO	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A66371 3D829	00	112
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE (Precede with security classification code)

(U) Field Studies on Drug Resistant Malaria (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 003500 Clinical Medicine;010'00 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. CUMULATIVE 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) 72	8.0	360
FISCAL YEAR (CURRENT) 73	4.0	240

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3551

20. GENERAL USE

Foreign Intelligence Not Considered

19b. PERFORMING ORGANIZATION

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ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Provide SEAN if U.S. Academic Institution)

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PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Colwell, LTC E. J.

NAME: Hickman, MAJ R. L.

21. KEYWORDS (Provide at least three keywords or phrases)

(U)Drug Resistant Malaria; (U)Chloroquine; (U)In vitro Models; (U)Tetracycline; (U)Glucose-6-Phosphate Dehydrogenase;(U)Quinine

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

23. (U) To define epidemiologic and entomologic variables which influence the prevalence of chloroquine resistant malaria. To evaluate in vitro models for determining chloroquine sensitivity of P. falciparum. To evaluate alternative treatments for drugs resistant malaria.

24. (U) Longitudinal epidemiologic and entomologic studies of malaria transmission were conducted in Central Thailand. In vitro drug sensitivity models were evaluated for reliability in Southern Thailand. Alternative treatments were evaluated in Central and Southeastern Thailand. Vector populations and climatic conditions are being longitudinally monitored at study sites.

25. (U) 71 07 - 72 06 An. balabacensis is still the primary vector in malarious areas under study. The in vitro models appear to be very reliable for detection of chloroquine resistance. A combination of quinine and tetracycline is highly effective for control of symptoms and radical cure of falciparum malaria. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 Jun 72.

(Available to contractors upon established 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 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 112, Field Studies on drug resistant malaria

Investigators

Principal: Ben F. Castaneda, SFC; William Chin, M.D.; Edward J. Colwell, LTC, MC; Douglas J. Gould, Ph.D; Robert L. Hickman, MAJ,VC; Peter K.Iber, MAJ, MSC; Walter W. Noll, MAJ, MC; Katchrinnee Pavanand, M.D.; Pung Phintuyothin, MG, MC, RTA(ret.); Herbert E. Segal, MAJ, MC; Rowland N. Wilkinson, CPT, MSC; Vithune Yuthasastr-Kosol, M.D.

Associate: David M.Bear, M.D.; Attaya Boonyakanist; Sasikorn Chulakanchana, M.D.; Ravivan Intraprasert, M.D.; Sanong Kosakal, M.D.; Suphat Neoypatimanondh, M.D.; Chalard Tirabutana, M.D.

Assistant: Samroeng Bamnetpandh M.T.; Prajim Boonyakanist; Dumrong Charoendhum; William Gresso, SFC; Miss Jarouy Grutaraniyom; Suwath Hanchalay; Ampon Nanakorn M.T.; Kol Mongkolpanya; Mrs. Vacharee Panalaks, RN; Nopakol Pooyindee; Prachar Pooyindee; Larp Punthusiri; Chawanit Sinsawat; Peter W. Smyth, SSG; Lek Somchit; Boonplook Sornklin; Mrs. Pannee Srangsomwong , RN; Vorashart Suphakarn, B.S. M.T. (ASCP); Withoon Thiemanun; Kosol Vetbutanapibul, M.T., H.T.(ASCP).

Minocycline and Tetracycline Treatment of Acute Falciparum Malaria in Thailand

Principal Investigator: Edward J. Colwell, LTC, MC

Associate Investigators: Robert L. Hickman, MAJ, VC
Ravivan Intraprasert, MD
Chalard Tirabutana, MD

OBJECTIVE: Recent investigations of both natural and experimentally induced falciparum malaria in man have shown that tetracycline is highly effective in terminating infections of chloroquine resistant strains of this parasite. However, its relatively delayed onset of action and prolonged course of administration limit its value as a suitable antimalarial agent.

Minocycline(7-dimethylamino-6-demethyl-6-deoxytetracycline) is a new synthetic addition to the tetracycline family of antibiotics. Its preparation, structure and some of its properties have been reported by Martell and Booth(1967). Preliminary investigations of this new analogue have shown that minocycline is more potent and has a broader antibacterial spectrum than tetracycline. The purpose of our studies was to evaluate the therapeutic efficacy of minocycline in acutely ill subjects naturally infected with Plasmodium falciparum.

DESCRIPTION: The study was conducted at Prapokklao Hospital in the capital city of Chantaburi Province, which is located approximately 300 km southeast of Bangkok and 10 km north of the Gulf of Thailand. This area was selected because of the occurrence of year round transmission of falciparum malaria. During 1970, over 3,000 patients with confirmed P. falciparum infections were treated and followed on the wards or in the outpatient department. Personal observations of the staff physicians indicated that radical cures of P.falciparum infections following conventional doses of chloroquine were infrequent. Moreover, chloroquine resistant strains of P.falciparum are highly endemic in Trat Province which is only 35 km east of Chantaburi City.

Acutely ill male subjects, aged 14-60 years, infected with P.falciparum were considered for study. Those with renal or cerebral complications and those with a history of antimalarial drug intake during the preceding 7 days were excluded. Selected subjects were alternately assigned to one of 2 treatment groups. Individuals in one group were given 640 mg of quinine sulfate, thrice daily for 3 days, followed by 100 mg of oral minocycline twice daily for 7 days. Those in the other treatment group were administered

a similar course of quinine followed by 250 mg of tetracycline, 4 times daily for 10 days. Control of initial symptoms with quinine was indicated because of the relatively delayed onset of action of tetracycline. Seven subjects in the quinine-minocycline(QM) group and 8 in the quinine-tetracycline(QT) group were initially hospitalized for 6-8 days. The others were treated and followed on an outpatient basis.

During the treatment phase, all subjects were examined daily for clinical manifestations and appropriate medications were dispensed. Blood smear examinations in subjects among both treatment groups were performed daily for the first ten days and then at weekly intervals for the next 3 weeks. When subjects were available, blood smears were also obtained 2 months after commencement of therapy. Quantification of asexual and sexual parasitemias was performed by the method of Earle and Perez(1932). Serum specimens for determinations of bilirubin, and alkaline phosphatase and glutamate-oxaloacetate transaminase activities were obtained before treatment, and on the 10th and 31st days of observation.

PROGRESS: Twenty-nine subjects were chosen for the QM regimen. Treatment and follow-up examinations were successfully completed in 28, aged 15 to 50 years(mean 25.2). Asexual parasitemias among individuals in the QM group upon admission to the study ranged from 390 to 90,650 per cmm with a geometric mean of 8,670 per cmm. Of 33 subjects chosen for the QT regimen, treatment and follow-up examinations were successfully completed in 29, whose ages ranged from 14 to 57 years(mean 31.7). The levels of asexual parasitemias in subjects of the QT treatment group at the onset of study ranged from 425 to 238,700 per cmm with a geometric mean of 14,170 per cmm.

Table 1 shows the presumptive radical cure rates and the parasite clearance times obtained with both treatments. The single subject who experienced a treatment failure with the QM combination became symptomatic 24 days after commencement of therapy and exhibited asexual parasites 3 days later. Infections with P. vivax were observed on the 31st day of follow-up examination in 5 subjects treated with QT and in 3 treated with QM. Sixteen and eight individuals in the QM and QT groups, respectively, were available for follow-up blood smear examinations 2 months after commencement of therapy. No parasites of P. falciparum were observed.

None of the 28 subjects treated with QM and only 1 of 29 subjects treated with QT exhibited gametocytemia upon admission to the study and circulating gametocytes were observed during the follow-up period in specimens from 18 of the 57 subjects among both treatment groups. The levels of sexual parasites were generally low in density and persisted for a period longer than 12 days in only 6 subjects of whom 4 were treated with

QM and 2 with QT.

Daily doses of both tetracycline and minocycline were generally well tolerated. Transient complaints of mild nausea, vomiting and weakness were occasionally observed during minocycline treatment after clearance of patent parasitemia. Abnormalities in one or more of the liver function tests possibly attributable to minocycline or tetracycline were observed in 9 patients. Six patients in the QM group and 3 patients in the QT group, who had normal pretreatment serum values, had an abnormal elevation in the serum transaminase and/or alkaline phosphatase tests on the 10th day of observation. These abnormalities were mild and were still demonstrable on the 31st day in 4 patients treated with QM, but in none treated with QT. In 7 subjects among both treatment groups who had an abnormal liver function test(s) on the first day of observation, none exhibited significant increases during the treatment and follow-up period.

SUMMARY: The purpose of this study was to evaluate the antimalarial action of a new tetracycline analogue, minocycline, which is more potent and has a broader antibacterial spectrum than tetracycline. The study was conducted in an endemic area for chloroquine resistant falciparum malaria. Acutely ill residents infected with P. falciparum were alternately assigned to 1 of 2 treatment groups. Subjects in one group were given 540 mg of quinine base, thrice daily for 3 days followed by 100 mg of minocycline, twice daily for 7 days. Subjects in the other group were given a similar course of quinine followed by 250 mg of tetracycline, 4 times daily for 10 days. Presumptive radical cures were achieved in all 29 patients treated with quinine-tetracycline and in 27 of 28 treated with quinine-minocycline. No significant toxic side effects were observed. Although both treatments were highly effective, further studies are warranted to determine the optimal duration and dosage of minocycline and its potential human toxicity.

Table I

Presumptive radical cure rates and mean parasite clearance times with 2 treatments of acute falciparum malaria

Treatment*	No. subjects	% cure	Mean parasite clearance
Quinine-Tetracycline	29	100.0	3.0
Quinine-Minocycline	28	96.4	3.1

* See test for dosage.

Quinine-Tetracycline and Quinine-Bactrim Treatment of Acute Falciparum Malaria in Thailand

Principal Investigator: Edward J. Colwell, LTC, MC

Associate Investigators: Robert L. Hickman, MAJ, VC
Sanong Kosakol, MD
Pung Phintuyothin, MG, MC, RTA(ret.)
Suphat Neopatimanond, MD

OBJECTIVE: Tetracycline and a combination of sulphamethoxazole and trimethoprim have been shown to be highly effective in terminating infections with chloroquine resistant strains of Plasmodium falciparum. Investigations by Rieckmann et al.(1971), Clyde et al.(1971) and Colwell et al.(1972) have demonstrated that tetracycline treatment alone was sufficient to produce radical cures of both experimentally and naturally acquired P.falciparum infections in subjects who exhibited low density parasitemias and mild degrees of illness. Benjapongs and associates(1970) reported that a combination of sulphamethoxazole-trimethoprim(Septrin^{R}, Burroughs Wellcome and Co.), which was originally formulated for treatment of bacterial infections, effected radical cures of falciparum malaria in patients residing in an area highly endemic for chloroquine resistant strains.

However, the slow rate of these two alternative regimens, reflected by delayed clearances of patent parasitemia, does not permit their use for treatment of patients with moderate to severe degrees of illness. Subsequently, the efficacy of a combination of quinine and tetracycline was examined in acutely ill Thai patients residing in areas of chloroquine resistant falciparum malaria. A preliminary subcurative course of quinine was employed as an adjunctive agent for initial control of symptoms and parasitemia. These investigations showed that the quinine-tetracycline regimen was highly effective in controlling the symptoms and in producing a radical cure of the infection. A major disadvantage of this combination treatment was the prolonged course of drug administration(i.e., 13 days). The purpose of our investigations was to examine the efficacy of shorter courses of these alternative regimens in patients with acute falciparum malaria, with respect to onset of action, cure rate, cost and patient acceptability.

DESCRIPTION: The investigations were initiated in 1971 during the summer peak of malaria transmission at Phrabuddhabat, Saraburi Province in Central Thailand. When transmission subsided, the studies were relocated to Trat

Province in Southeast Thailand. Both areas are highly endemic for chloroquine resistant strains of P. falciparum.

Subjects considered for admission to the study were acutely ill patients, aged 12 to 60 years, with single P. falciparum infections. Those with a history of antimalarial drug intake during the preceding four days and those with evidence of renal and cerebral complications were excluded. Selected subjects were assigned to one of two treatment groups and the drug combinations were concurrently initiated on the first day of observation. Subjects assigned to one group were given 540 mg of quinine base, orally, every eight hours for only one day and 250 mg of tetracycline, orally, every six hours for seven days. Subjects assigned to the other treatment group were administered a similar course of quinine and two tablets of sulphamethoxazole-trimethoprim(Bactrim, Roche Co.) every 12 hours for five days. Each tablet of Bactrim contains 400 mg of a long acting sulfonamide, sulphamethoxazole, and 80 mg of a folate antagonist, trimethoprim.

Blood smear examinations were obtained daily for the first seven days and at weekly intervals for the next three weeks. When patients were available, blood smears were also obtained two months after commencement of therapy. The method of Earle and Perez(1932) was employed for quantification of asexual and sexual forms of P. falciparum. A radical cure was presumed if asexual parasitemia cleared during the first week and follow-up blood smear examinations remained negative for asexual parasites during the next three weeks.

More than 90% of the patients were hospitalized during the treatment period and they were examined daily during this period for clinical manifestations and dispensation of medications. The few non-hospitalized subjects were also examined daily during the treatment period, either at the outpatient department or at home visitation. There were no differences between the treatment groups which might lead to differences in re-exposure to malaria infections during the follow-up period.

PROGRESS: Forty-one subjects residing at both locations were chosen for the quinine-tetracycline(QT) regimen. Treatment and follow-up examinations were successfully completed in 32. Thirty-nine subjects at both locations were chosen for the quinine-Bactrim(QB) regimen, and treatment and follow-up examinations were completed in 31.

Tables 1 and 2 show the age, sex and pretreatment levels of patent asexual parasitemia among patients of both groups in whom examinations were completed. There were 22 males and 10 females, ranging in age from 12 to 44 years(mean 24.1) in the QT group. The levels of asexual parasites in this group ranged from 1,200 to 192,700 per mm^3 with a

geometric mean of 21,190 per mm^3. In the QB group, there were 22 males and 9 females, ranging in age from 12 to 54 years (mean 26.2). The pre-treatment levels of asexual parasites ranged from 1,100 to 414,480 per mm^3 with a geometric mean of 18,480 per mm^3.

The clinical and asexual parasite responses among subjects in both treatment groups are summarized in table 3. Presumptive radical cures were demonstrated in 84% of 32 subjects treated with QT and in 81% of 31 patients administered the QB regimen. These rates are not significantly different. The mean clearance times for fever in both groups were similar and almost as rapid as the fever clearance times for chloroquine and quinine treatment of susceptible strains of P.falciparum. However, the mean clearance of asexual parasitemia in the QT group was significantly longer in comparison with the mean clearance of patent parasitemia in the QB group. The latter clearance compares favorably with asexual parasite clearances observed with chloroquine and quinine treatment of susceptible strains. Blood smears were also obtained two months after commencement of therapy in 17 patients given QT and in 20 given QB. Only two of these patients, treated with QT, exhibited asexual parasites of P.falciparum at this time.

Circulating gametocytes were never observed in 16 of 52 patients who demonstrated presumptive radical cures. In addition, gametocytemias were low in density and transient in duration(i.e., less than 8 days) in 27 of the remaining 36 patients. These observations could reflect an inhibition or reduction of gametocyte production by early institution of the QT and QB treatments. The latter phenomenon has also been reported with the early administration (i.e., within first week of patent asexual parasitemia) of chloroquinine and quinine for susceptible strains of P.falciparum. High levels of circulating gametocytes which persisted for longer than 2 weeks were observed in 9 successfully treated patients of whom 5 and 4 were given the QT and QB regimens, respectively.

With regard to cost and patient acceptibility, the QT regimen appears to be superior to the QB regimen. The cost of the latter treatment was thrice that of the QT treatment. Although no serious toxic reactions were observed with either regimen, nausea and vomiting were observed more frequently during the treatment period among patients given the QB combination regimen.

SUMMARY: Two antimalarial treatments were assessed for onset of action, cure rate, cost and patient acceptibility. Thirty-two subjects were given a quinine-tetracycline(QT) combination consisting of 540 mg of quinine base every eight hours for only one day and, concurrently, 250 mg of tetracycline, four times daily for 7 days. Thirty-one patients were given a

quinine-Bactrim(QB) combination consisting of a similar course of quinine and, concurrently, 2 tablets of Bactrim every 12 hours for 5 days. Each tablet of Bactrim contains 400 mg of sulphamethoxazole and 80 mg of trimethoprim.

Presumptive radical cures were demonstrated in 84% treated with QT and in 81% given the QB regimen. All patients exhibited initial clearance of fever and asexual parasites. The mean fever clearance times in both groups were similar and almost as rapid as that observed with chloroquine and quinine treatment of susceptible P.falciparum strains. However, the mean clearance of asexual parasitemia in the QT group was significantly delayed (4.0 vs 3.0 days) in comparison with the mean clearance of the QB group. Neither treatment appeared to exert a gametocytocidal effect.

With regard to cost and patient acceptibility, the QT regimen was superior to the QB regimen. The latter costs thrice as much as the QT regimen. Although no serious toxic side effects were observed, nausea and vomiting were more frequently observed in the QB group.

Table 1

Pretreatment observations in subjects administered quinine-tetracycline*

Age(years)	Sex	Asexual parasite count (per mm^3)
12	Female	192,720
36	Male	191,400
38	Female	189,000
26	Male	159,250
25	Female	101,400
19	Male	81,900
14	Female	79,170
36	Male	69,960
16	Female	61,160
25	Male	51,320
15	Male	50,600
14	Male	47,860
31	Female	37,100
15	Female	28,620
17	Male	24,000
16	Male	21,800
23	Male	20,520
25	Male	19,800
18	Male	17,180
20	Male	14,760
18	Female	13,560
42	Male	13,220
14	Male	11,200
43	Male	8,820
27	Male	8,820
20	Male	6,600
17	Male	4,640
14	Male	2,540
18	Female	2,460
38	Male	2,440
44	Female	1,410
34	Male	1,200

*Simultaneous initiation of quinine(540 mg every 8 hours for 1 day) and tetracycline (250 mg every 6 hours for 7 days).

Table 2

Pretreatment observations in subjects administered quinine-Bactrim[R] *

12	Male	414,480
45	Male	251,700
12	Male	127,600
15	Female	80,100
16	Male	67,160
52	Female	52,860
41	Male	51,280
12	Female	50,600
14	Female	47,160
19	Female	33,360
50	Male	31,040
14	Male	28,440
49	Male	22,720
15	Female	21,600
17	Male	21,040
16	Male	20,350
14	Male	16,360
23	Male	12,360
45	Male	11,860
54	Male	10,860
24	Male	10,640
19	Male	10,590
32	Male	9,720
27	Male	7,120
23	Male	6,880
18	Male	5,300
19	Female	4,320
18	Female	3,300
27	Male	2,920
33	Male	1,940
34	Female	1,100

* Sumultanecus initiation of quinine(540 mg every 8 hours for 1 day) and Bactrim[R](2 tablets every 12 hours for 5 days).

Table 3.

Clinical and asexual parasite response to quinine-tetracycline or quinine-Bactrim[R] treatment of acute falciparum malaria.

Treatment	No. treated	Radical cure		Mean clearance time (Range) days	
		No.	%	Fever	Asexual parasites
Quinine-tetracycline	32	27	84.3	2.4(1-6)	4.0(1-7)
Quinine-Bactrim*	31	25	80.6	2.7(1-6)	3.0(1·5)

*Bactrim (Roche Co.) is a combination tablet containing sulphamethoxazole (400 mg) and trimethoprim (80 mg).

Quinine Treatment of Acute Falciparum Malaria in Central Thailand

Principal Investigator: Edward J. Colwell, LTC, MC

Associate Investigators: Suphat Neoypatimanondh, MD
Robert L. Hickman, MAJ, VC

OBJECTIVE: Chloroquine resistant falciparum malaria is distributed throughout all of Thailand and, in areas of intensive study, the majority of strains are resistant to conventional doses of chloroquine. Consequently, the treatment of choice for many hospitalized patients with moderate to severe degrees of Plasmodium falciparum infections is a 7 to 10 day course of quinine, In 1965, Pinswasdi and Charoenkwan observed an adult Thai male infected with P. falciparum who was unresponsive to chloroquine therapy and who exhibited a poor response to multiple courses of quinine administration. During the 12 months prior to our study, there have been several, isolated, unconfirmed reports of patients who showed a poor or no response to quinine administration. Because quinine is the treatment of choice for moderate to severe P. falciparum infections at most provincial hospitals in Thailand, it was considered highly desirable to examine the quinine sensivity of this parasite and attempt to confirm resistant strains.

DESCRIPTION: The investigation was conducted at Phrabuddhabat Hospital, Saraburi Province in Central Thailand, which was one of the locations for unconfirmed reports of quinine resistance. This area has been shown to be highly endemic for chloroquine resistant falciparum malaria. Patients considered for admission to the study were acutely ill males and females, aged 14 to 60 years, exhibiting moderate to severe degrees of illness. Those patients with a history of antimalarial drug intake during the preceeding 3 days were excluded.

Subjects so selected were hospitalized and given oral quinine, 540 mg base, thrice daily for 7 to 10 days. One patient presented with protracted emesis and was administered intravenous quinine(1.62 gm base a day) for the first 4 days. Blood smear examinations for quantification of asexual parasites were obtained daily during the treatment period and, subsequently, on the 17th, 24th and 31st days of observation.

PROGRESS: Thirty-three patients were selected for study. There were 28 males and 5 females, ranging in age from 14 to 57 years of age (mean was 26.5 years). The pretreatment levels of asexual parasitemias and the intervals for asexual parasite clearance for these patients are shown in Table 1. The

mean parasite clearance time for 31 subjects who showed initial clearing was 3.3 days(range 1 to 6 days). Parasite clearances for the other 2 patients were excluded from the mean computation because they expired during the early treatment period while still demonstrating patent parasitemia.

Twelve of 30 patients remained available for prolonged follow-up examinations. None showed a recrudescence.

Three of the 33 subjects originally selected for study expired during the treatment period. Two were males, aged 14 years(# 32 in Table 1) and 57 years(# 31), who died on the 3rd and 4th day, respectively. These two deaths were noted on early morning nursing rounds. The exact time and manner of demise was unknown. Postmortem examinations were not accomplished. Because these expirations occurred during the early treatment period, it is impossible to determine the major precipitating event; complication of the infection, intercurrent illness or drug toxicity. The extremely high degrees of erythrocytic parasitization in these two subjects would appear to indicate that the more probable event was a complication of the infection

The remaining patient who expired was a 57 year old wemen who was admitted with an asexual parasite count of 231,000 per cmm and no evidence or renal, hepatic or cerebral complications. On the 9th treatment day at which time fever and patent parasitemia were absent, she experienced sudden death in the early evening hours. Gross fingings at autopsy revealed only pulmonary congestion. It is our opinion that the most probable cause of death in this patient was a cardiotoxic event precipitated by quinine.

SUMMARY: Thirty-three Thai subjects acutely ill with falciparum malaria were given quinine treatment at a daily dose of 1.62 gm base for 7-10 days periods. The mean parasite clearance time in 31 of 33 patients, who exhibited initial clearing of asexual parasites, was 3.3 days. Prolonged follow-up examinations were accomplished in 12 patients and none showed a recrudescence. Three patients expired during the treatment period. Quinine cardiotoxicity was believed to be the cause of death in 1 of the 3 mortalities.

Table 1

Results of Quinine Treatment of acute falciparum malaria in Central Thailand

Patient #	Age (years)	Sex	Pretreatment Asexual parasitemia*	Duration of Asexual parasitemia (days)	Comment
1	17	M	397,360	5	Cure**
2	18	M	373,120	4	NF**
3	53	M	231,740	4	Hepatitis, NF
4	21	M	140,140	4	NF
5	25	M	136,400	4	NF
6	43	M	127,770	4	NF
7	18	M	104,470	3	Hepatitis, NF
8	27	M	96,230	4	Hepatitis, NF
9	42	F	52,980	2	Cure
10	15	M	44,880	3	NF
11	20	M	44,040	3	Cure
12	17	M	36,260	3	Cure
13	40	F	33,000	3	NF
14	20	M	32,040	3	Cure
15	16	M	30,840	4	NF
16	15	M	25,280	4	Hepatitis, NF
17	18	M	24,120	3	Cure
18	16	M	23,320	3	Cure
19	18	M	23,000	4	NF
20	18	M	21,929	4	Cure

(cont.)

(cont.) Table 1

Patient #	Age (years)	Sex	Pretreatment Asexual parasitemia*	Duration of Asexual parasitemia (days)	Comment
21	19	M	20,800	3	NF
22	16	M	17,850	4	NF
23	37	M	11,240	3	NF
24	40	M	11,000	3	Cure
25	49	M	7,360	2	NF
26	25	M	5,360	4	Cure
27	24	M	5,240	3	Cure
28	16	F	2,120	2	Cure
29	46	F	200	2	NF
30	19	M	20	1	NF
31	37	M	495,000	-	Expired
32	14	M	353,320	-	Expired
33	57	F	231,140	4	Expired

* Per cmm
** Cure indicates no recrudescence within 31 days of follow-up
*** No follow-up after treatment

A Comparative Evaluation of Sulfalene-Trimethoprim and Fanasil-Pyrimethamine Against Falciparum Malaria in Thailand

Principal Investigator: William Chin, MD

Associate Investigators: David M. Bear, MD
Edward J. Colwell, LTC, MC
Sanong Kosakol

OBJECTIVE: Chloroquine resistant falciparum malaria was first reported from Thailand in 1962. Subsequent surveys performed in this country have indicated a rate ranging from 50-100%.

Because of the problem of drug resistance and because the administration of quinine to large numbers of patients in the field is operationally impractical, the need in Thailand for a simple and effective regimen against falciparum malaria is most urgent. Presently, 2 sulfonamide combinations have the potential for meeting this critical need. These are:

1. Sulfalene-trimethoprim and
2. Fanasil-pyrimethamine.

The purpose of this study was to assess these 2 combinations against falciparum malaria in adult Thai males, with respect to rapidity of action, cure rate, and possible side effects, especially side effects in individuals deficient in erythrocytic glucose-6-phosphate dehydrogenase (G-6-PD) activity.

DESCRIPTION: Subjects for this evaluation were chosen from acutely ill individuals seeking treatment at the Trad Provincial Hospital located in Southeast Thailand. Selected were adult males, 15 years of age or older, with a positive blood smear for falciparum malaria who, by history and physical examination, were found to be free of obvious evidence of renal, cerebral, or hepatic complications.

Initial laboratory determinations consisted of hematocrit and leukocyte count. A venous blood sample was obtained for the assessment of possible G6PD deficiency by the Hyland G-6-PD spot test. Subjects were then alternately assigned to one of 2 treatment groups listed below.

Treatment I: S-T (Sulfalene 1 gm and trimethoprim 0.5 gm).
Treatment II: F-P (Fanasil 1 gm and pyrimethamine 0.050 gm).

Following treatment, patients were admitted to the medical ward

where they were observed initially for vomiting and, when required, given supportive care.

As patients became asymptomatic they were discharged after at least 3 consecutive days of negative blood smears.

Hematocrits, leukocyte counts, and thick-thin blood smears were taken at least daily during the patients's hospitalization and weekly thereafter on days 7, 14,21 and 28 following discharge.(The day of treatment was designated day 0). The smears were treated with Giemsa stain. Parasites per cmm were calculated by multiplying the number of parasites per 100 WBC by the factor obtained from each patients's white blood(WBC) count taken on the same day. (Factor=WBC/cmm of blood ÷ 100) If 5 minutes of examination failed to disclose parasites, a smear was considered negative.

In this study, cure was defined as the clearance of patent asexual parasitemia by day 7 with no recrudescence during the remaining weekly follow-up examinations to day 28. Failure was the lack of clearance of patent parasitemia by day 7 or reappearance of patent asexual forms during any of the weekly follow-up examinations after initial clearance.

PROGRESS: Eighty-eight patients were selected for inclusion in the study. Adequate history from 75 of the patients indicated that 73% took some antimalarials prior to their hospital admission. The medication most frequently utilized was chloroquine, although occasionally intramuscular quinine had been administered.

More than 90% of the subjects had been ill for an average of 6 days prior to hospital admission. Fewer than 10% stated that they had come to the hospital on the first day of illness or that they had been ill for greater than one month. Approximately 60% of the patients had a history of one or more malaria infections.

The subjects were divided on an alternating basis into 2 groups. S-T was given to 45 cases and F-P given to the other 43. The pre-treatment observations of the 2 groups were quite comparable with respect to age, weight and initial parasite density(Table 1).

The results of the immediate response to treatment are summarized in table 2. The results observed in the 2 groups were again comparable. There was one case in each of the treatment groups in which patent parasitemia was not cleared within 7 days.

Follow-up for one month was possible in 65 of the 88 cases. The results of these 65 cases are summarized in Table 3. Although the F-P regimen

produced a better cure rate than that of S-T, the difference was not significant, the Chi square value for the observed differences being only 0.261. In the cases of delayed treatment failure, patent parasitemia was cleared initially within 2-4 days in 4 S-T treated individuals and within 6-7 days in 2 cases given F-P.

The WBC count and hematocrit values of all subjects were within the usual range found in patients with malaria. The lowest WBC count, 1,320/cmm, was recorded on day 21 from one case given F-P treatment. This individuals's WBC count rose to 7,700/cmm on day 28.

G-6-PD deficiency was found in 11(12.5%) of the 88 subjects. Comparison of the post-treatment hematocrit values of these 11 individuals with the other subjects disclosed no detectable differences.

The effect of treatment of gametocytemia in 63 of the cases observed for 28 days is shown in table 4. In both treatment groups, gametocytes usually developed approximately 6 days after treatment. In general, gametocyte densities were found to be less than 1,000/cmm. In one exceptional case, a maximal density of 18,778/cmm was observed 7 days after treatment with F-P.

Two patients given S-T vomited soon after treatment. The other subjects in both groups tolerated the medications well, and no adverse side reactions were observed.

SUMMARY: In areas of the world where chloroquine resistant falciparum malaria is a significant problem, long-acting sulfonamide combinations have a definite place as an alternative treatment in proven drug resistant cases. Experience in Thailand indicates that the rational field use of such combinations should be guided by the following considerations:

1. The recurrence of patent infection with asexual forms of falciparum malaria within one month following treatment with a presumptive dose of chloroquine(600 mg base) is sufficient indication of chloroquine failure, suggesting the intervention of long-acting sulfonamide combintions as the next treatment.

2. Until more information is available regarding the possible sporontocidal property of long-acting sulfonamide combinations, the additional use of primaquine as a gametocide is recommended.

3. Prior to contemplated mass use, an evaluation should be undertaken to determine susceptibility of local strains to the long-acting sulfonamide combinations.

4. Following failures to cure with such combinations, treatment with quinine should be instituted.

It is apparent that sulfonamide combinations, because of their known limitations, are not the final solution to the problem of drug resistant falciparum malaria. Until a better treatment is developed such combinations can, if used judiciously, fill existing needs.

Table 1

Pre-treatment observations of study subjects

Rx	No. Cases	Age in yrs. Range (average)	Wt. in kg Range (average)	Parasite count/cmm Range (geometric mean)
Sulfalene-trimethoprim	45	15-50 (26.1)	25-90 (50.9)	59-120,054 (6,473)
Fanasil-pyrimethamine	43	15-51 (25.5)	31-63 (48.6)	271-125,400(7,010)

Table 2

Immediate response to treatment

Rx	No. cases	Clearance of patent parasitemia by day 7 No. (%)	Average No. days to clear parasitemia	Average No. days to clear fever(100°F)
Sulfalene-trimethoprim	45	44 (97.8%)	2.2	2.4
Fanasil-pyrimethamine	43	42 (97.6%)	2.6	2.5

Table 3

Treatment results in patients followed one month

Rx	No. cases*	No. cured(%)	Day of recrudescence in delayed failures
Sulfalene-trimethoprim	31	26(83.9%)	14, 28, 21, 28
Fanasil-pyrimethamine	34	31(91.2%)	28, 28

* Also includes the 2 immediate treatment failures noted in table 2.

Table 4

The effect of S-T and F-P treatment on gametocytemia in cases followed for one month.

Rx	Total cases	Gametocytes present on initial smear No. (%)	Gametocytes not observed during 28 days No. (%)	Gametocytes developing after treatment No. (%)
Sulfalene-trimethoprim	30	3 (10%)	7 (23.3%)	20 (66.6%) range = 1-14 days average= 5.9 days
Fanasil-pyrimethamine	33	1 (3%)	4 (12.1%)	28 (84.8%) range = 1-23 days average= 6.2 days

Investigations of in vitro and in vivo Chloroquine Sensitivity of Plasmodium falciparum in Yala Province.

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Associate Investigators: Edward J. Colwell, LTC, MC
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OBJECTIVE: Chloroquine-resistant Plasmodium falciparum malaria is widespread in Thailand, approaching 100% prevalence in some areas. In the provinces of Yala, Satul and Narathiwat, Bourke, et al. (1966) reported finding a prevalence of chloroquine-resistant P. falciparum infections as high as 81%. This rate was based on the persistance of asexual parasitemias 3 days after the administration of 4 mg. chloroquine base per lb body weight. Although this dose is now considered subcurative and the follow-up period brief, Sandosham, et al. (1964), in reviewing results of earlier investigations, provided evidence that 4 mg per lb usually produced clearance of asexual chloroquine-sensitive parasites within 3 days.

Recently Andre, et al. (1972) reported that the prevalence of chloroquine-resistant P. falciparum in Kelantin province of Malaysia, adjacent to the study sites employed by Bourke and associates appeared to be much lower. Only 5 of 87 infected school children, (6%) had demonstrable parasitemias on the 7th day following conventional treatment with chloroquine (25 mg per kg). These results are identical to those obtained by McElvey, et al. (1971) 7 days after similar treatment of commonwealth troops stationed in Malaysia. Further in vivo studies by McElvey, et al., and limited in vitro investigations by Andre, et al., indicated the true prevalence was higher but less than has been reported in Thailand.

There is, therefore, some evidence that the prevalence rates of chloroquine-resistant falciparum malaria in Thailand and Malaysia are different. Furthermore, because of the proximity of the study sites used by Andre, et al., and Bourke, et al., it is conceivable that a transition zone between the higher and lower rates coincides with the location of the Thailand-Malaysia border. This would not be too surprising since the border is more than just a political and religious boundry separating Bhuddist Thailand and Moslem Malaysia. It is also the approximate position of a transition zone between 2 types of climate and is a widely recognized zoo-geographical boundary. On either side of the zone marked differences in flora and fauna exist. If a difference in rates of chloroquine resistance does exist, the border area would be a unique site to study the bionomics of chloroquine-resistant falciparum malaria.

The present investigations were initiated in an effort to obtain more recent data regarding the in vivo and in vitro chloroquine sensitivity of P. falciparum in southern Thailand and to compare the results with those obtained previously in both southern Thailand and Malaysia.

DESCRIPTION: Yala city was chosen as a study site because previous studies were conducted near by and because of the support available from the provincial hospital and the district Malaria Eradication Center. Malaria infected subjects were referred for study from both sources. Other patients reported directly to the laboratory for diagnosis. Blood smears were made on all patients and checked for the presence of asexual parasites, their identification and the degree of parasitemia. Only subjects with P. falciparum infections were selected. Patients with parasitemias less than 1000 per cmm and those with mixed infections were excluded. Others excluded were patients under 6 years of age, those with a history of antimalarial treatment during the preceding 96 hours and those with renal or cerebral complications.

A total of 64 selected subjects were administered conventional doses of chloroquine (10 mg chloroquine base per lb. body weight) over 48 hours. Blood smears were examined daily for 7 days to determine parasitemias. If parasite levels and/or symptoms were not controlled by chloroquine, quinine was administered. Further follow-up examinations on days 14 and 21 were performed on all subjects who were parasite free on the previous examination. All infections which recrudesced were treated with alternative antimalarial drugs.

At the time of subject selection, the mean level of trophozoite maturity was assessed. If the parasites were judged to be of adequate maturity, venous blood was drawn for an in vitro determination of the parasite sensitivity to chloroquine. The method used was that described by Rieckmann, et al. (1968) and employed by Colwell, et al. (1972). The test was considered successful if 5% or more of the trophozoites in the control vial matured to schizonts containing 3 or more nuclei. The parasites were arbitrarily considered to be chloroquine resistant if any schizonts were found in vials containing 0.6 millimicromoles or more of chloroquine base.

PROGRESS: The results of post-treatment examinations to determine in vivo chloroquine sensitivity of P. falciparum in Yala province are summarized in Table I. A total of 42 patients were examined daily for 7 days. Of these, 3 failed to respond to treatment either clinically or parasitologically and 14, although they did respond, were parasitized on day 7. Thus, 40.5% of the infections demonstrated RII-RIII type chloroquine resistance. Of the 25 subjects who were parasite free on day 7, 21 were examined on day 14 and if negative again on day 21. Asexual parasitemias of P. falciparum, suggestive of RI type chloroquine resistance recurred in all but one. The cumulative prevalence of chloroquine-resistant P. falciparum was 97%. Since patients were rarely hospitalized more than 5 days, it is possible that some of the later recrudescences were, in fact, reinfections.

The prevalence of chloroquine-resistant P. falciparum in Yala as determined by the in vitro technique was similar to that obtained in vivo. The in vitro test was successfully conducted with the blood of 42 patients, 25 of whom subsequently participated in the in vivo portion of the study. The corresponding in vivo and in vitro results are presented in Tables II and III. With regard to chloroquine resistance, the results are in complete agreement, both being 100%. As in previous studies (Colwell, et al., 1972), however, the degree of resistance as determined clinically (RI or RII) could not be correlated with the growth patterns that occurred in vials containing different concentrations of chloroquine. Individual results of in vitro tests where in vivo data was not obtainable are presented in Table IV. The prevalence, overall, of in vitro chloroquine resistant P. falciparum was 97%, the same as was determined using the in vivo technique.

The in vivo results compare favorably with those obtained previously in southern Thailand (Bourke, et al., 1966) and are markedly higher than those obtained more recently in Malaysia (McElvey, et al., 1971; Andre, et al., 1972). In the latter studies, only 6% of the subjects studied had infections that could be classified as chloroquine resistant 7 days after treatment was initiated. Further studies by McElvey, et al., showed that 47% of the patients that were parasite free on day 7 recrudesced before day 32 resulting in an overall prevalence of chloroquine resistance of 50%. The possibility of reinfection was excluded in the latter study.

Differences in the number of patent infections found on day 7 and in the overall prevalence of resistance suggest that there is a difference in the prevalence and degree of chloroquine resistance in the extreme south of Thailand and in Malaysia. It is conceivable that the differences may be somewhat exaggerated by reinfections which, if they occurred, were unavoidable in the current investigation.

It also true that the subjects studied in Yala province represented a different population that did those studied in Malaysia. The fact that subjects selected at Yala were seeking treatment implies that they may have been more ill than those studied in Malaysia and some may have already been treated unsuccessfully with chloroquine. Both variables may have influenced the results in such a way as to suggest a higher prevalence of chloroquine resistance. On the other hand, the level of acquired immunity in the Yala patients who were predominatedly young adults (mean age: 25.1 years) was probably greater than that in either the school children examined by Andre, et al. (1972) or the commonwealth troops studied by McElvey, et al. (1971). Higher levels of immunity may improve the efficacy of chloroquine and, thus, result in a reduced prevalence of chloroquine-resistant falciparum malaria. Obviously further study will be required to more fully evaluate the situation, but these preliminary results strongly suggest that a gradient in the prevalence of chloroquine-resistant P. falciparum does exist in the Thailand-Malaysia border area.

SUMMARY: In vivo and in vitro studies were conducted in Yala province to determine the chloroquine sensitivity of P. falciparum in southern Thailand. By the seventh day after conventional treatment with chloroquine was initiated, 40.5% of the infections studied were

either still patent or had been treated with other drugs because of an unsatisfactory patient response. By day 21, all but one of the remaining infections studied had presumably recrudesced although the possibility of reinfection could not be entirely excluded. The overall prevalence of both in vivo and in vitro chloroquine resistance was 97.4%. This is comparable to the prevalence found earlier in Thailand (Bourke, et al., 1966) and is higher than reported recently in Malaysia (McElvey, et al., 1971; Andre, et al., 1972). The evidence suggests that there may be a prevalence gradient in the area of the Thailand-Malaysia border. If true, the area may be a unique site in which to study the bionomics of chloroquine-resistant falciparum malaria.

Table I. Clinical Response of *P. falciparum* infections to conventional chloroquine therapy

Response	No. of subjects	Per cent
RIII	3	7.1
RII	14	33.3
RI	20	47.6
S-7*	4	9.5
S-21**	1	2.4

* No recrudescence through day 7. No further follow-up.

** No recrudescence through day 21. No further follow-up.

TABLE II. EFFECT OF CHLOROQUINE UPON IN VITRO MATURATION OF P. FALCIPARUM IN SUBJECTS WITH AN RII - RIII CLINICAL RESPONSE TO CHLOROQUINE.

Subject Number	Parasite Count (per cmm)	Chloroquine Concentration *						
		0	0.4	0.6	0.9	1.35	2.02	3.04
1	16,350	84**	73	65	53	34	5	1
2	1,560	76	70	66	61	47	11	5
3	14,070	68	52	47	0	1	0	0
4	3,460	27	12	24	30	10	15	2
5	296,600	21	17	15	8	4	3	0
6	55,200	18	12	12	5	4	3	2
7	39,170	9	5	4	4	4	3	2

* Millimicromoles per ml of added inoculum.

** % Schizonts with 3 or more nuclei.

TABLE III. EFFECT EFFECT OF CHLOROQUINE UPON *IN VITRO* MATURATION OF *P. FALCIPARUM* IN SUBJECTS WITH AN RI CLINICAL RESPONSE TO CHLOROQUINE.

Subject Number	Parasits Count (per cmm)	Chloroquine Concentration						
		0	0.4	0.6	0.9	1.35	2.02	3.04
8	6,650	89	80	34	57	38	4	1
9	1,175	81	79	76	74	40	57	1
10	2,250	56	25	28	1	0	0	0
11	7,080	50	20	14	1	0	0	0
12	1,990	50	42	39	24	10	3	1
13	9,470	48	51	43	21	10	13	0
14	2,660	40	28	35	20	6	2	0
15	24,450	35	23	29	31	6	6	1
16	13,820	22	0	15	2	0	1	0
17	13,640	14	13	10	5	1	0	0
18	27,070	12	9	8	7	4	3	5
19	13,210	9	9	7	6	1	1	0
20	40,760	8	8	5	5	3	1	0
21	1,780	8	4	5	8	2	1	0
22	44,200	7	7	6	5	1	0	0
23	5,930	7	3	7	6	3	2	1
24	21,640	5	4	5	3	3	0	0
25	9,070	5	5	5	3	1	3	1

TABLE IV. EFFECT OF CHLOROQUINE UPON IN VITRO MATURATION OF P. FALCIPARUM IN SUBJECTS WITH AN UNKNOWN CLINICAL RESPONSE TO CHLOROQUINE.

Subject Number	Parasits Count (per cmm)	Chloroquine Concentration						
		0	0.4	0.6	0.9	1.35	2.02	3.04
26	3,220	75	73	85	36	10	6	0
27	3,410	74	60	64	13	1	0	0
28	2,400	60	50	48	52	0	6	0
29	27,550	44	11	21	7	2	2	0
30	17,490	44	17	19	10	2	0	1
31	6,970	43	44	40	18	7	5	0
32	12,380	30	13	23	20	7	8	1
33	7,840	28	19	16	10	1	1	0
34	7,490	26	24	20	18	12	7	2
35	25,140	25	10	25	10	2	0	1
36	5,640	19	11	9	9	6	7	0
37	36,630	16	17	12	13	6	2	0
38	29,060	16	4	4	1	0	0	0
39	34,010	8	0	0	0	0	0	0
40	14,640	7	5	7	7	4	2	1
41	56,230	6	3	4	1	1	1	1
42	5,540	6	4	6	0	0	0	0

Investigations of the Fluorescent Spot Test for Erythrocyte Glucose -6-Phosphate Dehydrogenase Deficiency in Southeast Thailand

Principal Investigator: Ben F. Castaneda, SFC

Associate Investigators: Edward J. Colwell, LTC, MC
Pung Phintuyothin, MG, MC, (ret.)
Robert L. Hickman, MAJ, VC

OBJECTIVE: Several investigations of the prevalence of erythrocytic glucose -6-phosphate dehydrogenase(G6PD) deficiency have been reported among male residents of the northern, central and southeastern areas of Thailand. No studies of this genetically determined, sex-linked enzyme deficiency have been accomplished in the remote southeastern regions bordering Cambodia. Both falciparum and vivax malaria are highly endemic in this region and infected patients are often administered primaquine therapy. Since primaquine among other drugs is known to precipitate acute intravascular hemolysis in subjects whose red blood cells are deficient in G6PD activity, it was considered desirable to obtain information on the prevalence of this enzyme deficiency in Southeast Thailand. This report also describes studies on the reproducibility and reliability of the screening technique employed.

DESCRIPTION: The surveys were conducted in Trad Province at the 2 localities shown in Fig.1. These locations were chosen because of the relative stability of their population and the endemicity of vivax and falciparum malaria in both communities. The capital city of Trad Province has a population of approximately 10,000 people. Takum, a typical rural village with an approximate population of 400 people, is located 18 km northeast of Trad City.

Blood specimens were obtained from the majority of the total male population(i.e.,200) at Takum during a civic action medical patrol. Trad City, which has an estimated male population of 5,000 was arbitrarily divided into 4 geographic regions. Blood specimens were obtained by house visitation from approximately 10% of the estimated male population in each region. All subjects were between the ages of 6 to 60 years and did not exhibit symptoms of clinical illness at the time of specimen collection. Specimens consisted of a heparinized capillary tube of blood obtained by digital puncture. Those obtained at Takum were transported immediately on wet ice to a base laboratory located at the provincial hospital in Trad City. Processing of all specimens was accomplished within 2-3 hours after digital puncture.

The method employed for screening was a commercially available kit of the fluorescent spot technique(Hyland G-6-PD screen test). The test was conducted in strict accordance with the directions supplied with the kit. Preliminary studies were initially performed to determine the reproducibility of the test. Replicate specimens from 10 male subjects were coded and processed by 3 different technicians. The reactions of the test were observed independently by 3 individuals using the same ultraviolet light source and the results were recorded as strong (bright fluorescence), weak(barely detectable fluorescence) and negative (no fluorescence).

Investigations were also undertaken to assess the reliability of the visual observations of the fluorescent spot test. Thirty-eight specimens processed with the spot test were examined for quantitative erythrocytic G6PD activity(G-6-PD Statt Pack). In addition, the same specimens were examined for the proportion of G6PD normal and deficient erythrocytes by the methemoglobin elution test as modified by Gall, et al.(1965). In the latter technique, a mixture of normal and deficient erythrocytes can be differentially identified by a series of chemical treatments which include methemoglobin reduction and elution, and subsequent hematoxylin staining to identify the normal and deficient red blood cells.

PROGRESS: The results of the fluorescent spot test reactions among residents of Trad city and Takum are shown in Table 1. The instructions of the Hyland test kit specify that specimens having no or barely detectable fluorescence are indicative of erythrocytic G6PD deficiency. According to these criteria, the deficiency rates at Trad city and Takum combining weak and negative reactions, were 16.0 and 12.8%, respectively.

The results of replicate examinations for assessing the reproducibility of the fluorescent test are presented in Table 2. In general,there was satisfactory agreement among the results of the replicate examinations and among independent observations of the same reaction by different technicians.

In order to assess the reliability of the spot test results, 2 other methods for analysis of G-6-PD activity were conducted. Table 3 shows the results of the methemoglobin elution test and the quantitative assay for assessing the reliability of the fluorescent spot test reactions in paired specimens. All specimens exhibiting either a strong or negative reaction in the spot test demonstrated corresponding normal or deficient reactions in the elution test and the quantitative assay. However, of 14 specimens exhibiting weak fluorescent reactions in the spot test, 10 were normal and 4 were deficient in the elution test as well as the quantitative assay.

The results obtained in these alternative tests confirm the reliability of the negative and strong spot test reactions. However, they indicate that

the weak reactions are not necessarily indicative of G-6-PD deficiency. Therefore it is not possible to classify these reactions, and the results of our survey must be presented as a range: 2% to 12% at Takum and 8% to 16% at Trad city. The lower limits of the range denote the proportion of negative reactions in the spot test and the upper limits are the combined proportions of the negative and weak reactions. It is proposed that the G-6-PD spot test be re-evaluated in the laboratory before further use in the field is considered.

SUMMARY: A survey to determine the prevalence of G-6-PD deficiency was conducted in Trad city and the village of Takum situated in Trad Province, Thailand employing the fluorescent spot test as the screening technique. The results are reported as a range of 2 to 12% deficiency at Takum and 8 to 16% deficiency at Trad city. Expression as a range of G-6-PD deficiency was necessitated because of the unreliable interpretations of weak fluorescent reactions in the spot test. A very high degree of reliability of strong and negative fluorescent test reactions was established by the confirmatory results of the quantitative assay and methemoglobin elution tests.

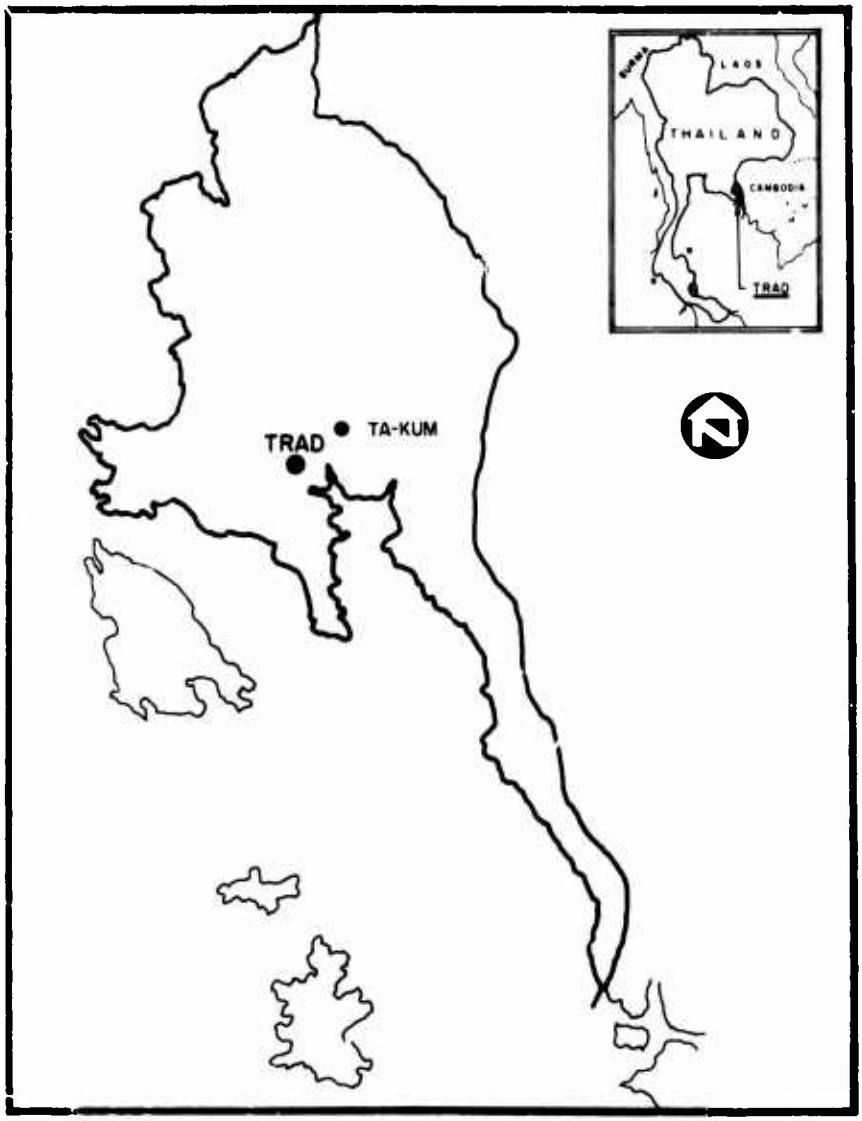

FIG. 1 MAP OF TRAD PROVINCE IN SOUTHEAST THAILAND SHOWING THE STUDY SITES.

Table 1

Results of the fluorescent spot test reactions in Trad Province.

Location	No. males examined	Rates(%) of spot test reactions		
		Negative	Weak	Strong
Trad City	518	8.3	7.7	84.1
Takum	125	2.4	10.4	87.2
Total	643	7.2	8.1	84.8

Table 2

Results of replicate examinations to access the reproducibility of the fluorescent spot test.

	Replicate A			Replicate B			Replicate C		
Patient	R1*	R2	R3	R1	R2	R3	R1	R2	R3
1	S**	S	S	S	S	S	S	S	S
2	W	W	W	W	W	W	W	W	W
3	S	S	S	S	S	S	S	S	S
4	S	S	S	S	S	S	S	S	S
5	S	S	S	S	S	S	S	S	S
6	W	W	W	S	S	S	W	W	-
7	S	S	S	S	S	S	S	S	S
8	S	S	S	S	S	S	S	S	S
9	-	-	-	-	-	-	-	-	-
10	S	S	S	S	S	S	S	S	S

* Reading of spot test by different technicians

** (S,W,-) indicate strong, weak or no fluorescence, respectively.

Table 3

Comparison of the results of the fluorescent spot test, methemoglobin elution test and quantitative assay tests.

Spot test		Elution test		Assay test	
Reaction	No. reacting	Normal	Deficient	Normal	Deficient
Strong	21	21	0	21	0
Weak	14	10	4	10	4
Negative	3	0	3	0	3
	38				

Evaluation of In vitro Drug Sensitivity of Human Plasmodium falciparum by Incorporation of Radioactive Isoleucine.

Principal Investigators:	Peter K. Iber, MAJ, MSC Katchrinnee Pavanand, M.D.
Associate Investigators:	Norman E. Wilks, LTC, MSC Edward J. Colwell, LTC, MC

OBJECTIVE: This investigation was an attempt to assess chloroquine sensitivity of P. falciparum isolated from naturally infected Thai nationals by employing both the in vitro culture system described by Diggs et al and the protein incorporation of C^{14}-isoleucine as a measure of plasmodial growth. If successful, such a system could be adapted to the field to detect resistant malaria in less than the 28 day observation period prescribed by WHO[8].

BACKGROUND: Cultivation of malaria parasites in vitro has been restricted generally to experimentation with animal plasmodia. Despite the successful culture of Plasmodium falciparum as early as 1912[1], the culture of native falciparum malaria for extended periods of time has been hampered by a lack of knowledge of parasite metabolism and biochemical requirements from the host red cell. Siddiqui et al[2] reported successful culture for 48 hours but only on rare occasion did they achieve multiplication rates where the number of parasitized cells greatly increased after schizogony. More recently, Diggs and associates[3] have reported a culture system in which five to eight fold increase in parasitized cells were observed repeatedly and growth periods of greater than 72 hours were routine through the use of media T-199 and heat treated human AB serum. The parasite multiplication was determined by the use of fetal cells as markers for merozoite penetration. By virtue of the increase in survival time and parasite populations this system lends itself to extensive research opportunities including metabolic and pharmacologic studies of Plasmodium falciparum.

Polet and Conrad[4] have reported isoleucine as an essential amino acid for P. knowlesi. Canfield et al[5] have described an in vitro technique for testing potential antimalarial drugs using methyl C^{14}-methionine incorporation into parasite protein as measurement of drug effect. McCormick[6] was successful in using C^{14}-isoleucine as a growth marker for P. falciparum in the test described by Rieckmann[7].

DESCRIPTION: The basic procedures for cultures of Plasmodium falciparum were those previously described[3]. For drug sensitivity studies the following modifications were necessary.

Parasites: Patients from the Yala Provincial Hospital and the Malaria Eradication Center, Yala, Thailand were used as donors for parasites of Plasmodium falciparum. Only patients with parasitemias greater than 0.1% were accepted. The blood was collected in a heparinized syringe and the cells were later separated from the plasma by centrifugation at 210 X G for 10 minutes. The cells were then washed twice with physiological saline, centrifuging each time at 210 X G for 10 minutes.

Media: The packed cells from the second washing were then placed into media prepared for each row of a microtitration plate as follows:

Media T-199	0.6	ml
Heated Serum (AB Group)	0.6	ml
Washed packed cells	0.125	ml
C^{14}-isoleucine*	0.050	ml (0.048 ug = 2.21×10^5 dpm)
Total volume	1.375	ml (enough for 6 wells)

The media-cell suspension was then distributed into the wells of one row in the microtitration plate in the array of drug concentrations shown in Table I (each well contained 0.2 ml of the media-RBC suspension).

Harvesting Cultures: The culture suspension was quantitatively transferred from each well in one column at the harvest time into a 13 x 100 mm test tube. A few milliliters of normal saline was needed to effect quantitative transfer. The suspension was centrifuged at 6000 x G and the supernatant was discarded. Packed cells were washed twice with 5 milliliters of normal saline, and centrifuged as above, and then 1 ml of 10% trichloroacetic acid was added, mixed well, and centrifuged at 10,000 x G. The supernatant was removed, the precipitate was washed with 1 ml of ethanol-ether (1:1), mixed well, centrifuged, and the supernatant was discarded. Three drops of 30% hydrogen peroxide were added, mixed and incubated at 56°C for 15 minutes. Two milliliters of Soluene (TM 100 Packard) was added to the precipitate which was then incubated 30 minutes at 60°C and transferred to a counting vial using fluor as a rinsing agent for the test tube. The final volume was 12 milliliters.

Drug growth studies were performed using two series of Chloroquine concentrations. The first series, covering Yala patients Y-36 to Y-48 (See Table II), was cultured utilizing a drug concentration of 8 milli-

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* Carbon-14 Isoleucine. New England Nuclear Corp., NEC 278, Lot # 605-072 with Specific Activity 273 millicurie/millimole.

micromole chloroquine base per ml blood[7]. This concentration was based on a 12 hour serum level in Thai volunteers (Figure 1) and the partition ratio RBC: Serum of approximately 4:1 to 5:1.

With the 8 millimicromole concentration of chloroquine, although pharmacological, it is difficult to evaluate the culture results. Since the 12 hour serum concentration is transient, it was decided that the 72 hours serum concentration was more realistic for the culture period. Therefore, we revised the chloroquine concentrations in the subsequent series. In Table III, the results of 16 cultures utilizing the new concentrations are summarized.

Initially we used only chloroquine and a control which yielded culture data as shown in Figure 2, but it was later realized that a reference culture was required. Since P. falciparum in Thailand is generally considered to be sensitive to quinine[8], a culture containing 8 millimicromoles of quinine base per ml blood was added to the culture system. This simplified culture interpretation and resulted in the following system of grading the resistance of the parasites:

(1) If both concentrations of chloroquine produce growth curves near the quinine growth curve the culture is rated sensitive (S) (Figure 3).

(2) If the 0.2 millimicromole chloroquine curve approximates the control curve and the 0.4 millimicromole chloroquine curve approximates the quinine curve, the culture is rated R+.

(3) If both concentrations approximate the control growth curve the culture is rated R++ (See Figure 4).

In vivo studies were possible in 50% of the malaria cases seen. Follow-up visits were requested for days 7, 14 and 21 after being admitted to the study program. In only a few cases was this schedule actually achieved.

A reproducibility study was performed by utilizing three identical wells for each harvest time and drug concentration. All wells were processed independently and counted to provide a 99% probability of 1% counting error.

RESULTS: The in vitro growth multiplication rate of P. falciparum with C^{14} isoleucine as a marker confirmed a minimum multiplication of five fold through one cycle of schizogony. This was determined by comparing the slopes of the growth curve before and after schizogony. This determination was facilitated by the fact that during schizogony no C^{14} isoleucine is incorporated as reported earlier by Cohen et al for H^3leucine

incorporation[9,10]. When maximum growth was desired, the media was changed after 48 hr. to remove harmful metabolic products from the system. The culture system designed for drug testing showed slightly lower multiplication rates (i.e. 2-3 fold) because the media could not be changed.

In all cultures containing quinine, a depressed growth curve was consistently evident as compared to the control, indicating the susceptibility of parasites to the antimalarial agent. In case of resistance, the growth curve approximated that of the control. This was also confirmed by morphological studies in three of these cultures (Table IV) of which one in vivo follow-up was possible, and in this case there was good correlation between in vivo, in vitro and morphological observations. It appeared that in the presence of antimalarial agents, susceptible parasites could not survive through schizogony, while resistant parasites escaped the antimalarial drug action and the penetration of merozoites into new red cells was evident.

The maximum variance observed in the reproducibility study was 10%, the normal variance was 3 to 5%. Counting efficiency was determined to be 58 to 64% using the channels ratio counting method.

In vivo follow-up studies were disappointing in that few patients returned for examination beyond day 7. The in vitro results compare favorably with in vivo parasitemias recorded on day 7, but less so for those few patients who returned for examination after that time. Patients Y-39 and Y-41 in Table II supported the in vitro findings of resistance with positive blood slides by day 21 but Y-44 did not bear out the prediction of susceptibility by becoming positive by day 14. Of those 5 patients shown in Table III as having had follow-up studies two were resistant both in culture and clinically, while three which produced susceptible cultures became positive on the 14th or 21st day. The possibility of reinfection could not be ruled out during this season of active transmission.

DISCUSSION: The culture system reported upon consistently produces successful culture growth over a sufficient period of time to allow meaningful studies, in this case morphological and radiochemical observations. The number of culture failures has been less than 1% and were usually attributed to power failure or the administration of an antimalarial compound prior to patient study. The technic lends itself to the field because it does not require elaborate and sophisticated equipment during the initial culturing procedures nor must the technicians be as highly trained as with many in vivo culture systems. The cultures can be quenched at the field site and transported back to a base laboratory for channels ratio counting. Investigations underway

indicate that cultures can originate from outside a country with subsequent acceptable laboratory results.

The selection of a site in which to perform the in vitro cultures is based on the availability of laboratory space, electricity and an available patient population with malaria. The centrifuge, refrigerator, incubator, ultraviolet chamber, plates and reagents are readily transported in one vehicle. Frequently in areas where malaria persists as a major health problem, electricity is absent. At one of the early field sites, electricity was provided by a 6KW generator with emergency 300 W Honda generators for additional support, and successful cultures were obtained.

As was the case in this study, the available patients most usually comprise an agricultural population intent upon proceeding to their homes and farms and who are reluctant to return to the provincial medical facility unless they have actually relapsed or recrudesced. Thus, the validity of such an in vitro test is difficult to measure. This is particularly true during the rainy season when transmission is at its peak. To assure that a patient has not been reinfected is generally impossible. Too, the presence in most Thai villages of quack doctors who possess and dispense a wide assortment of substances, including chloroquine, may tend to confuse and distort the findings of patients who do return for further examination. It is possible that apparent susceptibility of parasites in vivo as determined by blood examination two or three weeks after initial detection could be due to drug pressure. Unless the patient can be closely observed for the full 28 days prescribed by WHO[8] there will generally be doubtful correlation between an in vivo study and in vitro data. It is tempting to use the field expedient suggested by WHO[8] whereby assessment of resistance is based on the response of asexual parasitemia during the first week of treatment, with further observations providing evidence of recurrence of parasitemia which could only be presumptive if re-infection cannot be ruled out. The series of patients referred to in Table II provide some correlation between clinical and culture results when the first week's response only is considered, with just 2 in vitro resistant cases being negative on day 7 (Y-39 and Y-41). When applied to the patients of Table III, there are 5 instances of agreement and 4 where the findings in the patient on day 7 differ with the conclusions obtained from the cultures. In the latter event all were resistant by the in vitro technic which could have presumably resulted in the prescribing of a drug other than chloroquine.

The culture results substantiate the findings of Colwell[11] that P. falciparum in Thailand is mostly resistant to chloroquine, a fact which impairs the evaluation process for any in vitro test. Complete evaluation of the technique incorporating the radioisotope C-14 requires that

a strain of malaria which is susceptible to chloroquine be investigated. If sufficient cases of sensitive P. falciparum can be found to establish confidence in the test procedure for non-resistant strains, an essential baseline could be described. The mean growth curve for susceptible strains would provide the necessary reference for comparison with the quinine growth curve, a control curve and the growth curve of the strain to be tested. To continue evaluation of this test and to provide the required characterization of the minimum response of a susceptible strain, investigations are currently underway at a research station outside of Thailand.

SUMMARY: A test for assessing the in vitro susceptibility of Plasmodium falciparum to chloroquine has been described. The culture system utilizes the protein incorporation of C^{14}-isoleucine as a growth marker. Culture failure was low, reproducibility was good and there was a multiplication rate of 2:1 or better in 90% of the study cases. The culture results obtained by measuring the C^{14} incorporation provide an excellent correlation with morphological observations. In this preliminary report it was not possible to follow sufficient cases of clinical malaria to establish the necessary in vitro - in vivo correlation. Further studies are in progress with parasites less resistant to standard chloroquine chemotherapy.

Table I

Microtitre Plate Scheme

Type of Culture	Harvest Time			
	23 hrs	40 hrs	48 hrs	63 hrs
Miscroscopic Control (No C^{14}-isoleucine)	0	0	0	0
C^{14} Growth Control	0 (XX)	0 (XX)	0 (XX)	0 (XX)
0.2 millimicromole Chloroquine base/ml	0 (XX)	0 (XX)	0 (XX)	0 (XX)
0.4 millimicromole Chloroquine base/ml	0 (XX)	0 (XX)	0 (XX)	0 (XX)
8 millimicromole Quinine base/ml	0 (XX)	0 (XX)	0 (XX)	0 (XX)

Microtitre Plate Scheme. 0 = Principal culture wells harvested to determine growth curve.

X = Culture wells harvested to assess reproducibility of culture procedure.

Table II

Comparison of *in vitro* and *in vivo* studies of 8 patients using chloroquine drug level 8 millimicromole base/ml blood* in patients from Yala, Thailand.

Patient No.	*In vitro* Result	Parasitemia		
		Day 7	Day 14	Day 21
Y-36	R	±		
Y-39	R	-	+	+
Y-41	R	-	-	+
Y-44	S	-	+	
Y-45	R	+		
Y-46	R	+		
Y-47	R	+		
Y-48	R	+		

* Based on serum level at 12 hours after commencing standard oral therapeutic course of Chloroquine diphosphate.

Table III

Comparison of *in vivo* and *in vitro* studies of 16 patients with *Plasmodium falciparum* from Yala, Thailand.

Patient No. Yala	*In vitro* Result	Parasitemia		
		Day 7	Day 14	Day 21
51	R	-	+	
52	PF*			
53	S	-	-	+
54	R			
55	R			
55B	R+			
56	S			
56B	R^{++}			
57	R^{++}			
58	R^{++}	+		
59	S	-	+	
60	S	-	+	
61	R^{++}	-	+	
62	R^{++}	+		
63	R^{++}	-		
64	R^{++}	-		

NOTE: PF* = power failure.

Table IV

Microscopic Evaluation of In vitro Culture Growth

Patient #	Time	Control				0.2 milli CQ/ml.				0.4 milli CQ/ml.				Quinine			
		R	M	S	Total	R	M	S	Total	R	M	S	Total	R	M	S	Total
Y56B	24	8	44	7	59	28	33	1	62	34	23	-	57	39	18	-	57
In vitro*	40	22	3	47	70	9	16	29	54	16	21	14	51	27	24	-	51
Resistant	47	44	19	53	116	37	26	35	98	32	22	27	81	26	30	-	56
Y59B	24	14	31	-	45	13	26	-	39	23	19	-	42	20	14	-	34
In vitro*	40	3	15	23	41	11	24	-	35	22	12	-	34	21	16	-	37
susceptible	47	14	11	30	55	9	22	8	39	20	9	-	29	16	22	-	38
Y60	24	3	14	5	22	13	9	-	22	10	13	-	23	12	12	-	24
In vitro**	40	20	5	13	38	17	6	-	23	12	11	-	23	10	15	-	25
susceptible	47	48	6	9	63	22	9	7	28	15	9	1	25	12	19	-	31

R = Young Ring

M = Mature Trophozoite

S = Schizont

Note: Counts are for 10, 000 RBC.

* No clinical follow-up.

** Parasitemia on day 14

1294

1294

AVERAGE SERUM CHLOROQUINE LEVELS OF THAI VOLUNTEERS
AFTER ORAL DOSAGE OF 1500 MG BASE

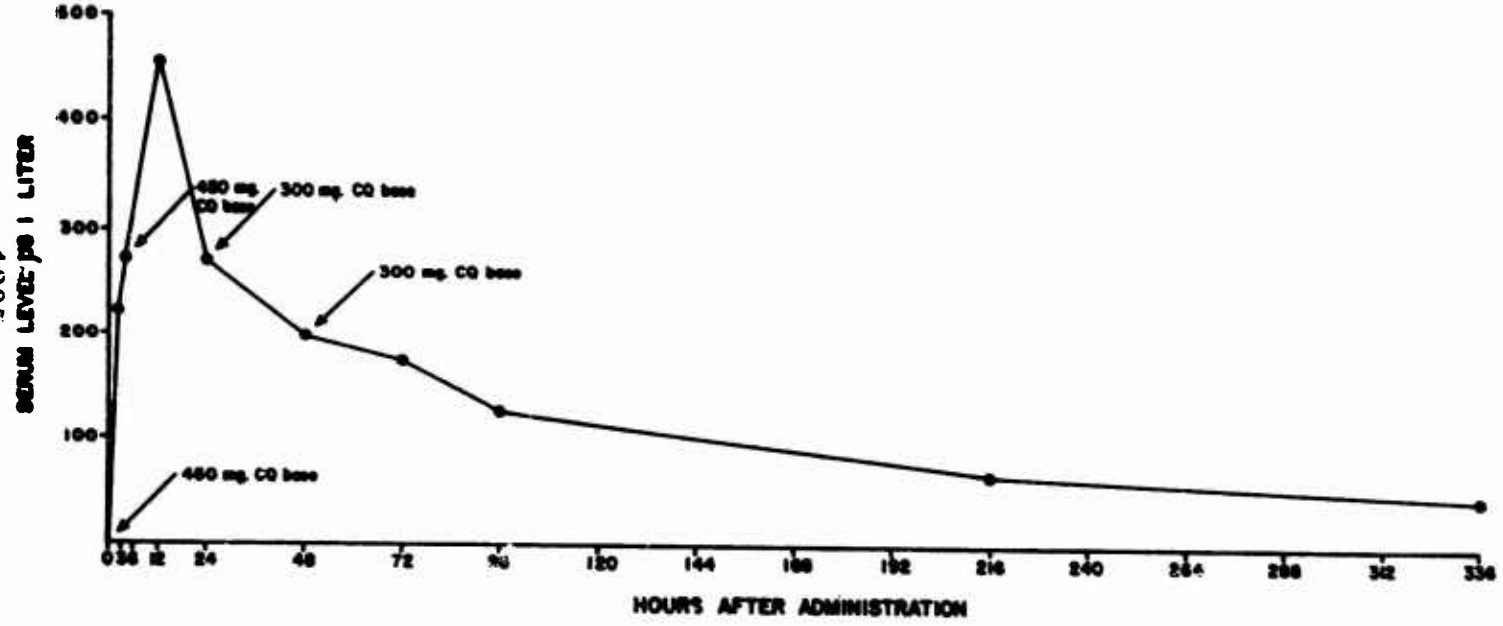

FIGURE 1

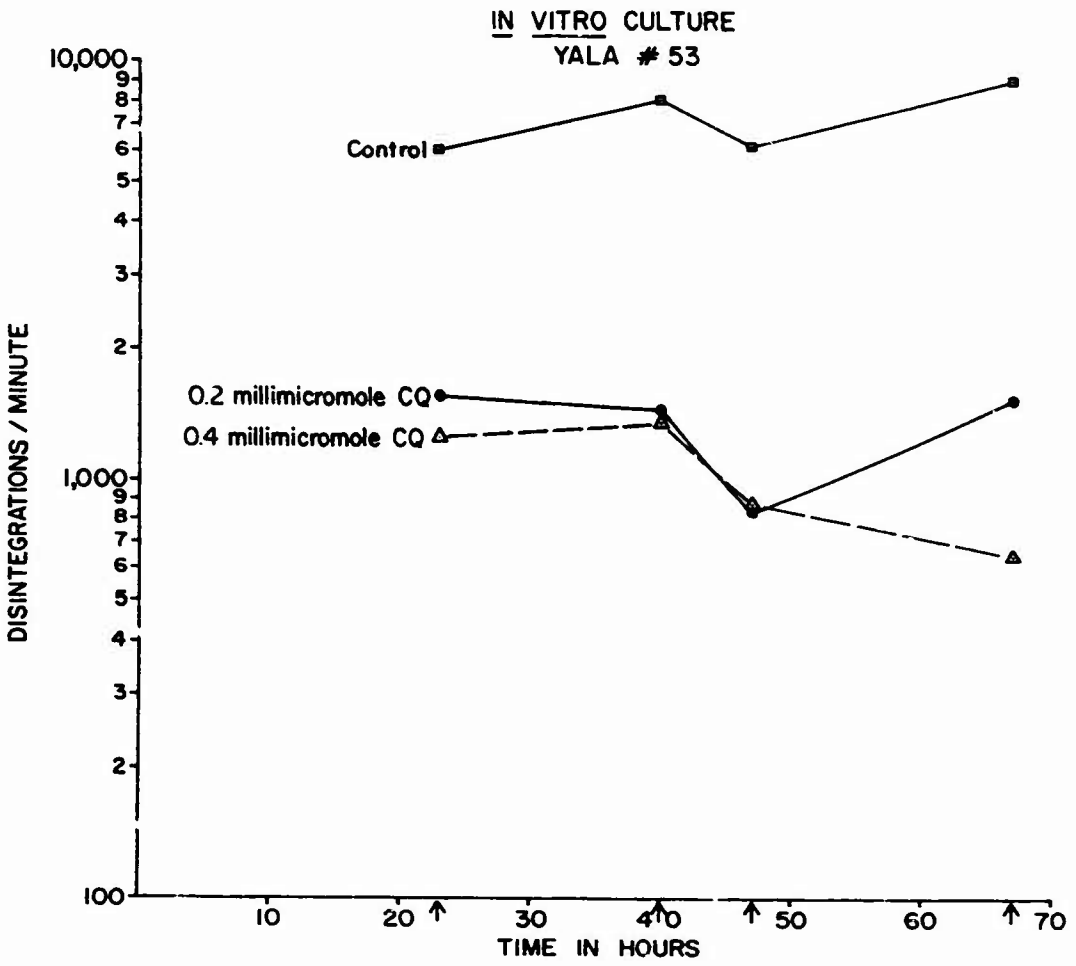
IN VITRO CULTURE
YALA # 53
10,000
9
8
7
6
5
4
3
2
1,000
9
8
7
6
5
4
3
2
100
DISINTEGRATIONS / MINUTE
Control
0.2 millimicromole CQ
0.4 millimicromole CQ
10
20
30
40
50
60
70
TIME IN HOURS

FIGURE 2.

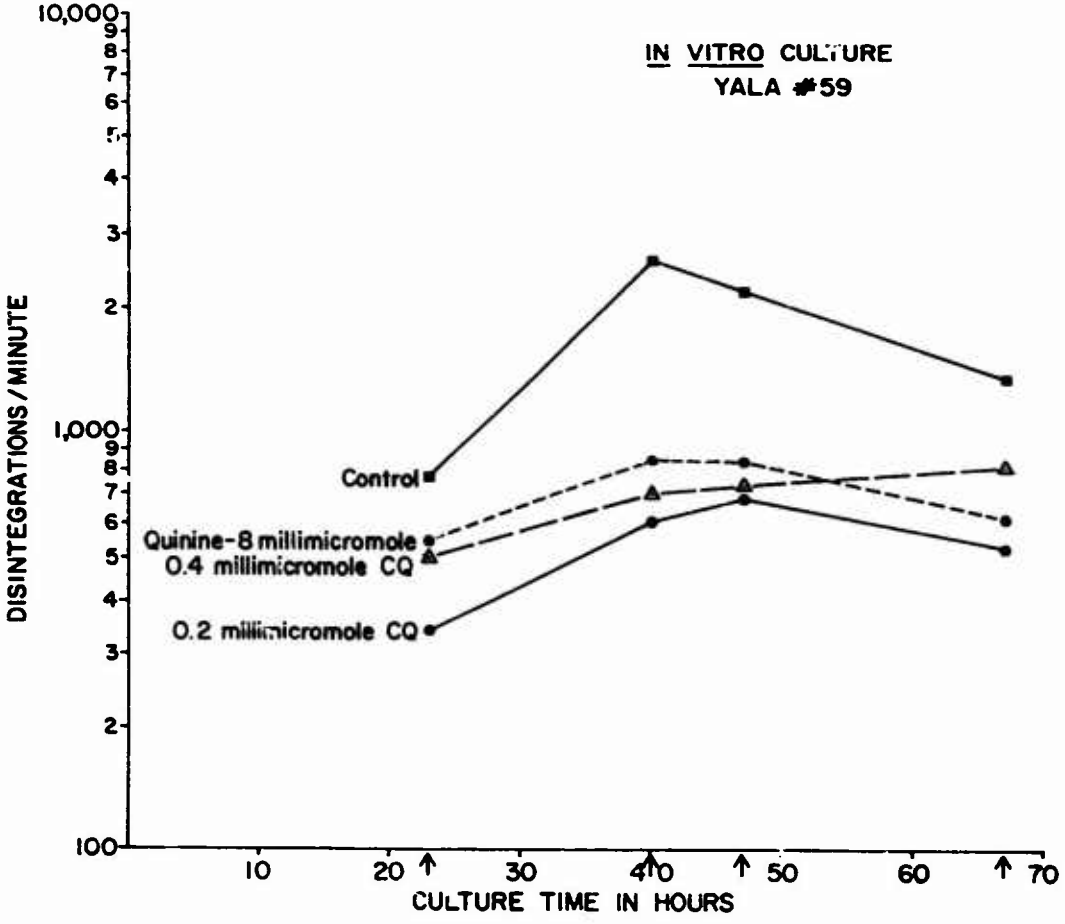
IN VITRO CULTURE
YALA #59
DISINTEGRATIONS / MINUTE
10,000
9
8
7
6
5
4
3
2
1,000
9
8
7
6
5
4
3
2
100
Control
Quinine-8 millimicromole
0.4 millimicromole CQ
0.2 millimicromole CQ
10
20
30
40
50
60
70
CULTURE TIME IN HOURS

FIGURE 3.

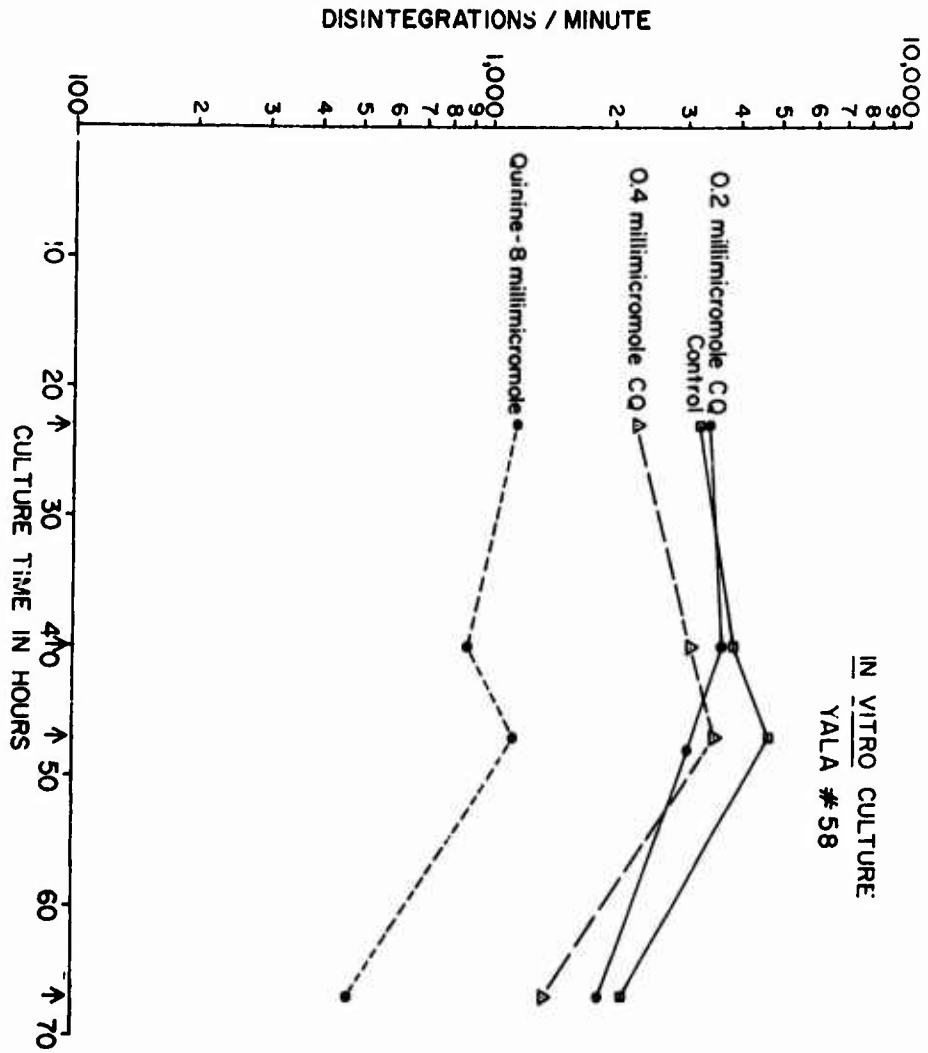
DISINTEGRATIONS / MINUTE
10,000
1,000
100
0.2 millimicromole CQ
Control
0.4 millimicromole CQ
Quinine-8 millimicromole
IN VITRO CULTURE
YALA #58
10
20
30
40
50
60
70
CULTURE TIME IN HOURS

FIGURE 4.

Hemolytic Activity in Human Malaria Infections.

Principal Investigator: Vithune Yuthasastr-Kosol, M.D.

Associate Investigator: Norman E. Wilks, LTC, MSC
Peter K. Iber, MAJ, MSC

OBJECTIVE: To investigate the hemolytic factors associated with human malarial infections with particular emphasis on host complement activity in Chloroquine resistant and non-resistant malaria and immunological phenomena producing host cell lysis.

DESCRIPTION: Previous studies at SEATO Medical Research Laboratory (1968-69), using P. inui and P. coatneyi in monkeys showed marked decrease in erythrocyte survival time in the course of chronic infections with scanty parasitemia, or even in the absence of parasites. Studies also showed that inappropriate erythrocyte destruction was mediated by some humoral factor associated with chronic infection. Equally striking is the marked decrease of complement activity in the infected monkeys using the spectrophotometric method for complement assay as described by Hook and Muschel (1964) and Fogel et al at WRAIR (1966).

A study of complement activity levels of acute malaria patients (1970-1971) found this activity to decrease during infection and rise rapidly to normal levels after successful chemotherapy. Preliminary studies of patients infected with both P. falciparum and P. vivax indicate that there is a pronounced depletion of C' activity in human malaria infections in Thailand.

PROGRESS: All patients studied in this report were selected from Ayuthaya Province, about 120 kilometers north of Bangkok. Four females and ten males aged between 18-41 years and infected with P. falciparum were followed from the day of treatment through the complete course and for as long as 144-150 days. There were another 18 patients who were hospitalized for a short period of 1 to 9 days, and could not be followed further. One female and 7 males of the 18-41 years age group were infected with P. vivax and were also followed for as long as 150 days. A cumulative total of forty normal Thais aged between 18-27 years were used as controls, with only two being from the Bangkok area.

All patients were treated with intramuscular Chloroquine, 150 mg. base, or intramuscular quinine, 300 - 600 mg, and then continued with chloroquine orally for a total of 1500 mg base. Primaquine was administered to P. vivax patients and the oral course of chloroquine was repeated upon relapse.

Blood specimens were collected in number and volume permitted by the patients. Specimens were obtained, if possible, each day during the first week, on the 7th, 10th or 14th day, and then every 14 days for as long as possible. Serum was separated from all blood specimens after clotting and immediately frozen. The micro-complement fixation technic was used. The extent of sheep erythrocytes sensitized with rabbit antibody to yield 80 to 90% lysis after 30 minutes at 37°C was estimated by absorption measurements at 412 mu = Δ OD_{412} spectrophotometrically on supernatant fluids from the reaction mixtures. The results are expressed as logarithemic values at Δ OD_{412}.

The distribution of C' activity levels in malarial patients during acute illness, after radical cure and recrudescence are shown in Fig. 1. Normal Thai values and those of patients with other diseases are shown for comparison. In one control group of 10 normal Thai randomly selected and followed during the same period as some of the patients, and 15 normal Thais randomly selected for determination of activity by one test range from 3.0 - 9.989 (75 - 92.2% hemolysis of sensitized sheep erythrocytes). The sera from patients with infections other than malaria did not produce low C' values.

In Figure 2 are shown the mean C' activity values for patients who were radically cured, some who were incompletely cured (i.e. the parasitemia was persistent) and of normal patients who were followed extensively. The Δ OD_{412} values of the C'activity on the day of admission or during the acute attack were between 0.0 and 0.433 (7% - 30% hemolysis) with a mean value of 0.232 for P. falciparum and 0.267 in P. vivax infections. The ranges in parasitemia were from 0.2 to 1.4% in P. falciparum cases and from .11 to .35% in P. vivax infections. The C' levels rose rapidly in response to chloroquine chemotherapy, and returned to normal in 3 - 5 days in P. vivax patients and in about 7 days in P. falciparum cases. After that time the levels were consistently above 3.0, and equivalent to normal.

In those demonstrating chloroquine resistance, the levels did not return to normal for several weeks. When such activity did increase to beyond 3.0 it remained at the higher level. Whether the infection relapsed or remained patent at a very low parasitemia, in those patients for whom the chemotherapy was inadequate the C' activity level persisted below 3.0.

The studies performed to detect hemolytic factors associated with an immunological phenomenon yielded no abnormal results. None of the patients examined presented with chronic infections similar to those observed in previously studied experimental monkeys. No cases of

blackwater fever were discovered during the reporting period.

SUMMARY: The measurement of C' activity levels during and following infections with P. falciparum and P. vivax has been performed on patients for as long as 150 days. During the infection the complement level was markedly decreased and returned to normal rapidly after successful chemotherapy. Throughout persistent infections and recrudescence the C' activity remained depressed, regardless of the species of malaria. In other infectious diseases the complement was not detectably reduced.

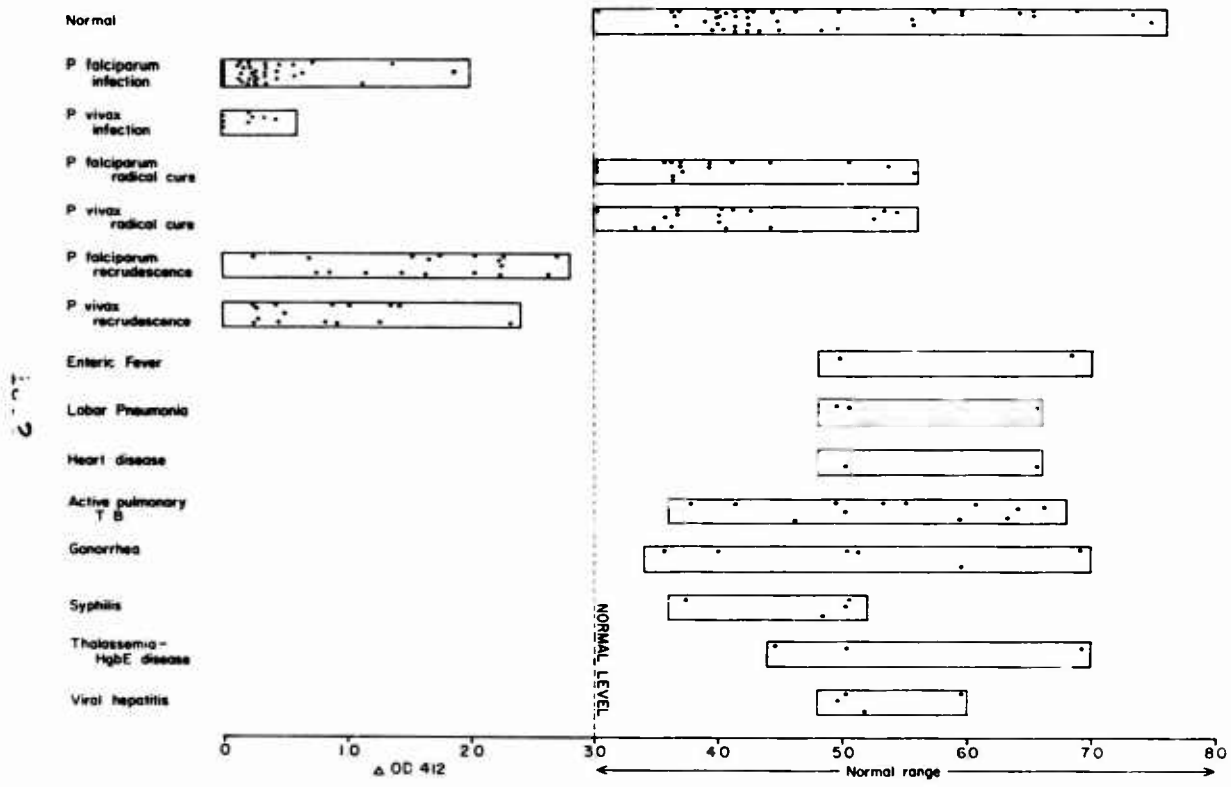

FIGURE 1 COMPARISON OF DISTRIBUTION OF C' ACTIVITY IN RANDOMLY SELECTED NORMAL THAI, MALARIAL PATIENTS AND PATIENTS WITH OTHER DISEASES

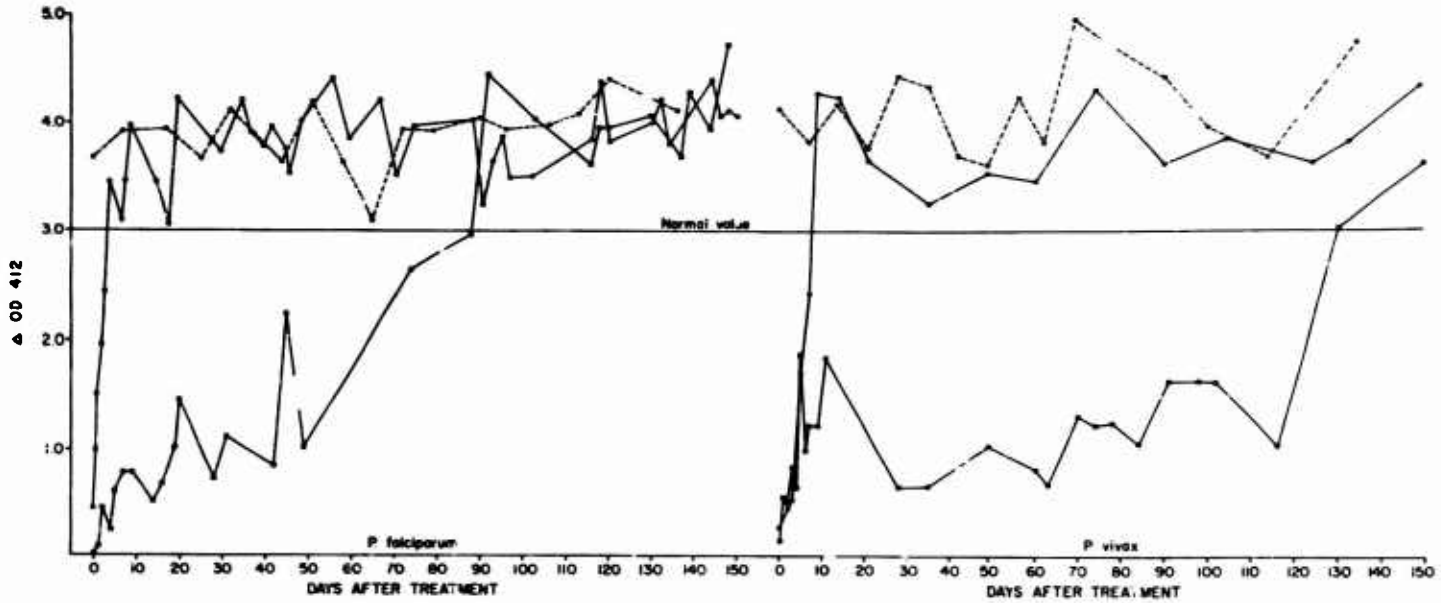

Figure 2. Mean C' Activity in Patients with malaria. _____ = Radical Cure (11 P.f. and 2 P.v.) and Incomplete cure (3 P.f. and 2 P.v.) ------ = Normal controls (6 and 6).

Malaria Field Studies Khok Salung, Lopburi.

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Associate Investigators:	Norman E. Wilks, LTC, MSC Rowland N. Wilkinson, CPT, MSC
Assistant Investigators:	Chamroen Chetty, B.Sc. Kitti Watanasirmkit, B.Sc.

OBJECTIVES: 1. To examine a population reportedly infected with a susceptible strain of P. falciparum possibly transmitted by a vector other than A. balabacensis. 2. To obtain parasites and sera from patients parasitized with P. falciparum for comparative studies using C^{14}-isoleucine protein-incorporation culture method.

BACKGROUND: From unpublished data obtained at Khok Salung in August 1965 it appeared that this village was sufficiently solated and large enough to perform extensive field malaria studies. Because of its geographical location, Khok Salung was expected to provide a study population undisturbed by migration and external population pressure.

In August 1965, a sick-call survey of 166 villagers provided 46 cases of malaria; 37 cases of P. falciparum and 9 cases of P. vivax. Seven cases were studied and treated with a standard therapeutic course of chloroquine diphosphate. All cases responded to the therapy and remained smear-negative for a minimum period of 7 days. This indicated that there was a possibility to observe an incidence of chloroquine sensitive P. falciparum which is not known to exist elsewhere in Thailand. The geographic characteristics of this area do not appear to provide a suitable breeding environment for A. balabacensis, and a potential vector/parasite relationships which might contribute to our knowledge of the biology of susceptible and resistant malaria seemed possible.

DESCRIPTION: The initial step in this field study was to survey the school children and map the village to obtain baseline infection rates and the location of any malaria present in the village (Fig. 1 and Table I). Throughout the study period P. falciparum infection rates at sick-call were maintained. In addition periodic visitations were made to the agricultural sites used by the villagers.

School visitations and sick-calls were held in smaller villages located throughout the agricultural area (Table II). The agricultural area is bounded on all sides by low hills thereby isolating it from neighbouring areas. The study sites were located approximately 15-20 km from the low hills bordering the area. School surveys were again performed toward the end of the study period.

Mosquito biting collections were made during a three-month-period in the agricultural areas of interest (Table III). Sites for biting collection were selected on the basic of incidence of malaria in villagers residing or working in the area.

Twenty-one patients parasitized with P. falciparum were treated and followed clinically for twenty-one days. C^{14}-isoleucine protein-incorporation studies were attempted on blood samples from five of these subjects.

RESULTS: A school survey of 1218 students in Khok Salung showed no parasitemic children. Since school was in session there was probably little or no migration of the students to the agricultural areas. It therefore seemed likely that transmission was not occurring in Khok Salung.

Surveys of 431 school children within the agricultural areas showed 8 positive cases of P. falciparum. Sick-calls held in the agricultural areas showed 41 (4.4%) cases of P. falciparum among 926 patients examined for malaria.

During the period of 10 July - 28 October 1971, sick-call was held daily at the Khok Salung Health Center. Sixty cases (17.3%) of P. falciparum were detected among 347 patients screened for malaria. All of the positive smears except one were from persons who freely admitted travelling regularly to the agricultural area surrounding Khok Salung. Extensive questioning of villagers indicated little travel outside the village and agricultural area.

Mosquito biting collections made from 28 July - 4 September 1971, showed an extremely low density of Anopheles mosquitoes and no evidence for the presence of A. balabacensis (Table III). Among the Anopheles mosquitoes collected the only known malaria vector was A. minimus. No mosquitoe dissections to determine infectious were performed.

Among the 21 malaria patients studied 9 cases were lost to complete follow-up. Of the remaining 12 cases, 7 infections were sensitive to chloroquine, 2 showed an R-I type resistance, recrudescence occurred between days 8 and 14, and 3 an R-II type resistance. No R-III resistance was observed.

Although several attempts were made to culture Plasmodium falciparum by the in vitro system incorporating C^{-14} tagged isoleucine, none were successfully concluded. During the period of transmission in the agricultural regions an erratic and undependable electrical power system was the only source available, and the cultures overheated or could not be properly incubated. A reliable generator was obtained at the beginning of the dry season when transmission declined abruptly. Contamination was only a minor problem when an ultra-violet light could be used. Dust, pollen and occasionally a fungus contaminated some of the culture wells. A continuing impediment was the lack of assurance that the patients had not visited one of the 4 quacks who practised in the village. Since no western medicine was available to them prior to the visit of the SMRL team, they depended largely upon the services of these unlicensed practitioners. Patients could not be included in the study group if suspection arose that they were receiving other compounds.

The isoleucine uptake test proved to be practical as a field expedient, despite the occurrence of culture failures, as was later confirmed by studies in Yala.

Of particular interest was the change in malarial incidence and in the local terrain since the initial study in 1965. The low hills surrounding the region have been practically denuded of vegetation in the interests of the charcoal industry. Crops have been changed from exclusively rice to broad fields of corn. The forest around Khok Salung proper has been removed and low scrub shrubbery only persists. The river is 3 km away and the only additional available water is stored rainwater. Mosquitoes are not abundant in the village, and only near the new corn fields and the river can potential vectors be obtained. The activities of the villagers have altered the environment in such manner as to deprive the major vector of Thailand, A. balabacensis, with suitable breeding sites.

SUMMARY: A malaria field study has been performed in a geographically isolated village where migration and population movement pressure was minimum. Surveys showed that all malaria was contracted

in the agricultural area surrounding the village, most of which was not accessible to the investigators. Mosquito biting collections showed an absence of A. balabacensis, that combined with the geographical conditions tends to indicate the possibility of transmission by a vector other than A. balabacensis.

Among 12 P. falciparum patients studied, 7 were sensitive to standard susceptible chloroquine therapy. A field-trial of the C^{14}-isoleucine culture system showed it could be performed in the field if a proper electrical power supply can be provided.

Table I

School Survey Khok Salung and Surrounding Areas

School	No. Students	P. falciparum positive cases	
		May 1971	October 1971
Village (Khok Salung)	934	0	0
Monastery (Wat Nong - Taming, Khok-Salung)	284	0	0
Village (Manau Wan I)	121	1	0
Village (Manau Wan II)	199	0	0
Village (Suan Madua)	112	7	ND*

* Not done.

Table II

Sick-Call Observations

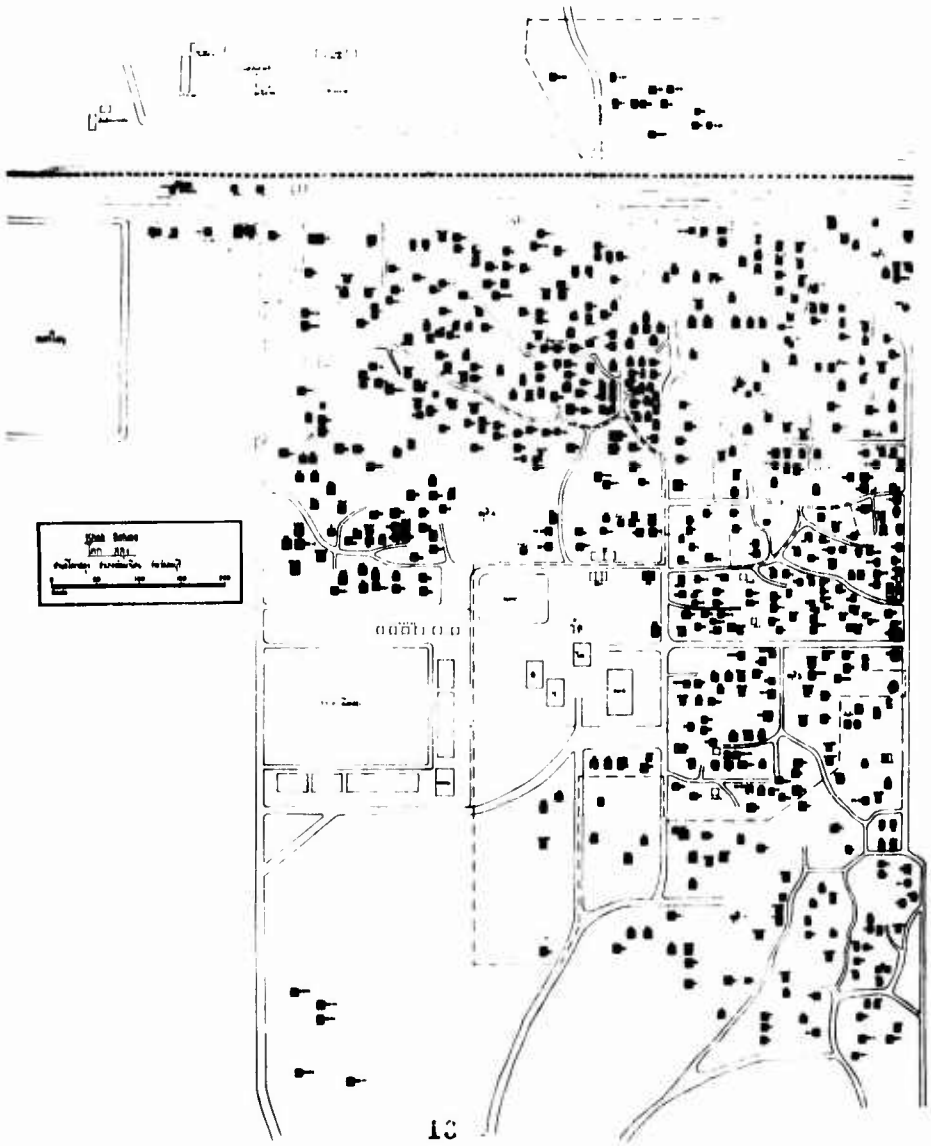
Area	No. Patients	P. vivax	P. falciparum
Khok Salung Health Center	324	7	53
District Manau Wan	291	3	10
Village of Suan Madua	635	1	31
Total	1,253	11	94

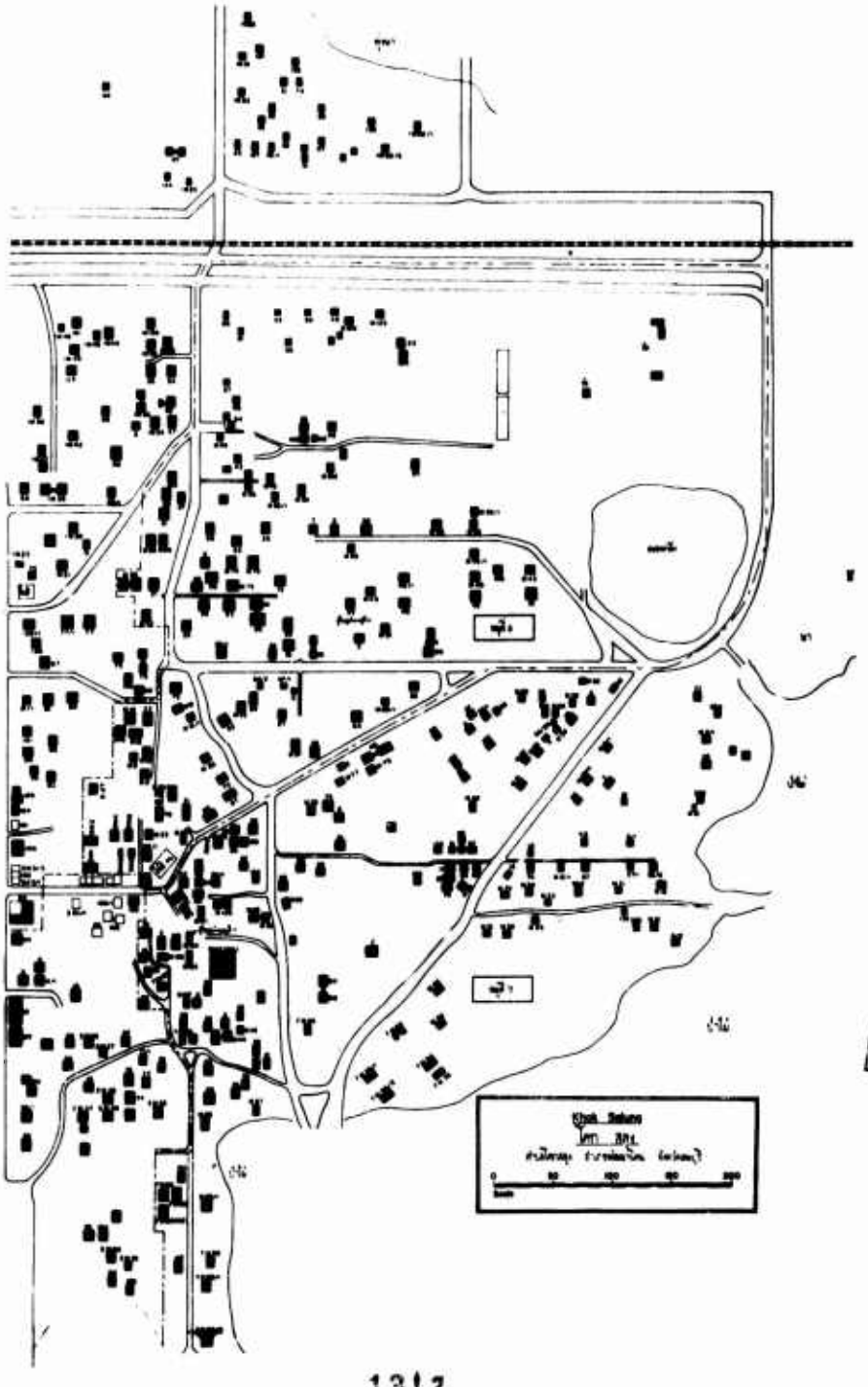
No mixed infections were observed during the study period.

Table III

Mosquito Biting Collections

Mosquito Species	Date of Collection			
	28 July'71	10 Aug'71	31 Aug'71	4 Sept'71
Aedes (stegomyia) sp.	101	104	41	0
Aedes (neomelaniconion) sp.	2	0	20	16
Aedes (ochlerotatus) sp.	0	0	66	0
Aedes (adimorphus) sp.	1	0	44	206
Culex (culex) sp.	24	54	58	44
Mansonia sp.	2	4	0	2
Anopheles (cellia) minimus	0	6	0	0
Anopheles (cellia) vagus	0	3	3	0
Anopheles (cellia) subpictus	0	2	0	0
Anopheles (cellia) nivipes	0	1	0	1
Anopheles (cellia) tessellatus	0	1	0	0
Anopheles (anopheles) sp.	0	0	2	28
Total	130	175	234	197





Plasmodium Falciparum Infection Rates in Normal and Enzyme-Deficient Erythrocytes of Glucose-6-Phosphate Dehydrogenase Deficient Heterozygotes

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Associate Investigators: Chalard Tirabutana, M.D.
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Assistant Investigators: Chowanit Sinsawat
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OBJECTIVE: P. falciparum infection rates in normal and enzyme-deficient erythrocytes of Thai women, heterozygous for glucose-6-phosphate dehydrogenase (G-6-PD) deficiency are being determined and compared.

DESCRIPTION: Women, heterozygous for G-6-PD deficiency (an X-chromosome-linked trait), are mosaics: approximately half of their red blood cells are normal, the other half are G-6-PD deficient. The two cell populations can be distinguished histochemically by the methemoglobin elution method (Gall et al, 1965). This technique is being applied to blood from Thai women who have malaria and are heterozygous for G-6-PD deficiency. Infection rates in both normal and enzyme-deficient red blood cells are being determined and compared. Hematocrit, reticulocyte count, red blood cell morphology, hemoglobin type, and G-6-PD activity (spectrophotometric assay) are also being determined.

PROGRESS: To date, 376 women and girls with malaria, who presented at the Provincial Hospital or Malaria Eradication Center in Chantaburi, have been tested by the methemoglobin elution technique. Approximately ten per cent have been classified as heterozygous for G-6-PD deficiency. Parasite counts have been made in both normal and enzyme-deficient cells and these data are now being analyzed.

Longitudinal Malaria Studies in Rural Northeast Thailand

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Associate Investigators:	Walter W. Noll, MAJ, MC Rowland N. Wilkinson, CPT, MSC
Assistant Investigators:	William E. Gresso, SFC Pannee Srangsomwong, RN Vacharee Panalaks, RN Withoon Thiemanun Jarouy Grutaraniyom

INTRODUCTION: A prospective longitudinal study of the transmission of Plasmodium falciparum malaria was began at Ban Bu Phram and Ban Tablan, Tambon Tungpo, Prachinburi Province. Initially, attention was to be focused on the patterns of parasitemias and clinical disease associated with chloroquine-sensitive and chloroquine-resistant strains of the falciparum parasite. All parasitemias in Bu Phram were to be treated with chloroquine in an effort to determine whether intensive chloroquine pressure could be shown to measurably increase the proportion of chloroquine-resistant infections. Most falciparum infections were found to be resistant to chloroquine treatment from the outset (see below). The objectives of the study were therefore restated as follows:

OBJECTIVES:

(1) To study the variables associated with the acquistion, duration, and density of P. falciparum parasitemias.

(2) To measure the prevalence of chloroquine-resistant falciparum infections and evaluate the usefulness of chloroquine as a control measure.

(3) To assess the feasibility of successfully performing a prophylactic drug trial.

DESCRIPTION: Two Northeast Thai villages, Ban Bu Phram and Ban Tablan, Prachinburi Province, were selected for study. They are located in a heavily-forested mountainous region approximately 100 kilometers south of Nakorn Ratchasima(Korat) on Route 304. On the basis of hospital reports, chloroquine-resistant falciparum malaria and vivax malaria were considered to be endemic. The villages were mapped and censused in mid-April 1971. There were 380 persons resident in Bu Phram and 605 in Tablan at that time. A random sample of households was drawn and included 252 persons in both villages. Both village samples("Family Group") were representative of the entire village populations and comparable to one another.

Investigators visited each family sixteen times, at approximately two-week intervals, between late April 1971 and early January , 1972. During each visit they inquired about a history of fever and/or headache and collected capillary blood specimens for quantitative parasite counts and, on one occasion, for glucose-6-phosphate dehydrogenase(G-6-PD) determination and hemoglobin electrophoresis. In Bu Phram, all Family Group subjects with falciparum or vivax parasitemia, symptomatic or asymptomatic, were treated initially with chloroquine(Comer et al, 1968). In Tablan, only those with symptomatic parasitemias were treated.

Sickcall was held weekly and non-Family Group persons("Sickcall Group") with complaints related or unrelated to malaria were seen and treated. All parasitemias found in members of this group were treated with chloroquine.

Parasite densities were determined by relating the parasite-leukocyte ratio on the blood film to the total leukocyte count. The methemoglobin elution test(Gall et al, 1965) was used to identify G-6-PD deficient, heterozygous, and normal persons. Hemoglobin electrophoresis, carried out on cellulose acetate strips, was used for family pedigree analysis.

The data accumulated permitted study of the association of falciparum and vivax infection rates, average number of parasitemic and gametocytemic episodes per infected subject, parasitemia-associated morbidity, and parasite densities with variables under consideration. A history of fever preceding or fever coincident with documented parasitemia was recorded as evidence of symptomatic infection since other symptoms, complications, and mortality from malaria were not observed. Data from the two study villages were pooled for this report, since chloroquine treatment of asymptomatic parasitemias (Ban Bu Phram) was without demonstrable effect (see below).

PROGRESS: Variables Associated with Infection: The associations of P. falciparum and P. vivax infections with the variables of age and sex, time and place of parasite acquisition, and G-6-PD deficiency were studied. Forty-six percent of subjects studied were parasitemic with P. falciparum and 23 percent with P. vivax during the study period(Table I). Parasitemia rates were similar in all subjects except for those under one year of age. Males were infected in greater proportion than females (Table II).

Peak transmission occured during May, June, and July, with lower levels occuring during the remainder of the study period. In the ten week period from mid-May through July, 61 percent of the "new" falciparum infections and 47 percent of the "new" vivax infections were found. The

largest collections of Anopheles balabacensis were also made during this period and four mosquitoes were found infected (Gould and Wilkinson, 1972).

Twenty-nine percent of Family Group subjects resided in shelters in fields away from the village for some part of the study period (Table III). These subjects were at greater risk of acquiring infection than subjects denying any overnight absences from the village. Whether subjects presumably infected in the fields were infected early in the season and introduced infection into the village is under study.

The 72 percent of subjects who gave no history of overnight absence were presumably infected in the village proper. The night-time patterns of activity of 22 Family Groups were observed (Table IV) and compared with vector mosquito collections (Gould and Wilkinson, 1972). Seventy-four percent of An. balabacensis collected were caught after 2300 hours, when all subjects were inside the house, asleep, and those who had mosquito nets were using them. Infection rates in those subjects using nets(infants and females) were lower than rates in non-users. (Two cycles of house-spraying with residual DDT were completed by National Malaria Eradication Program [NMEP] personnel during the study period).

The prevalence of the gene for G-6-PD deficiency was 16.1% in male Family Group subjects. Thirteen males and two females were found to be enzyme-deficient. Fifteen (female) heterozygotes were identified by a combination of the methemoglobin elution test and/or family pedigree. Enzyme-deficient and heterozygous Family Group subjects were not infected with P. falciparum in lesser proportion and were not parasitemic less often than comparable enzyme-normal relatives or the remainder of the Family Group (Table V) (Segal et al, 1972).

Usefulness of Chloroquine in Treatment of P. falciparum: The usefulness of chloroquine treatment of both symptomatic and symptomatic falciparum parasitemias was studied. (All vivax parasitemias were sensitive to chloroquine). Prior use of chloroquine in the villages was assessed by determining what proportion of villagers suspected of being parasitemic might have (ideally) been treated by the NMEP house visitor. (The house visitor dispensed chloroquine based on a history of fever). Data accumulated (Table VI) suggested that he might have treated six percent of falciparum parasitemias, twelve percent of vivax parasitemias, and few non-parasitemic persons. On three occasions, self-treatment with chloroquine (for fever) was observed by the investigators.

In Bu Phram, all parasitemias found in Family Group subjects were treated; in Tablan, only symptomatic parasitemias were treated. Parasitemic subjects in Bu Phram were treated approximately three times as

often (Table VII). Nevertheless, the average number of episodes of parasitemia experienced was similar to that of Tablan. The average number of episodes of gametocytemia and parasite densities experienced were also similar.

Subjects from either the Family Group or Sickcall Group under twenty years of age and with falciparum parasitemias higher than 1000 asexual parasites per cubic millimeter of blood were treated with chloroquine to determine the proportion of chloroquine-resistant P. falciparum strains. Thirty-eight such subjects were found, treated, and followed for 28 days (Table VIII). Only five of the 38(0.13) were cured of their infection. The majority of strain reponses were of the R2 pattern. Subjects cured of their infections were somewhat older(median age 11.0 years) than those with resistant infections(median age 6.0). Subjects with either sensitive or resistant infections were equally distributed by sex and village.

Subjects not responding to chloroquine and having continuing symptoms(R3 pattern) were treated with quinine; all were cured. Subjects from among those described above, if not symptomatic, were retreated with chloroquine if they had received no other treatment and if their parasite density was still greater than 1000 per cubic millimeter on day 28 of follow-up. Of five such subjects, three demonstrated falciparum infections "more sensitive" to chloroquine on retreatment (Table IX).

Sickcall was held weekly to identify parasitemic persons outside the Family Group. The majority of persons presented with complaints seemingly unrelated to parasitemia. Parasitemia was suspected and capillary blood for smear taken from 303(0.18) of the 1963 persons seen. One hundred fourteen(0.38) of these 303 persons were infected with P. falciparum and 33(0.11) with P. vivax. All parasitemic persons found at sickcall were treated with chloroquine.

Feasibility of Prophylactic Antimalarial Drug Trials: The prospects for successful completion of a prophylactic antimalarial drug trial were assessed using census data, participation rate data, and infection rate data obtained during the study period. In a trial restricted to persons ten years of age and older, 937 persons(0.73 of the population) would be eligible for study. The minimum number of subjects required was calculated, based on the following assumptions: 1) parasitemia rates similar to those observed during the previous study, 2) A two-fold reduction of falciparum parasitemia by the drug under study, and 3) participation rates higher than the lowest rate attained during the previous study(55 percent). A minimum of 103 subjects, in each of the study groups(drug and placebo) would be required. Thus, such a drug trial is feasible.

Table I. Age-specific incidence rates for falciparum and vivax parasitemias among Family Group subjects.

Age group	Population at risk	Falciparum parasitemia	Vivax parasitemia	Parasitemias combined
< 1	17	2(0.12)*	1(0.06)	3(0.18)
1-4	35	14(0.40)	10(0.29)	24(0.69)
5-9	37	20(0.30)	11(0.30)	31(0.84)
10-19	50	30(0.60)	14(0.28)	44(0.88)
20-29	45	22(0.49)	11(0.24)	33(0.73)
30-39	33	11(0.33)	5(0.15)	16(0.48)
≥ 40	35	18(0.51)	6(0.17)	24(0.69)
All ages	252	117(0.46)	58(0.23)	175(0.69)

*Number (proportion) infected.

Table II. Sex-specific incidence rates for falciparum and vivax parasitemias among Family Group subjects.

Sex	Population at risk	falciparum parasitemia	Vivax parasitemia	Parasitemias combined
Male	127	69(0.54)*	33(0.26)	102(0.80)
Female	125	48(0.38)	25(0.20)	73(0.58)
Both sexes	252	117(0.46)	58(0.23)	175(0.69)

* Number (proportion) infected

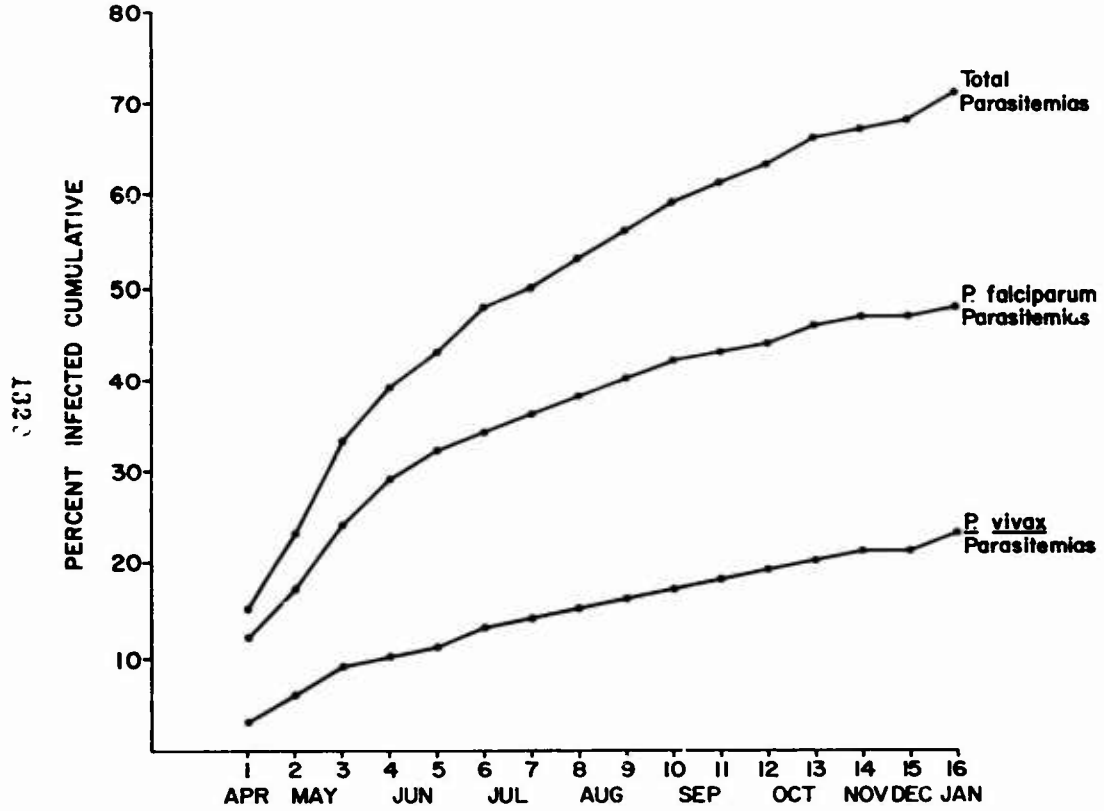

FIGURE 1. CUMULATIVE PERCENT FAMILY GROUP SUBJECTS INFECTED.

Table III. The relationship of a history of overnight absence from the village to falciparum and vivax parasitemias in Family Group subjects.

Absence history	Population	No. (proportion) infected
Absent	58	42(0.72)
Not absent	146	76(0.52)
Total	204	118(0.58)

Table IV. Nighttime activities of 22 Family Groups* observed during biting collections.

Time period	Activity		
	Inside house	Asleep	Under mosq. net
1800-1900	0.67+	0.05	0.01
1900-2000	0.88	0.28	0.14
2000-2100	0.95	0.75	0.48
2100-2200	0.96	0.92	0.57
2200-2300	0.97	0.93	0.60
2300-0500	1.00	1.00	0.55
0500-0600	0.98	0.72	0.64

* These represent 50% of the Family Groups

+ proportion of persons observed.

Table V. P.falciparum Infection rates and average number of parasitemic episodes in G-6-PD deficient, heterosygous, and normal Family Group members.

Population	Sex	No. persons	No. (proportion) infected	No. of parasitemic episodes	
				Total	Average +
Deficient	Male	13	9(0.69)	45	5.0
Deficient	Female	2	0	0	0
Heterozygous(test)	Female	9	2(0.22)	4	2.0
Heterozygous(all) *	Female	15	4(0.27)	10	2.5
Enzyme-normal relatives	Male	24	9(0.38	31	3.4
	Female	20	4(0.20)	8	2.0
Remainder of Family Group	Male	73	39(0.53)	105	2.7
	Female	72	30(0.42)	129	4.3

* This group includes individuals classified by pedigree as well as by the methoglobin elution test.

+ Number of parasitemic episodes per infected person.

Table VI. Parasitemic and non-parasitemic-associated fevers among Family Group subjects

Smear	No. infected subjects	No. parasitemic episodes	No. febrile episodes	Proportion febrile episodes
P. falciparum	118	421	36	0.06
P. vivax	57	104	12	0.12
Negative	0	0	48	0.01*

*Based on 3219 negative smears

Table VII. The effect of chloroquine treatment on the number of episodes of falciparum parasitemia.

Village	No. subjects infected, total	No. treatments		No. episodes of parasitemia	
		total	per infected subject	total	per infected subject
Bu Phram	62	172	2.8	229	3.7
Tablan	56	50	0.9	190	3.4

Table VIII. Response to chloroquine treatment of selected Family and Sickcell Group subjects with falciparum parasitemias.

Response pattern	Number	Proportion
Sensitive	5	0.13
R1	6	0.16
R2	22	0.58
R3	5	0.13
Total	38	1.00

Table IX. Response to chloroquine retreatment of selected Family and Sickcall Group Subjects with falciparum parasitemias.

Patien.(sex, age)	Initial response	Retreatment response
B 48 (F,8)	R1	R1
SB 34 (F,3)	R3	R2
B 12 (F,7)	R2	R2
SB 59 (F,6)	R3	S
SB 45 (F,6)	R3	R1

Ecology of Malaria Vectors

Principal Investigators : Douglas J. Gould, Ph.D.
R. N. Wilkinson, CPT, MSC

Associate Investigators : Kol Mongkolpanya
Larp Panthusiri
Prajim Boonyakanist

OBJECTIVE : To investigate the bionomics and population dynamics of known and potential vectors of human malaria in Southeast Asia and their relationship to the dissemination of chloroquine resistant strains of P. falciparum.

DESCRIPTION : Specific factors being studied in the process of defining actual and potential vector species in Thailand include the following: incidence of malarial oocysts and sporozoites in wild anopheline populations, susceptibility of colonized strains of Anopheles to infection with P. falciparum, patterns of biting activity of vector species, ovipositional habits of anopheline mosquitoes and the viability their eggs under varying environmental conditions.

PROGRESS :

1) Malaria field studies in Prachinburi province. Entomological field studies were carried out in Prachinburi province in support of a longitudinal study of the epidemiology of malaria in the area. The study site was located in a forested valley in the mountains about 100 Km south of Korat and, malaria is endemic in the human population of the area. Periodic collections of adult and larval anophelines were made in and near the villages of Ban Bu Phram and Ban Thap Lan. Initially, a comparison was made of the number of anopheline adults attracted to CDC light-traps and lard-can traps, both baited with a CO_2 attractant (dry-ice), and those obtained from concurrent human-bait collections. Use of the two types of traps was discontinued because they yielded few mosquitoes, and the collection of anophelines resting inside and outside houses and biting human subjects was relied upon thereafter. A total of 35 night time collections, made between dusk and dawn, were carried out at weekly intervals in the two villages from June 1971 through March 1972. Weekly collections were terminated at the end of the rainy season in November, and begun again in February 1972 during the peak of

the dry season. During the latter period collections were also made at a logging camp in the forest 2 miles NW of Ban Bu Phram. A total of 571 A. balabacensis females were collected over the whole period (Table 1). Smaller numbers of A. minimus, A. aconitus and A. maculatus females were also collected. The largest number of balabacensis were collected during June and July; both villages were sprayed with DDT at the end of July and comparatively few balabacensis adults were collected thereafter (Table 2). A. balabacensis was the only anopheline found infected during this period, and the overall infection rate for the 571 A. balabacensis collected during the study period was 1.0%. Two infected mosquitoes were collected in each of the following situations: biting indoors, biting outdoors, and resting outdoors. Biting activity by balabacensis began outdoors as early as 1900 hours and indoors by 2000 hrs. Biting activity reached a peak between 2300-2400 hrs, however the differences in hourly collections between 2100 and 0400 outdoors and between 2200 and 0500 indoors were small. A. balabacensis were collected resting outdoors from 1900 to 0500 and indoors from 1900 to 0600. Peak numbers were found resting outdoors between 2200-2300 hours and between 2000 and 2300 hours indoors.

Efforts were made during this period to locate the daytime resting places of adult A. balabacensis, but only three males and two females were collected during daylight hours resting in an abandoned charcoal pit near Ban Bu Phram. No A. balabacensis adults were collected resting in vegetation during daylight hours, even though collections were begun at daybreak in areas where A. balabacensis had been collected the previous night or when these collections were conducted near known ovipositional sites.

Larval habitats of A. balabacensis in the vicinity of Ban Bu Phram and Ban Thap Lan were located and described. During this period balabacensis larvae were found in 43 ground-pools in 18 locations. Once discovered these pools were visited weekly and sampled for larvae. The temperatures and pH of water in these pools were measured weekly. The majority of these pools were formed by elephant footprints, however larvae were also collected from water which collected in buffalo hoofprints, wheel ruts, natural depressions and in fallen tree trunks. All these sites were shaded from the sun during part of the day. The daytime water temperatures ranged from 26 to 31°C and the pH values ranged from 6.0 to 7.0 throughout the study period. Larvae were present in one or more of these sites from June until November. Because of irregular rainfall,

the majority of the larval pools were intermittently dry, and none had balabacensis larvae continuously during the period of observation. Larvae of at least 8 other anopheline species were collected in association with A. balabacensis, including A. barbirostris, members of the A. hyrcanus complex, A. barbumbrosus, A. hodgkini, A. insulaeflorum, A. kochi, A. tesselatus and A. vagus. Throughout the rainy season samples of moist soil were taken from edges and bottoms of dry pools from which balabacensis larvae had been previously collected. These samples were returned to the laboratory and immersed in pans of water. One hundred twenty such samples were obtained and first stage balabacensis larvae were recovered from 10. Premumably these larvae hatched from eggs present in the soil samples, although it is also possible they represented larvae trapped in the mud at the bottoms of the drying pools. However, the former assumption is supported by the observation that gravid balabacensis females from the SMRL colony, given the opportunity to oviposit in pans containing pools of water in a sand substrate, deposited their eggs only on the sand at the margins of the water rather than on the surface of the water. In addition, eggs of colonized A. balabacensis have remained viable for up to 56 days when kept on moist filter paper in SMRL laboratory.

Rainfall ceased in Ban Bu Phram valley on 4 November, and all larval pools under observation on the valley floor were dry by 15 November. During the remainder of November and in December efforts were made to locate balabacensis breeding sites at the margins of the forested hills surrounding the valley where water was still present in stream beds and seepage areas. Larvae of A. balabacensis were found in such sites during November and December, but no larvae were collected between January and March 1972, although suitable larval habitats were still present. Larvae of A. balabacensis were obtained from soil collected from two former breeding sites during December, but samples collected between January and March were negative.

2) Susceptibility of A. balabacensis and A. minimus to infection with P. falciparum. A study was undertaken to determine if there is a difference in the susceptibility of colonized strains of A. balabacensis and A. minimus to infection with naturally occurring P. falciparum in an area where chloroquine resistant falciparum is endemic. This study was conducted in the town of Phra Phutthabat, Saraburi province approximately 130 km north of Bangkok. Laboratory reared A. balabacensis and A. minimus from SMRL colonies were fed

simultaneously on 36 persons with P. falciparum infections. These subjects came from a wide area of central Thailand for treatment of acute malaria at either the local hospital or Malaria Eradication Center in Phra Phutthabat. Only those with single infections of P. falciparum and gametocyte densities of at least 100 per cmm were selected for study. Mosquitoes which engorged on these subjects were dissected eight to ten days later, and the oocysts present on their guts counted. The proportions of mosquitoes which had oocysts (per cent positive) and the mean number of oocysts per infected female (oocyst index) were used to compare the susceptibility of the two mosquito species. The results of the 36 attempts to infect the two mosquito species are summarized in Table 3. More A. balabacensis fed and were dissected than A. minimus. Sixteen of the 36 subjects were infectious for A. minimus, while A. balabacensis were infected by these and an additional eight subjects. Twelve of the subjects were non-infectious for both mosquito species. The results of the 16 paired feedings which resulted in infection of both mosquito species are summarized in Table 4. The medians of per cent positive and oocyst numbers were 2.5 and 4.5 greater, respectively, for A. balabacensis than for A. minimus. The results of the feedings on the eight subjects which infected only A. balabacensis are presented in Table 5. There were no noticeable differences in size or morphology of the oocysts which developed in the two species.

The A. balabacensis and A. minimus oocyst indices from 13 subjects are shown in Figure 1. The results were highly correlated ($r = .80$, $p < .01$). In Figure 2 are presented the percent positive results for these 13 subjects; association was less marked ($r = .39$, $p < .15$). The data from the additional three subjects which infected both species are not included in Figures 1 and 2 because in each case less than ten A. minimus were dissected and, of these, only one was infected.

The percents positive for both mosquito species were also compared with the gametocyte densities in the subjects. When mosquitoes were infected, larger proportions of A. balabacensis were infected at each gametocyte density. However, the association between gametocytemia and proportion infected for the above mentioned 13 subjects was not appreciable for either A. balabacensis ($r = 0.36$, $p < .15$) or A. minimus ($r = .15$, $p < .50$). Similar results were obtained for both mosquitoes when oocyst indices were compared with levels of gametocytemia. The gametocyte densities and percentages of mosquitoes infected for all 36 subjects are shown in Figure 3.

Table 1. Numbers of A. balabacensis Collected in Ban Bu Phram Valley.

Location	Biting		Resting		Total
	Outdoors	Indoors	Outdoors	Indoors	
Village	161	193	111	45	510
Forest	11	1	44	5	61
Totals	172	194	155	50	571

Table 2. Summary of the A. balabacensis collected at Prachinburi study site. (June 71- March 72)

Location	Month	Biting				Resting	
		Total Numbers Collected		Number Collected /man/night		Total Numbers Collected	
		Outdoors	Indoors	Outdoors	Indoors	Outdoors	Indoors
Village	June	69	90	4.3	6.4	60	4
"	July	53	72	2.8	2.7	37	40
"	Aug	1	21	0.2	3.5	2	1
"	Sept	20	9	0.8	0.3	8	0
"	Oct	16	1	0.5	0.04	1	NC*
"	Nov	2	0	0.7	0.0	0	NC
"	Feb	0	0	0	0.0	3	0
Forest	Feb	11	1	1.8	0.5	43	5
"	Mar	0	0	0	0	1	NC
TOTAL	8	172	194	-	-	155	50

* NC : No. collection made

Table 3. The median and range for the number of engorged and dissected mosquitoes (groups of 50) for 36 attempted infections.

		A. balabacensis		*A. minimus*	
		Infected	Not Infected	Infected	Not Infected
No. groups		24	12	16	20
No. fed	Median	41.5	38.0	25.5	22.5
	Range	31 - 49	18 - 48	7 - 42	7 - 44
No. dissected (survived)	Median	37.5	32	20.0	17.5
	Range	30 - 47	15 - 47	6 - 38	6 - 33
Percent fed dissected		90.3	84.2	78.4	77.7

Table 4. Results of dissections of 16 groups of A. balabacensis and A. minimus when both groups were oocyst positive.

		A. balabacensis	A. minimus
Percent positive	Median	83.3	33.2
	Range	6.5 - 100	3.9 - 69
Oocyst index	Median	13.4	3
	Range	1.4 - 248	2 - 42

Table 5. Results of dissections of eight groups of A. balabacensis when the corresponding A. minimus group negative.

Percent positive	Median - 36 Range - 2.3 - 100
Oocyst index	Median - 1.5 Range - 1 - 34.8

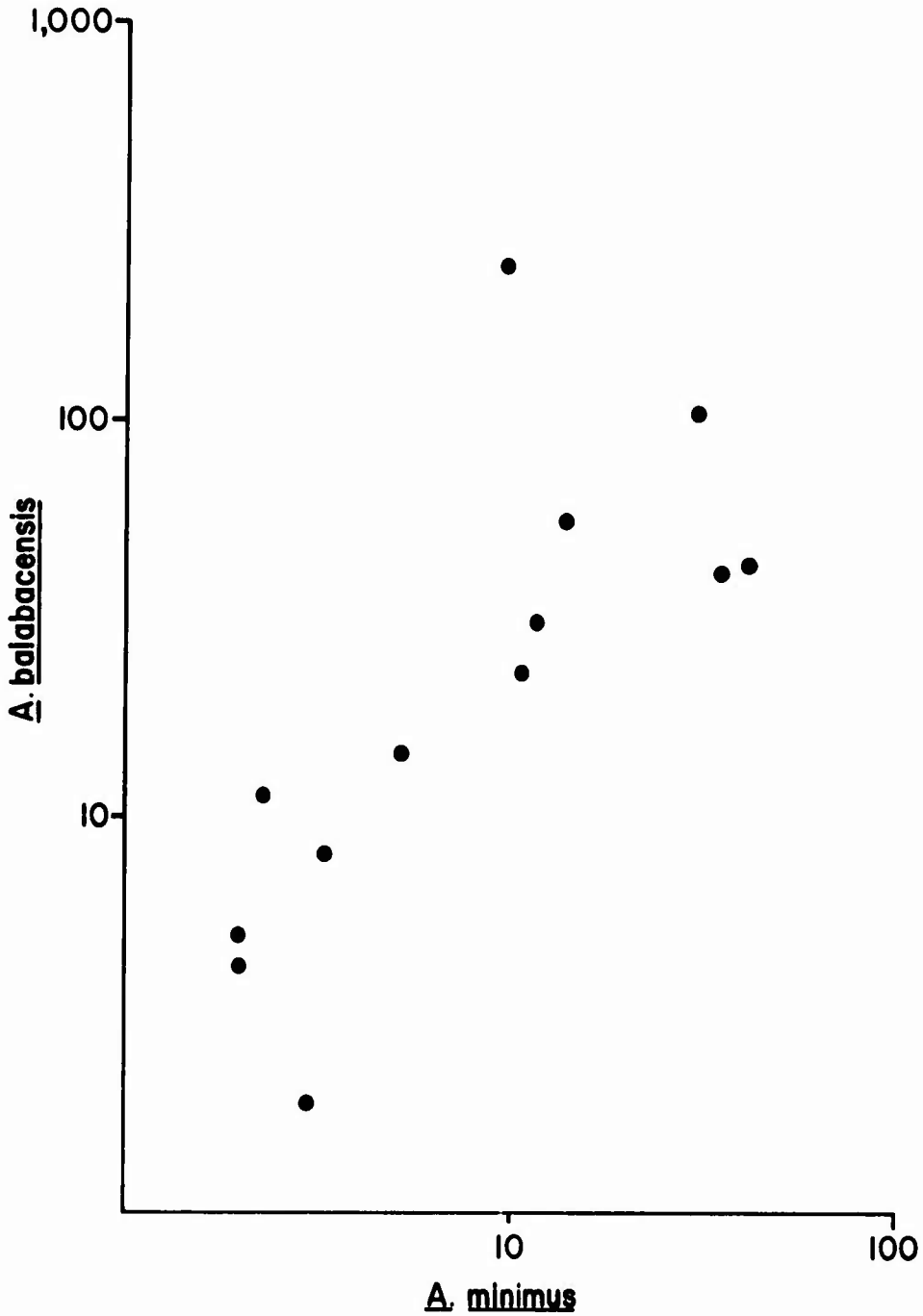

Fig. 1. Oocyst index for A. balabacensis and A. minimus by subject.

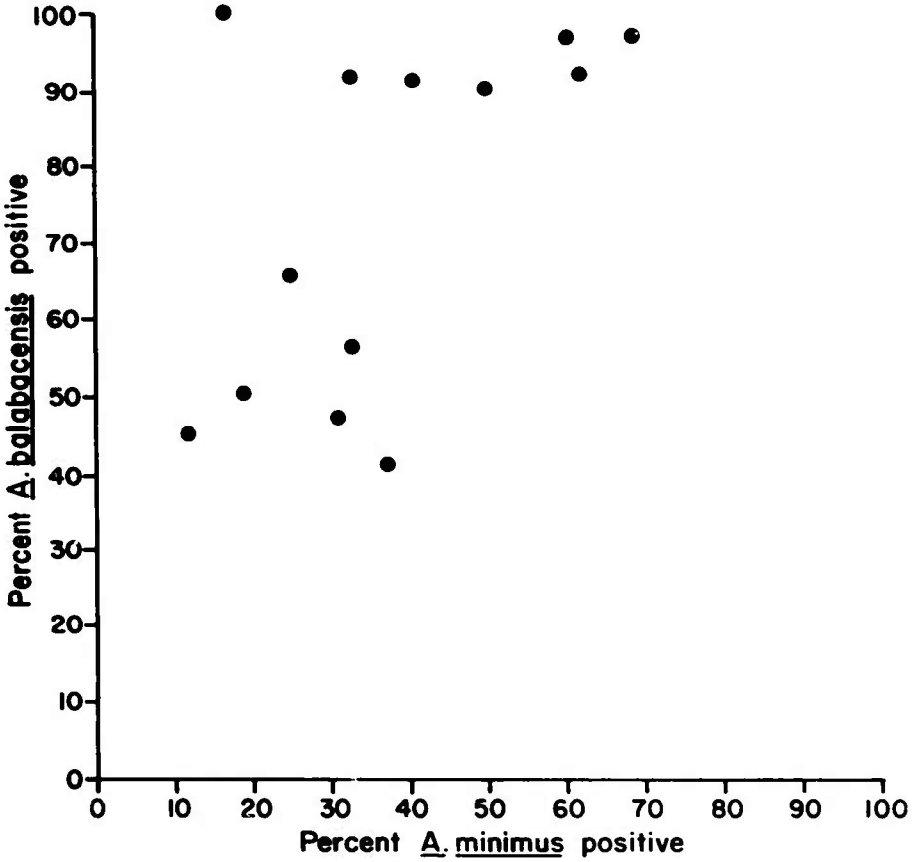

Fig. 2. Percent positive A. balabacensis and A. minimus by subject

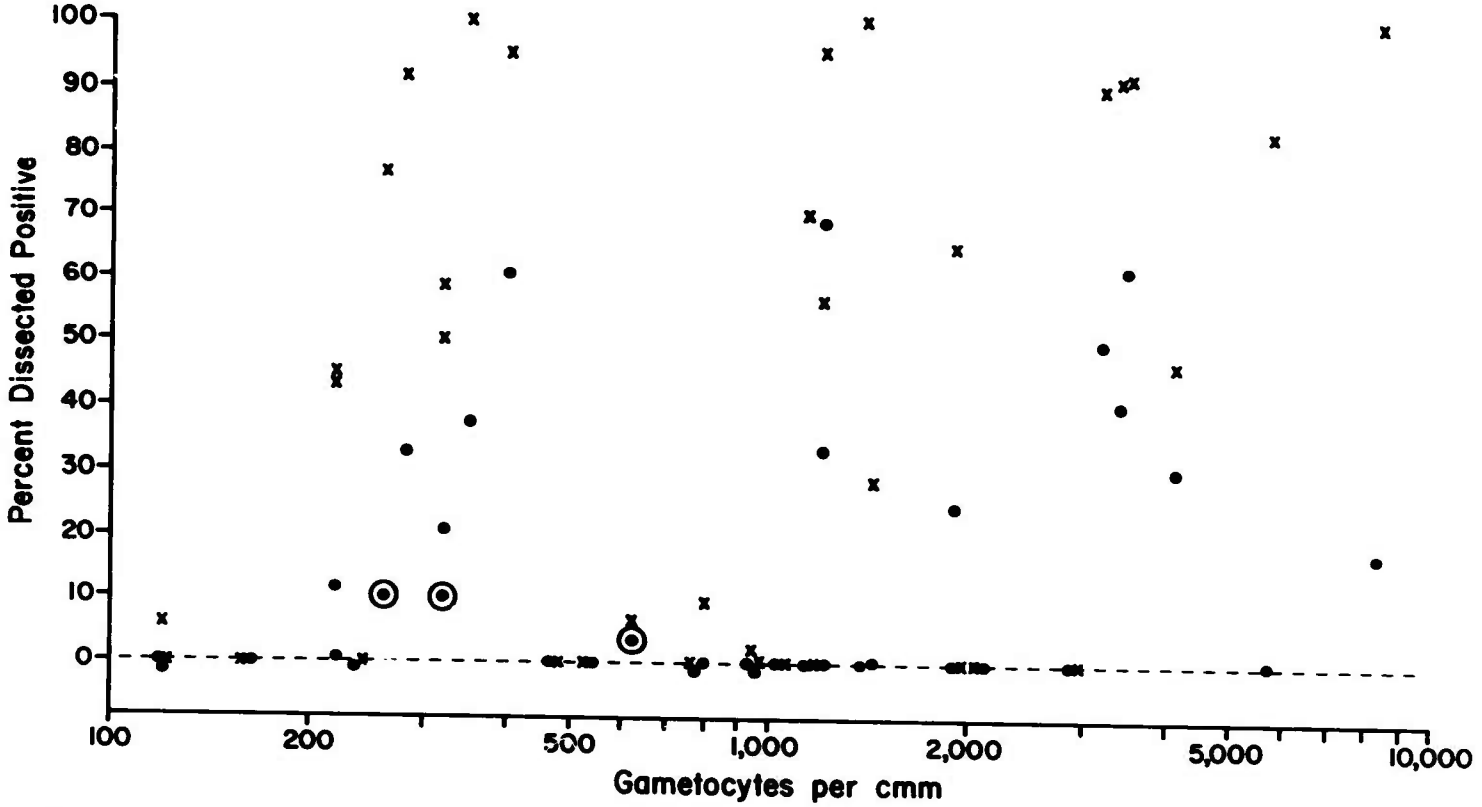

Fig. 3. Percent of A. balabacensis and A. minimus infected by feeding simultaneously on 36 subjects with P. falciparum, as related to gametocyte density. (x = A. balabacensis, • = A. minimus, ⊙ = A. minimus, less than 10 dissected.)

Effect of Sulphamethoxazole-trimethoprim on the Viability of Plasmodium falciparum Gametocytes.

Principal Investigators : Rowland N. Wilkinson
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Associate Investigators : Attaya Boonyakanist
Dumrong Charoendhum

OBJECTIVES : Previous investigations have shown that a combination of sulphamethoxazole, a long active sulfanimide, and trimethoprim, a folic acid antagonist, was effective in the treatment of P. falciparum asexual parasitemias. However, this combination was not gametocyticidal and no investigations were conducted to examine its sporonticidal efficacy. The purpose of this investigation was to determine the sporonticidal effects of sulphamethoxazole-trimethoprim on the gametocytes of natural infections of P. falciparum.

DESCRIPTION : Subjects who were ill with falciparum malaria and whose pretreatment blood smear examination demonstrated gametocytes were hospitalized at the Phra Phutthabat District Hospital and were administered 2 tablets consisting of 400 mg sulphamethoxazole and 80 mg trimethoprim twice daily for 7 1/2 days. Groups of 50, colonized, A. balabacensis were fed on subjects who had at least 100 gametocytes per cmm after completion of therapy. These mosquitoes were dissected for oocysts and/or sporozoites 7-18 days after feeding on the patients.

PROGRESS : Gametocytes persisted in the blood of all patients during and after treatment. A. balabacensis were fed on 10 subjects which had been treated with the 7 1/2 day course of sulphamethoxazole-trimethoprim. Oocysts and/or sporozoites were demonstrated in 6 of the 10 lots of mosquitoes indicating that the treatment had no sporonticidal effects.

Mosquito Fauna of Thailand

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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 field.

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 Smithsonian Institution for study by specialists in the Southeast Asia Mosquito Project (SEAMP).

PROGRESS : During this year 234 mosquito collections were made in 5 provinces of Thailand. The majority of the collections were made in Prachinburi province. These collections resulted in 1344 pinned adults, 2630 slide mounts of larvae, larval and pupal skins and 83 slide mounts of terminalia. Results of the mosquito collections made during this period are as follows :

Anopheles - During this period emphasis was placed on collecting Anopheles (Cellia) balabacensis. Larvae of this species were collected

* SEAMP, Smithsonian Institution, Washington, D.C.

from small shaded pools in scrub and deciduous forest, these pools were formed in elephant foot-prints, wheel ruts, seepage areas, hollow logs, crab holes and fallen tree trunks. Anopheles balabacensis larvae were collected in association with Aedes (Aedimorphus), Aedes (Neomacleaya), and Culex (Culiciomyia) as well as other Anopheles species.

Aedes - Taxonomic studies on species of the subgenus Stegomyia were continued. Aedes (S.) w-albus was collected in a water jar in Chanthaburi province in associatio- with Aedes (S) albopictus and Culex (Culiciomyia) fragilis. The only previous record of the species in Thailand was from Khon Kaen in 1966. Aedes (S) s. malayensis was collected from rock pools along the coastal areas of Koh Samui, Surat Thani province, and from bamboo cups in Nonthaburi province. Attempts to establish colonies of Aedes (Aedimorphus) mediolineatus and Aedes (Neomelaniconion) lineatopennis by natural mating failed in the second generation. Two possible new species of Aedes were collected in Prachinburi province. Aedes (Paraedes) sp. larvae were collected in an elephant footprint and in a crab hole. Adults of this species were collected both in light traps and biting man. Aedes (Neomacleaya)sp. larvae were collected from an elephant footprint. Twenty one other species of this subgenus had been previously collected in Thailand in 1968.

Culex - Studies of subgenus Culex were continued. Culex alienus a member of Culex vishnui subgroup which had been recorded only in Trat province was collected from a ground pool in Chanthaburi province. Culex whitei, another species of the Culex vishnui subgroup, was collected from stream pools in the Ban Bu Phram area in association with Culex fuscocephala, Culex gelidus and Culex tritaeniorhynchus.

A Review of the Drug Sensitivity of Plasmodium Falciparum in Thailand

by: LTC Edward J. Colwell, MC

OBJECTIVE: Twenty-five years ago, the introduction of chloroquine for suppression and cure of the human malarias appeared to offer a highly effective tool, in conjunction with vector control, for malaria eradication programs. However, the emergence of chloroquine resistant strains of Plasmodium falciparum had made the goal of eradication less tangible than that anticipated in earlier forecasts and has stimulated intensive studies on a search for alternative regimens for chloroquine. The purpose of this report is to review the historical aspects of chloroquine resistant falciparum malaria in Thailand with particular reference to its prevalence, geographic distribution and alternative treatment programs.

DESCRIPTION: The history of chloroquine resistant falciparum malaria in Thailand begins with the report of Professor Tranakchit Harinasuta and associates (1962) from the Faculty of Tropical Medicine in Bangkok, in which they reported chloroquine treatment failures in nine patients infected with P. falciparum. At approximately the same time, an American serviceman on temporary duty in Thailand contracted falciparum malaria. During the next 7 months, he received multiple courses of chloroquine, each ranging from 1.5 to 2.1 gm base, without achieving a radical cure (Young et al., 1963). Infected blood from this patient was intravenously inoculated into non-immune volunteers and subsequent drug sensitivity studies demonstrated that this strain, called the JHK strain, was also resistant to mepacrine, proguanil, pyrimethamine and amodiaquine (Young et al., 1964). Quinine treatment was ultimately necessary to produce a radical cure in both the naturally and experimentally infected subjects.

During the next decade, studies of the prevalence and geographic distribution of P. falciparum strains resistant to chloroquine have been reported by investigators from the Faculty of Tropical Medicine and the SEATO Medical Research Laboratory, and the results are illustrated in Figure 1.

Harinasuta and colleagues (1965) administered a conventional course of chloroquine (i.e., 25 per kg) to infected patients who were hospitalized in Bangkok. Under the conditions of their study, prolonged follow-up examinations were possible and natural transmission was unlikely.

Only 2(5%) of 42 patients experienced a radical cure. In the remaining 40 subjects, 28 exhibited an initial disappearance of parasitemia and symptoms followed by a recrudescence and 12 had only partical clearing of asexual parasites. In a subsequent report by Harinasuta et al. (1967), conventional chloroquine therapy was given to 65 hospitalized patients in Bangkok, who were acutely ill with P. falciparum infections. Not a single radical cure was achieved. Incomplete clearance of asexual parasites was observed in 40 patients and a recrudescence occurred in the remaining 25 patients who had experienced initial clearing. The subjects examined in the 2 reports by Harinasuta et al. resided in many areas of Thailand and these investigators suggested that strains of P. falciparum resistant to chloroquine were distributed throughout the entire nation.

While personnel of the Faculty of Tropical Medicine were concentrating their efforts on hospital based patients in Bangkok, investigators from the SEATO Medical Research Laboratory were conducting studies on the prevalence and geographical distribution of strains resistant to chloroquiune in rural areas of Thailand. In 1966, Bourke and associates reported a reduced sensitivity of P. falciparum to chloroquine among residents of 3 southern provinces bordering Malaysia. They observed that 80% of 307 infected residents who were given a single dose of chloroquine (10 mg per kg) still exhibited asexual parasites 3 days later. Previous investigations in Southeast Asia which were reviewed by Sandosham et al. (1964) showed that a single dose of 10 mg per kg was generally sufficient to clear asexual parasitemias within 3 days.

These field studies were extended by Cadigan and colleagues(1968) to other provinces in the southern, central and northcentral areas of Thailand. Infected subjects were given 25 mg per kg of chloroquine base and follow-up blood smears were obtained one week after commencement of therapy. The proportions of unsatisfactory parasite responses ranged from 6% in the central province of Saraburi to 85% in the northcentral province of Loei.

During the 1970 summer peak of malaria transmission in Central Thailand, investigations of chloroquine resistant malaria were renewed at Phrabuddhabat Hospital, Saraburi Province, which was an area previously surveyed by Cadigan and associates(1968). The objective of these studies was to assess the reliability and reproducibility of a rapid in vitro technique described by Rieckmann et al.(1968) for detection of chloroquine resistant strains of P. falciparum. This technique consists simply of an in vitro cultivation of parasitized blood with glucose as the only added nutrient. The

indicator response is the degree of trophozoite maturation to schizonts in vials containing zero or graded concentrations of chloroquine.

Concurrent in vivo and in vitro responses to chloroquine were measured in 57 patients infected with P. falciparum (Colwell et al., 1972 a). Treatment failures following conventional chloroquine administration were observed in 55(96%) of the 57 subjects. All 57 individuals had parasites which exhibited chloroquine resistance in vitro. It is conceivable that in the two subjects who were cured, the additive effects of host immunity and treatment were sufficient to produce a radical cure even though the parasites were relatively resistant in vitro.

Investigations of the in vitro sensitivity of P. falciparum have also been conducted in Nong Khai(northeast), Trat(southeast) and Yala (south) provinces in Thailand. The subjects examined in these 3 areas consisted primarily of acutely ill subjects seeking treatment at a local government health facility in the provincial capital city. However, 20 of the 43 subjects examined at Nong Khai were asymptomatic, infected residents of a remote rural village(Phon Phi Sai), who had not actively sought treatment from a government health facility. The proportions of resistant strains detected by the in vitro Rieckmann test at Nongkahi and Trat were approximately 90% (Hickman et al., 1971). Utilizing the same technique in Yala Province(south), our current studies have yielded a similar high rate of chloroquine resistant strains.

Among the alternative treatments for chloroquine which have been investigated in Thailand are quinine, atabrine, sulfonamides, folic acid antagonists, tetracyclines and various combinations of these agents. Table 1. summarizes the radical cure rates obtained with these alternate regimens reported during the past decade.

Quinine still appears highly efficacious in controlling symptoms and promoting rapid clearance of asexual parasitemias of P. falciparum in acutely ill patients. However, few reports are available concerning the efficacy of quinine therapy alone in effecting radical cures of falciparum malaria. In addition, the optimal duration of quinine administration for effecting radical cures is still open to question. In Southeast Thailand, Colwell and associates(1972 b) examined the efficacy of a combination of quinine and chloroquine in acutely ill subjects. Thirty-six infected subjects were given a 3 day course of quinine at daily dose of 1.62 gm base, followed by a conventional (i.e., 25 mg kg) course of chloroquine. During the one month follow-up period, only 41% of these patients experienced a radical cure. Harinasuta

et al.(1965) examined the efficacy of a 4 and 5 day course of quinine at the same daily dose. Although their patients exhibited initial clearing of asexual parasitemias, all experienced a recrudescence. More recently, the efficacy of a 7-10 day course of quinine was investigated in residents in of Saraburi Province (Colwell et al. 1972 c). Thirty-four acutely ill patients, many of whom were severely ill with high densities of asexual parasites, were administered a 7-10 day course of quinine at daily dose of 1.62 gm base. All subjects experienced initial clearing of asexual parasitemias ranging from from 1 to 6 days(mear clearance was 3.3 days). In 12 patients, blood smear examinations were obtained for one month after commencement of therapy and none exhibited a recrudescence of P. falciparum. These limited investigations suggest that quinine remains highly effective for treatment of falciparum malaria in Thailand. Only 1 isolated case of a poor response to quinine administration had been reported in Thailand(Pinswasdi and Charoenkwan, 1965).

Several investigations have been conducted to examine the effects of a single dose of a long acting sulfonamide, alone or in combination with pyrimethamine, against P. falciparum infections. Harinasuta et al.(1967) administered a single dose of sulphormethoxine (1000 mg) to 18 infected subjects who had recently experienced chloroquine treatment failures. Radical cures were reported in 11 of these individuals. When 50 mg of pyrimethamine was added to the sulfonamide, 17 of 19 infected individuals experienced a radical cure. The latter combination was associated with a more rapid onset of action than the sulfonamide alone, as reflected by mean parasite clearance times of 2.9 and 5.7 days, respectively.

Tawaramorn and his naval medical colleagues (1970) also examined the effects of a single dose administration of sulphormethoxine and pyrimethamine at the same doses employed by Harinasuta et al(1967). The majority of their infected subjects were marine and naval personnel who contracted P. falciparum in a single dose and radical cures were reported in all patients. The mean clearances of fever and asexual parasitemia in this study were considerably loner in comparison with the eralier report of Professor Harinasuta and associates (1967).

A comparative evaluation of a single dose administration of sulphormethoxine-pyrimethamine and sulfalene-trimethoprim was recently completed at Trat Provincial Hospital in Southeast Thailand (Chin et al., 1971). Adult males were administered a single dose of sulphormethoxine (1000 mg) - pyrimethamine (50 mg) or sulfalene(1000 mg) - trimethoprim (500 mg). Radical cures were achieved in 91% of 34 subjects treated with the sulphormethozine-pyrimethamine regimen and in 84% of 31 patients administered the

sulfalene-trimethoprim regimen. These rates were not significantly different. The mean clearances of fever and asexual parasites in both treatment groups were similar and were comparable to those reported by Harinasuta et al. (1967).

In Central Thailand, Benjapong et al. (1970) evaluated the antimalarial activity of a combination tablet of sulphamethoxazole (400 mg) and trimethoprim (80 mg) in doses recommended for treatment of bacterial infections. Administration of 2 tablets twice daily for one week resulted in a presumptive radical cure in 17 of 18 patients of whom two had experienced recent chloroquine treatment failures. Gametocidal effects were not observed and no significant toxic complications were reported. Although a single dose administration of a sulfonamide and folic acid inhibitor has its obvious advantages over multiple doses, further studies are warranted to assess therapeutic effectiveness and untoward side effects of these alternative regimens.

In addition to investigations of conventional antimalarial drugs, the therapeutic efficacy of the tetracycline family of antibiotics had been examined in subjects residing at Trat Province in Southeast Thailand (Colwell et al., 1971 b). Sixteen asymptomatic subjects of whom 8 had experienced treatment failures with chloroquine were given a 10 day course of oral tetracycline (250 mg every 6 hours) and blood smear examinations were accomplished for one month after commencement of therapy. Presumptive radical cures were demonstrated in 12 of 16. In the other 4 patients, tetracycline was discontinued within 72 hours because of the development of fever, headache and chills. The mean parasite clearance time of 4.7 days in the successfully treated patients is significantly longer than the clearance times associated with quinine and chloroquine treatment of susceptible strains (Handfield-Jones, 1949; Jefferey et al., 1956).

The antimalarial activity of tetracycline was also evaluated in acutely ill subjects with P. falciparum infections in a subsequent phase of the preceding study (Colwell et al., 1971 b). Because of the slow rate of tetracycline action, all subjects were given a 3 day course of conventional quinine therapy to control initial symptoms, followed by either a 10 day course of tetracycline or a conventional course of chloroquine. Treatment failures were observed in 15 (58%) of 36 patients treated with quinine-chloroquine, but in only one (3%) of 31 patients administered quinine-tetracycline. The difference in the failure rates was very highly significant. The single subject who experienced a treatment failure with quinine-tetracycline exhibited initial clearance of asexual parasites followed by a reappearance of trophozoites on the 28th day of observation, possibly reflecting reinfection rather than resistance. Although the prolonged course of tetracycline and its relatively delayed rate of action limit its antimalarial

efficacy, its lack of toxicity and high degree of effectiveness, particulary against resistant P.falciparum strains, warrant further study.

Minocycline, a new synthetic tetracycline analogue, was subsequently evaluated for its antimalarial efficacy at Prapokklao hospital, Chantaburi Province in Southeast Thailand (Colwell et al., 1971 d). The experiences of the staff physicians at this location indicate that this area is also highly endemic for chloroquine resistant falciparum malaria. Acutely ill subjects with P.falciparum infections were given a 3 day course of quinine to control initial symptoms, followed by the administration of 100 mg minocycline twice daily for one week. Presumptive radical cures were observed in all but one of 28 patients. The single subject with a treatment failure exhibited a reappearance of trophozoites on the 24th day of observation, and, hence, could have been reinfected. Additional studies are needed to evaluate the toxic side effects of this new tetracycline analogue in man. Rieckmann and associates (1971) have recently reported that tetracycline courses of 5 or 7 days were highly effective in terminating P.falciparum infections in partially immune volunteers who had low density asexual parasitemias. Similar to our studies, these investigators observed delayed clearances of fever and asexual parasitemias with tetracycline treatment alone. In an effort to reduce cost and duration of quinine-tetracyclines combinations, yet maintain effectiveness, we have renewed our in vivo drug sensitivity studies at Phrabuddhabat and Trat. Acutely ill adults infected with P. falciparum were given quinine (1.62 gm) for only one day and, at the same time, tetracycline therapy was initiated at a dose of 250 mg, 4 times a day, for only 7 days. A presumed radical cure was demonstrated in 25 of 29 patients who were followed for at least one month.

PROGRESS: Chloroquine resistant falciparum malaria appears to be distributed throughout all of Thailand and, in areas of intensive study, the majority of these strains are resistant to conventional doses of chloroquine. At localities in which low rates of resistance have been reported, these estimates may be conservative because subcurative doses (i.e., 10 mg base/kg) of chloroquine were administered and/or prolonged follow-up examinations were not accomplished. According to recommendations of the World Health Organization (1968), chloroquine sensitivity should not be presumed until blood smear examinations remain negative for asexual parasites of P.falciparum for at least 28 days after commencement of chloroquine treatment at a dose of 25 mg per kg body weight.

Other considerations, in addition to inadequate follow-up examinations, may obscure interpretation of in vivo drug sensitivity studies. These include

lack of drug intake supervision, exposure to reinfection and partial immunity of the sample population. These restrictions of in vivo studies can be eliminated by the utilization of a simplified in vitro technique for detection of drug resistance. Within certain limitations, the simplified in vitro technique described by Rieckmann and colleagues (1968) for detection of chloroquine resistant falciparum malaria proved in our investigations to be a rapid and reliable test that demonstrated a qualitative index of chloroquine resistance in Central Thailand(Colwell et al., 1971 a).

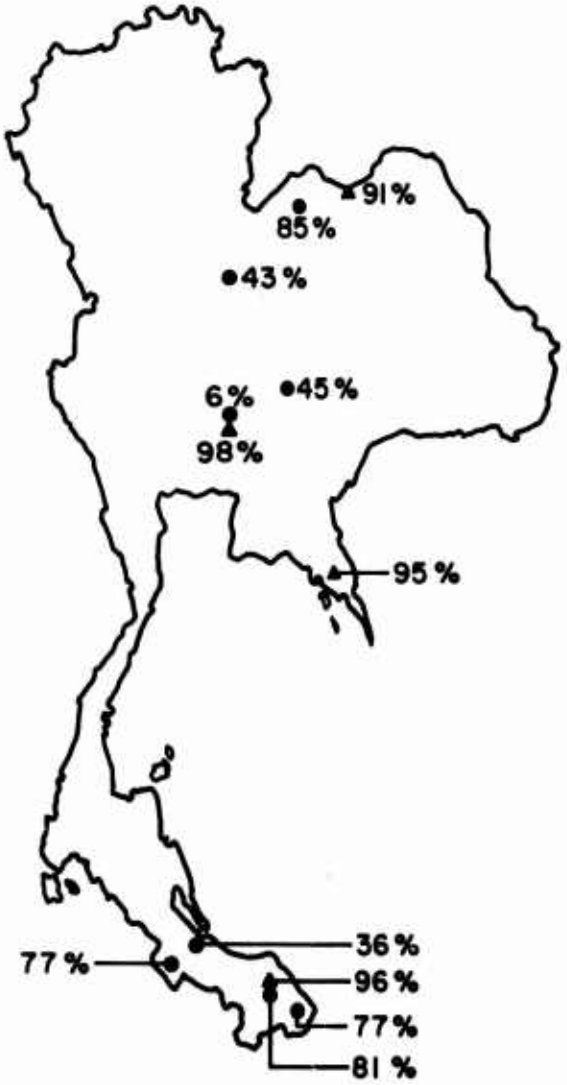
Among the alternative treatments for infected residents in endemic areas for chloroquine resistant falciparum malaria, the more promising regimens, with regard to cost, toxicity and efficacy are the single dose administration of sulphormethoxine-pyrimethamine and the multiple dose administration of quinine-tetracycline. The single dose schedule is particularly attractive for mass control campaigns wherein repeated supervision of drug intake is obviated. Although preliminary investigations of the efficacy of the sulfonamide-folate inhibitor combination in Thailand have demonstrated a high degree of efficacy, several disadvantages of this combination could preclude its mass use. Among the disadvantages are the lack of sporontocidal activity, the potential emergence of P. falciparum strains resistant to this regimen, the potential emergence of Neisseria meningitidis resistant to sulfonamides and toxic side effects. The latter possibilities associated with this regimen are bone marrow suppression, gastrointestinal irritability and the Stevens-Johnson syndrome.

For treatment of hospitalized patients, the combination of quinine and tetracycline is clearly superior, with regard to cost and toxicity, to the prolonged administration of quinine with or without pyrimethamine and sulpha drugs. Our studies clearly demonstrate that by controlling the initial symptoms with quinine, tetracycline is highly effective in producing a radical cure in acutely ill patients infected with chloroquine resistant falciparum malaria. No toxic side effects have been observed and recent studies have shown that the original 13 day course of quinine-tetracycline therapy can be reduced to 7 days without compromising the efficacy. A potential disadvantage of major importance in prospective investigations is the emergence of P. falciparum and pathogenic bacteria resistant to tetracycline.

SUMMARY: The historical aspects of chloroquine resistant falciparum malaria in Thailand with particular reference to its prevalence, geographic distribution and alternative treatments have been reviewed.

Chloroquine resistant strains of P. falciparum are distributed throughout all of Thailand and, in areas of intensive study, the majority of strains are resistant to conventional doses of chloroquine.

The most promising alternative treatments are a single dose administration of sulphormethoxine-pyrimethamine and a sultiple dose administration of quinine-tetracycline. The former combination is particularly attractive for mass control programs and the latter combination is suitable for hospitalized subjects with moderate to severe degress of illness.



DISTRIBUTION AND FREQUENCY OF CHLOROQUINE RESISTANT FALCIPARUM MALARIA IN THAILAND

Table 1

Radical cure rates associated with alternative regimens employed for treatment of falciparum malaria in Thailand

Drug(s)	Daily dose (mg)	Duration (days)	No. treated	Radical cure (%)	References
Mepacrine	200	7	12	66.7	Harinasuta et al. (1965)
Quinine	1,620	4	3	0	"
Quinine	1,620	5	3	0	"
Quinine	1,620	7	8	100.0	"
Quinine	1,620	7 - 10	12	100.0	Colwell et al. (1972)
Quinine Chloroquine	1,620 1,500	3 2	36	41.6	Colwell et al. (1972)
Fanasil	1,000	1	18	66.7	Harinasuta et al. (1967)
Fanasil Pyrimethamine	1,000 50	1 1	19	89.4	"
Fanasil Pyrimethamine	1,000 50	1	62	100.0	Tawaraman et al. (1970)

(cont.)

Table 1 (cont)

Drug(s)	Daily dose (mg)	Duration (days)	No. treated	Radical cure (%)	References
Fanasil Pyrimethamine	1,000 50	1 1	34	91.2	Chin et al. (1971)
Sulfalene Trimethoprim	1,000 500	1 1	31	83.9	"
Sulphamethoxazole Trimethoprim	1,600 320	7.5 7.5	19	89.4	Benjapongs et al. (1970)
Quinine Tetracycline	1,620 1,000	3 10	30	96.6	Colwell et al. (1972)
Quinine Minocycline	1,620 200	3 7	28	96.4	Colwell et al. (1972)

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 112, 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 OA 6537	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8b. DISP'N INSTR'N	8c. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	114
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (f)			

11. TITLE (Provide with security Classification Code)

(U) Malaria Program Supervision (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012100 Organic Chemistry

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 72	9	630
FISCAL YEAR CURRENT 73	9	630

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
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NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide KWOC with security classification code)

(U) Malaria; (U) Drugs; (U) Biology; (U) Chemistry

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

23. (U) To manage, to integrate, and provide quality control for the Drug Research Program on Malaria, both in-house and by contract.

24. (U) To define areas requiring investigation, to develop suitable contract proposals to follow progress by correspondence or site visits, to guide direction of investigation, to provide for exchange of information, and to continually check findings for verification through independent agencies (both in-house and contract). Two outside advisory groups are utilized.

25. (U) 71 07-72 06 Close supervision, through guidance and integrated evaluation of productivity, was continued for forty-nine contracts in the areas of chemical synthesis, drug preparation and data handling and for nineteen biological contracts concerned with the preclinical aspects of antimalarial efficacy and drug safety.

Three new Investigational New Drug (IND) applications and eleven IND supplements were submitted and approved, with preclinical work continuing on two IND basic documents and on six additional drugs of potential usefulness. Six meetings of advisory groups and selected contractors were organized and conducted to examine specific facets of antimalarial drug development and utilization. Expanded metabolism and safety studies continue following feed-back on drugs under active clinical investigation. For technical reports see Walter Reed Army Institute of Research Annual Progress Report. 1 Jul 71 - 30 Jun 72.

*(Applicable to contractors upon originating agency)

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaric Investigations

Work Unit 114, Malaria Program Supervision

Investigators
Principal: COL William E. Rothe, VC
Associates: Thomas R. Sweeney, Ph.D.; LTC David E. Davidson, VC: Melvin H. Heiffer, Ph.D.

General

The malaria program is a modern medicinal chemistry research effort designed to develop drugs to treat and/or prevent P. falciparum and P. vivax infections of man. The fields of investigation logically include synthetic organic chemistry, biology and pharmacology. Program supervision includes the ability to coordinate and direct efforts in each of these disciplines in a manner that produces a constant feedback of data from individual investigators and allows necessary program redirection. This research management culminates in the synthesis and biological evaluation of new antimalarial drugs for troop issue. The objectives are being attained through the combined efforts of a team of research managers with the necessary expertise, in-house laboratory support, and extramural consultation.

The Contract Chemical Synthesis Program

A breakdown of the contract synthesis program for FY-72, exclusive of preparations laboratories, is given in Table I.

In addition to the research synthesis contract there have been four contracts with preparations laboratories to resynthesize, on a larger scale, selected compounds that are needed for large animal testing, toxicological studies or clinical investigations. There has also been one contract to synthesize radioactively tagged compounds and one to analyze and check purity and identity of compounds and compositions to be used in preclinical and clinical studies.

TABLE I

Breakdown of Contract Synthesis, FY-72

Number of Contracts

Type of Organization	Active in FY-72	Expired in FY-72	Entering FY-73
Industry	8		7
University	24		17
Research House	13	1	12
TOTAL	45	9	36

During FY-72 there was a total of 998 compounds submitted by the synthesis contractors, 416 of which were target compounds. This figure, which is considerably lower than that for FY-71, reflects the shift in synthetic emphasis from the amino alcohols, where the chemistry had been well worked out, to the 6- and 8- aminoquinoline prophylactics where the chemistry proved to be intractable. It also reflects the loss from the program of the most prolific contractor. The average cost per target compound as of the end of FY-72, calculated from the inception of the program, is about $2,300. The preparations laboratories continued to be quite active. During the year the number of compounds requested from these laboratories was 16, 5, and 1 in small, medium and large quantities respectively. Compounds received numbered 7, 3, and 3 in these respective quantities. Publications and manuscripts generated by the synthesis contractors during the year totaled 68.

A number of miscellaneous areas, e.g., amino acids, peroxides, polysulfides, oxazolines, and solubilizing derivatives, all based upon solid leads, continue to be investigated.

Having reached the optimum development of the phenanthrene- and pyridine- aminoalcohols, and having selected compounds from these classes for clinical studies, synthesis of compounds in these classes, as in the quinolinemethanol class before them, ceased. Three other classes of aminoalcohols, less active than the above mentioned, viz., the fluorene.-, naphthothiophene- and polymethylene-bridged naphthalene- and quinoline-aminoalcohols have been abandoned. The naphthalene- and anthracene-aminoalcohols are quite active and it is planned to continue investigations in these areas.

Potential chemoprophylactic classes of compounds have continued to receive increased attention. The main thrust is in the 6- and 8-aminoquinolines and the substituted tetrahydrofurane and related compounds. Work on the nephthoquinones and RC-12-related compounds have been phased out. The related quinoline quinones are still being investigated.

The third broad area of sythesis has been the antimetabolites, which includes the antifols. After the research in the 2,4- diamino-6- arylthioquinazolines culminated in the emergence of compound WR-158122, work in this area reverted to probing action along a broad front; several of the types of compounds synthesized were, understandably, derived from the WR-158122 lead.

The Organic Chemistry Laboratory Synthesis Program

The in-house synthesis program has concerned two main areas. One is a series of substituted, bridged symmetrical triazines. The other is the development of methods for the conversion of the amino alcohol type antimalarials to the corresponding thiols, disulfides and thiosulfates. The conversion of quinine to the corresponding thiosulfate yielded a compound somewhat more active than quinine in the primary mouse screen.

Acquisition of Compounds

Slightly over 8800 compounds were acquired from all sources during the fiscal year; a little over one half of this number were submitted under the no-dollar agreement, and about one quarter were submitted as gifts. The remainder were the synthesis compounds, as mentioned above, and purchased compounds.

There was no single large submitter under the agreement during the year but rather a steady flow of compounds from multiple sources. Ten new no-dollar agreements were consumated during the year. The rate of acquision of compounds under the agreement appears to be roughly proportional to the effort that can be devoted to it; negotiations continue to be time consuming and complicated. The canvass of recently published compounds continues to be successful considering the limited effort that can be devoted to it.

Patents

During the fiscal year a final draft of a patent application has been developed on WR-158122 and a few related compounds and should be filed shortly. Allowance of U.S. patent application SN 23,506 on process improvement for the synthesis of aryl bromomethyl ketones now seems probable. Claims covering applications SN 150,745 and SN 150,746 in the carbinolamine area are under active investigation.

of the tetrahydrofuran series (WR 179305) were highly active, and are undergoing advanced study. In addition, 44 compounds were studied for comparative effectiveness by oral, intraperitoneal and subcutaneous routes of administration against blood-induced P. berghei in mice.

2. Compounds which are found active in the prophylactic chick and mouse tests are evaluated definitively by Dr. Harry Most of New York University to distinguish true causal prophylactic activity from activity which is merely a reflection of prolonged schizonticial action. Causal prophylactic activity is determined by quantitative microscopic examination of exoerythrocytic forms in the liver of rats infected with P. berghei sporozoite. Eight compounds were studied in the past year.

3. During the reporting period 101 compounds were evaluated by Dr. C. C. Smith of the University of Cincinnati for folic acid antagonism in three drug sensitive organisms, Streptococcus faecium, Lactobaciblus casei and Pediococcus cerevisiae, which comprise the standard antifolic screen. Of those compounds tested, 81 were active and 7 inactive. Fifty-two compounds were reversed by addition of folate and 8 were indeterminate. Strains of the above test bacteria resistant to cycloguanil, pyrimethamine or trimethoprim were tested for sensitivity to representative sulfonamides, sulfones, triazines, quinazolines and pteridines and against quinine, chloroguanide, quinacrine, WR 30090, WR 33063 and WR 122455. The sensitive and antifolate resistant strains of the above were checked for fermentation reactions and for purine and thymine requirements. These tests established that the strains gave typical reactions for their species and also that there were no detectable differences in the requirements of the parent sensitive and the resistant strains. Studies of the effects of antimalarials on growth and on the comparative drug reversing ability of folic acid and leucovorin with these bacteria have been partially completed It is hoped that the differences observed in the sensitive and resistant strains will provide leads to the mechanisms of resistance existing in these organisms.

4. Dr. Paul Thompson of the University of Georgia evaluated 122 orally administered experimental antimalarial drugs against drug sensitive. P. berghei, and against one or more strains resistant to chloroquine, chloroguanide, dapsone, or pyrimethamine. In a special mechanism study, it was found that the antimalarial activity of WR 30090, WR 33063, WR 122455 and WR 142490 was not reversed by the administration of PABA. A strain of P. berghei less highly resistant to chloroquine than the currently available strain is being developed.

5. Dr. Wallace Peters of the Liverpool School of Tropical Medicine evaluated the antimalarial activity of a series of Quinolinemethanols and phenanthrenemethanols against P. berghei.

A patent U.S. 3578692, on gem-dithiol compounds as antimalarials was issued during the year. A decision was made during Fiscal-72 to abandon patent coverage for the trifluoromethyl phenanthrenemethanols because the heavy workload with limited staff did not allow patent application within one year of public disclosure of the series.

Biology

All compounds received during this period were tested for antimalarial activity in mice infected with blood-induced Plasmodium berghei. In addition, selected compounds were screened for causal prophylactic activity against sporozoite-induced P. berghei in mice or against sporozoite-induced P. gallinaceum in chicks. Compounds found to be active in the primary screens were evaluated in secondary test systems to characterize their oral efficacy, their activity against drug resistant strains, their activity against simian malaria, and their activity against exoerythrocytic forms.

Progress

a. Primary Screens

1. The basic primary antimalarial screening system is the P. berghei-mouse mortality test performed by Dr. Leo Rane of the University of Miami. During the past year 14,886 compounds were tested in this system, of which 576 were active. The blood-induced P. gallinaceum-chick mortality test, also performed in Miami, was discontinued during the year in order to devote greater effort to the sporozoite-induced system. During 7 months of operation, 1615 compounds were evaluated in the chick test, including 90 which were active.

2. A sporozoite-induced prophylactic chick test utilizing Aedes aegypti mosquitoes for the production of P. gallinaceum sporozoites was made operational during the year. Prophylactic compounds produce an extension in survival time of the chicks when administered on the day of sporozoite inoculation. Control chicks die regularly between 5 and 11 days, with a mean survival time of 8.5 days. This test can be operated at a high rate of thruput for a prophylactic test and is expected to have a long-term capacity of 25-50 compounds per week. In 8 months of operation, 2168 compounds have been tested.

b. Secondary Tests

1. Selected antimalarial agents are tested for prophylactic activity in the sporozoite-induced Plasmodium berghei yoeli mouse test. During the reporting period, 242 compounds were tested, including at least 70 with potential causal prophylactic activity. An 8-aminoquinoline (WR 106147) with activity superior to primaquine, and an analogue

WR 142490, a quinolinemethanol was nearly as active against the highly resistant RD strain as against the moderately resistant NS strain, or against the sensitive N strain. In vitro studies demonstrated that the clumping of pigment in P. berghei after exposure to chloroquine could be prevented by inhibition of protein synthesis or inhibition of RNA synthesis. The relationship between the inhibitors studied and chloroquine was non-competitive. Further studies of mechanisms of drug resistance are in progress.

6. MAJ Dennis O. Johnsen of the SEATO Laboratory evaluated 18 new antimalarial compounds for curative activity against blood-induced P. cynomolgi infections in Rhesus monkeys. Further studies are in progress.

7. Aotus monkeys, infected with drug sensitive or resistant strains of P. falciparum, were used-routinely in the evaluation of candidate antimalarials for schizonticidal activity. Two new strains of P. falciparum are being adapted to the Aotus host: Honduras, a drug sensitive parasite; and Smith, a multiresistant organism. Use of the Smith strain-which has been fully characterized in man-should improve the correction of efficacy data generated in simian and human test systems. The drug response of two trophozoite-induced strains of P. vivax has been studied in Aotus monkeys. The Chesson strain from New Guinea is sensitive to standard antimalarials. A Viet Nam strain was found to be relatively resistant to both pyrimethamine and proguanil. Little progress has been made with a simian model using sporozoite-induced P. vivax. The major problems encompass low mosquito infectivity, variable prepatent periods and inconsistent relapse patterns. Work continues on a mosquito-vivax-monkey system. During this development phase candidate drugs are being tested for causal prophylatic activity in the standard P. cynomolgi/rhesus test of Schmidt.

Pharmacology and Clinical Program

Claims for Investigational Exemptions for New Drugs were filed for WR 4835, WR 142490 and WR 158122. Supplements to previous claims were filed for WR 6798; 122455; 30090; 40070; 81844; 4629/5949; 33063; 61112; 38839; and 171952. A document was prepared, but not filed for WR 113618 because of unresolved toxicity.

Summary and Conslusions

The successful use of human plasmodia-both P. falciparum and P. vivax-in animal systems continues to permit selection and appropriate chemical development of new structure leads which do not show cross resistance against several human strains of interest. Many of these candidate

antimalarials are undergoing active clinical and field evaluation. The manager-team approach in supervision of the malaria program has proved satisfactory, and the program continues in a balanced fashion. During the past year, the search for new causal prophylactic agents has been intensified.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 08 6480	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. GRADING	8A. REPRO	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (f)			

11. TITLE (Precede with Security Classification Code)

(U) Test System Design and Development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
70 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	CURRENT		
	72	3	105
	73	3	105

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202/576-3551

20. GENERAL USE

Foreign Intelligence Not Considered

19b. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicinal Chemistry

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic participation)

NAME: Kinnamon, K. E., LTC

TELEPHONE: 202/576-2292

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Smith, J. H., 1LT DA

NAME: Rich, R. R., 1LT

21. KEYWORDS (Precede each with Security Classification Code) (U) Chemical; (U) Chemistry; (U) Pharmaceutical; (U) Test System; (U) In vitro; (U) In vivo

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

23. (U) To design and develop in vitro and in vivo test systems for the evaluation of chemical compounds as prophylactic and therapeutic agents against diseases of major military significance. Infections such as malaria, which render troops ineffective for extended periods, receive principal emphasis.

24. (U) Appropriate in vitro and animal pilot models are designed to meet a specific need for testing chemical compounds. These models are expanded into operating test systems in-house and utilized to provide guidance to contractors when testing is to be done under contract. When necessary, modifications to existing test systems are designed and developed.

25. (U) 71 07 - 72 06. In vitro and in vivo studies of the effects of antimalarial drugs on the development of dental plaque and on the growth of plaque-producing bacteria are continuing. Nineteen of 62 compounds tested inhibited the in vitro growth of oral organisms, but none of these compounds inhibited plaque development in vivo when administered orally each day. Evaluation of these compounds when incorporated into the diet of guinea pigs is now underway. Studies aimed at development of a P. gallinaceum - causal prophylactic and radical curative test in chick embryos are continuing. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 to 30 July 72.

*Available to contractors upon satisfactory request.

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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 122 Test Systems Design and Development

Investigators:
Principal: LTC David E. Davidson, Jr.
Associates: LTC Kenneth E. Kinnamon
1LT Rolla R. Rich
1LT James H. Smith

Description

These investigations are designed to develop and improve biological test systems for the evaluation of antimalarial drugs both in-house and under contract.

Progress

1. A test system is being developed to screen candidate antimalarial drugs for activity against exoerythrocytic stages of Plasmodium gallinaceum utilizing embryonated chicken eggs as the vertebrate host. While embryos can be successfully infected by placing tissues infected with exoerythrocytic forms onto the chorioallantoic membrane, the methods tried to date have failed to produce sufficiently consistent infections to make drug testing feasible. Two methods have been used with limited success, (1) placement of infected tissue slices onto the chorioallantoic membrane, (2) placement of a homogenate of infected tissue onto the chorioallantoic membrane. Further refinement of the techniques of inoculation are clearly needed. Twenty experimental antimalarial drugs have been evaluated for toxicity to chick embryos in the expectation of eventual antimalarial studies.

2. Periodontal disease may be caused by bacteria which are common in the oral cavity of both humans and hamsters. Periodontal disease, characterized by accumulations of plaque on the gingiva of the molar teeth, may be induced experimentally in hamsters by feeding a high carbohydrate, low fat, non-abrasive diet and by inoculating plaque material from infected donors. This experimental system is being utilized to evaluate antimalarial drugs for possible activity in preventing or eliminating periodontal disease. Test drugs are first screened for antibacterial activity in vitro using drug saturated paper discs on nutrient agar inoculated with plaque cultures. Many of the antimalarial drugs are active in vitro and several have been selected for in vivo test in the hamster system. These compounds have been evaluated for their ability to prevent the development of plaque, and a few for their ability to eliminate plaque in hamsters with established disease.

Test drugs are dissolved or suspended in a suitable vehicle and delivered into the oral cavity once daily by pipette or syringe at a dose of 25 mg/day per hamster. The following compounds, all of which have antibacterial activity in the *in vitro* assay, have been evaluated *in vivo*:

WR 1544	Chloroquine
WR 2977	Amodiaquine
WR 2978	Pyrimethamine
WR 3091	Proguanil
WR 4629	Sulfalene
WR 4835	Amopyroquine
WR 6020	Isopentaquine
WR 6798	Diformyl Dapsone

Using these methods, no effect on the development of periodontal disease *in vivo* was observed. Further studies, in which the drugs are incorporated in the diet, are in progress.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6513	72 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
71 07 01	D. Change	U	U	NA	NA	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	123
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG114(F)			

11. TITLE (Precede with Security Classification Code)

(U) Biological studies on control of anopheline vectors of malaria

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: Not Applicable 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 72	2	30
FISCAL YEAR CURRENT 73	2	30

19. RESPONSIBLE DOD ORGANIZATION

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

NAME: Walter Reed Army Institute of Research
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TELEPHONE: 202 - 576-2553

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Eldridge, LTC B.F.

NAME: Schiefer, MAJ B.A. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Anopheles; (U) Biocontrol; (U) Colonize; (U) Mosquitoes; (U) Vectors

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 methods for the control of mosquito vectors of malaria which will have minimal impact upon the environment. Various mosquito pathogens will be studied to determine those types which can be utilized in a biological or integrated vector control program, which could decrease the incidence of malaria in OCONUS military personnel.

24. (U) Establishment of laboratory colonies of anophelines from areas of strategic importance. Evaluation of rearing procedures through alteration of environmental factors. Study of mosquito pathogens on mosquito behavior, especially as related to disease transmission.

25. (U) 71 07 - 72 06. Infection of Anopheles stephensi mosquitoes with a microsporidan parasite, Nosema algerae, reduced their susceptibility to infection with the simian malaria parasite, Plasmodium cynomolgi, as measured by malarial oocyst counts 6 days after an infective feed. Mortality from nosematosis was so great that 90 - 95 per cent of the exposed mosquitoes died before sporozoite levels could be assessed on day 14. It is concluded that the low incidence of Nosema in field populations of adult anophelines is related to the virulence of this pathogen. Additional studies will be made with Nosema and other anopheline species to determine whether virulence of the parasite is similar among all species of the genus Anopheles. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* 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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 123 Biological studies on anopheline vectors of malaria

Investigators.
Principal: Ronald A. Ward, Ph.D.
Associate: LTC Bruce F. Eldridge, MSC; MAJ Bernard A. Schiefer, MSC; SSG William D. Tracy; SP4 Paul F. Beeman; SP4 Jackie Williams; Leroy H. Bell, B.S.; David E. Hayes; Talmadge J. Neal, B.S.; Kenneth Savage, M.S.*

Description

Biological studies of anopheline mosquitoes are performed, using material maintained in the insectary at this Institute. Studies include evaluation of rearing techniques and their effect on the research results, development of rearing techniques, the effects of biological agents on anopheline mosquitoes and studies on anopheline behavior.

Progress

In the course of rearing anopheline mosquitoes for malaria transmission experiments it has been observed that the presence of microsporidan parasites can either decimate laboratory colonies to such an extent that transmission studies cannot be conducted or if the mosquitoes survive, the results of malaria transmission experiments are extremely variable, frequently with marked decreases in oocyst and sporozoite counts.

These studies started when it was observed that the WRAIR Anopheles stephensi colonies exhibited excessive larval and adult mortality during July 1968. The cause of the mortality was not known until dissection of adult mosquitoes infected with the simian malarial parasite, Plasmodium cynomolgi, revealed the presence of numerous spore-like structures in the fat body surrounding the midgut. They were tentatively identified as Nosema and further study by E.I. Hazard confirmed the diagnosis (Hazard, 1970). Records of the infection were kept and following elimination of the pathogen from the insectary, collaborative experiments were conducted with the U.S. Department of Agriculture Insects Affecting Man and Animals Laboratory, Gainesville, Florida. Their purpose was to determine whether a concurrent malaria and microsporidan infection in an anopheline vector could reduce the vector potential of an anopheline species or even prevent the species from transmitting malaria to a rhesus monkey. This information would be of value in designing strategies for the control of vector species with this pathogen.

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* Insects Affecting Man and Animals Laboratory, U.S. Dept. of Agriculture, Gainesville, Florida.

In the first series of experiments, Nosema spore suspensions were prepared in Gainesville and first stage Anopheles stephensi larvae were infected with the spores at WRAIR. Between April and July 1971 300 late 1st-stage and early 2nd-stage larvae were placed in pans containing a Nosema spore concentration of 5.6×10^3 spores/ml solution. They remained in these pans until pupation occurred. From August onward, 300 1st-stage larvae were exposed to spore concentrations (2.4×10^4 spores/ml solution) in small beakers for 5 - 21 hours and then transferred to larval rearing pans. Control pans of A. stephensi larvae were reared at similar densities adjacent to groups of treated larvae.

The results of dissections made 8 days after feeding on P. cynomolgi-infected rhesus monkeys indicated that there was a reduction in mean malarial oocyst counts in the majority of feedings from Nosema-infected pans (Table 1). 8/11 or 72.7% of the mean oocyst counts from female A. stephensi reared in infected larval pans were lower than the counts from uninfected controls. A similar comparison of mean oocyst counts between both groups showed a count of 48.49±3.56 oocysts from Nosema-infected pans while the controls averaged 56.60±3.59 oocysts. The anophelines from infected larval pans had a reduction in oocyst count of approximately 25% when compared to untreated controls. This difference was significant ($t = 2.59$, $p = 0.05$). During the 8 day post-emergence period, mortality in the controls ranged between 10 - 20% while that in the Nosema-infected cages varied from 25 - 75%.

Salivary gland dissections were made 6 days later from Nosema-treated cages which contained at least 6 live anophelines. There was a marked reduction in the incidence of Nosema in cages from the treated pans in the intervening period (Table 2). Four cages which initially had a Nosema incidence between 8 - 75% were negative for the parasite on the later date. Similarly, the other cages showed a marked reduction in parasite incidence. The sporozoite levels in the surviving anophelines from treated pans did not differ significantly from those observed in control groups. Among the cages which contained mosquitoes for dissection on the 14th day after the infective feed, mortality in the controls averaged 50% while that in treated A. stephensi ranged between 90 and 95%.

An attempt was made to determine whether a correlation existed between the level of Nosema infection in individual mosquitoes and the malarial oocyst count of that female. No significant differences were found between A. stephensi females which had Nosema infections scored as moderate (1+) as compared with those scored as very heavy (3+).

The above studies indicated that the concurrent infection of female Anopheles stephensi mosquitoes with a microsporidan parasite, Nosema algerae Vavra and Undeen, produced a reduction in the mean number of oocysts of the simian malarial parasite, Plasmodium cynomolgi, which developed on the mosquito midgut. Although the reduction was statistically significant no appreciable difference was observed in the salivary gland infection rate 6 days later. This was undoubtedly related to the

differential mortality of Nosema-infected females, the majority of which had died by day 14.

These observations closely paralled those of Hull* (1971) who investigated the concurrent effect of Nosema algerae in A. stephensi infected with the rodent malarial parasite, P. berghei (Nigerian strain, N-67). He noticed a reduction in oocyst count on Nosema-infect midguts and in addition a reduction in sporozoite counts. Titration of sporozoites from Nosema-infected mosquitoes in mice showed a significant decrease of infectivity of sporozoites as compared to uninfected controls.

Fox and Weiser (1959) pointed out that there is no evidence that Nosema is physiologically antagonistic to Plasmodium, or that the former attacks the latter. In heavily infected mosquitoes, the midgut wall was so disintegrated that suitable sites were not available for P. falciparum oocyst development. These general observations are valid for the dual Nosema-P. cynomolgi infections in Anopheles stephensi. The only exception is that A. stephensi females with extremely heavy Nosema-infections which reached the point of midgut disintegration, did not survive sufficiently long to permit midgut oocyst counts on day 6.

There are few records of microsporidan parasites from wild-caught anophelines. During the period June 1955 through April 1956 R.M. Fox dissected more than 7,000 Anopheles gambiae in Liberia yet only encountered a few mosquitoes with microsporidan infections (Fox and Weiser, 1959). This may indicate that natural infections of Nosema exist at very low frequencies or that adult mortality is fairly high when this parasite is present in a population. In view of laboratory experiments with the pathogen, the latter interpretation is probably the more valid.

Conclusions and Recommendations

1. Infection of Anopheles stephensi mosquitoes with a microsporidan parasite, Nosema algerae, reduced their susceptibility to infection with the simian malaria parasite, Plasmodium cynomolgi, as measured by malarial oocyst counts 6 days after an infective feed. Mortality from nosematosis was so great that 90-95% of the exposed mosquitoes died before sporozoite levels could be assessed (day 14). It is concluded that the low incidence of Nosema in adult anopheline populations in the field is related to the virulence of this pathogen.

2. Larval populations of anopheline mosquitoes should be surveyed in the field to determine the relative incidence of microsporidan parasites and their role in biotic control of vector populations. Additional laboratory studies should be made with Nosema and other anopheline species to determine whether virulence of the parasite is similar among all species of the genus Anopheles.

TABLE 1

Effect of Nosema infection of Anopheles stephensi (India strain) on susceptibility to Plasmodium cynomolgi bastianellii oocyst development

Date	Rhesus number	Percent mosquitoes with Nosema	Oocysts: Nosema		Oocysts: Control	
			No.	Mean ± SE	No.	Mean ± SE
14 Apr 71	H-776	50.0*	30	16.7±3.1	25	23.8±2.9
14 Apr 71	H-776	92.3*	13	11.6±4.7	25	25.4±2.5
6 May 71	H-711	83.3	12	215.3±42.2	12	103.8±37.2
12 Oct 71	K-822	50.0	10	0.9±0.6	6	1.3±1.2
13 Oct 71	K-822	25.0	12	39.3±8.9	12	87.7±18.6
13 Oct 71	K-821	75.0*	12	178.4±31.3	12	411.6±30.6
13 Oct 71	K-821	58.3*	12	352.8±49.2	10	384.1±48.3
18 Oct 71	K-822	8.3	12	57.8±10.9	12	46.7±17.5
21 Oct 71	K-821	83.3*	12	48.0±7.4	12	60.6±9.9
21 Oct 71	K-821	75.5*	11	33.6±9.1	10	61.1±26.3
22 Oct 71	K-821	50.0	12	21.1±2.5	12	18.3±3.6

* Mosquito cages derived from different larval rearing trays.

TABLE 2

Effect of Nosema infection of Anopheles stephensi on Plasmodium cynomolgi bastianellii sporozoite development

Date	Rhesus number	Percent mosquitoes with Nosema		Sporozoite level[1]	
		Day 8	Day 14	Nosema group	Control
13 Oct 71	K-821	75.0	0	2.3	3.1
13 Oct 71	K-821	58.3[2]	12.5	2.6	3.0
13 Oct 71	K-822	21.1	0	1.2	3.0
14 Oct 71	K-821	8.3	0	0	0.7
14 Oct 71	K-822	16.7	0	0.4	-
22 Oct 71	K-821	50.0	16.7	3.5	3.7
22 Oct 71	K-821	58.3[2]	33.3	3.0	-

[1] Mean of 6 dissected pairs of salivary glands; 1.00 =<100 sporozoites, 2.00 = 100 - 1,000, etc.

[2] Mosquito cages derived from different larval rearing trays.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 123 Biological studies on anopheline vectors of malaria (CD&I)

Literature Cited

References:

1. Fox, R.M., and Weiser, J.: A microsporidian parasite of Anopheles gambiae in Liberia. J. Parasitol. 45:21-30, 1959.

2. Hazard, E.I.: Microsporidian diseases in mosquito colonies: Nosema in two Anopheles colonies. Proc. 4th Intl. Colloq. on Insect Pathol., pp. 267-271, 1970.

3. Hulls, R.H.: The adverse effect of a microsporidan on sporogony and infectivity of Plasmodium berghei. Trans. Roy. Soc. Trop. Med. Hyg. 65:421-422, 1971.

Publications: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6514	72 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
71 07 01	D. Change	U	U	NA		☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	124
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Biological Studies of mosquito malaria infection and transmission (09)

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: Not Applicable 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 72	1	35
FISCAL YEAR CURRENT 73	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202 - 576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Div of CD and I
Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic participation)
NAME: Ward, Dr. R. A.
TELEPHONE: 202 - 576-2553
SOCIAL SECURITY ACCOUNT NUMBER: [redacted] PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Eldridge, LTC B.F.
NAME: Hayes, D.E. DA

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.

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) 71 07 - 72 06. The multi-resistant Smith strain of falciparum malaria from Vietnam was successfully adapted to splenectomized Aotus monkeys and is being used in the WRAIR contract program on malaria chemotherapy to collect data that only could previously be achieved in human volunteer studies. Mosquito susceptibility to Plasmodium falciparum in Aotus is related to the early passage history of a particular strain rather than to any inherent qualities of the strain. With increased blood passage, Aotus-adapted strains become more virulent and the infection is fatal before gametocyte maturity is achieved. Immunization of rhesus monkeys with irradiated sporozoites of P. cynomolgi did not protect monkeys from sporozoite challenge. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* 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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 124 Biological studies of mosquito malaria infection and transmission

Investigators
Principal: Ronald A. Ward, Ph.D.
Associate: LTC Bruce F. Eldridge, MSC; CPT J. Scott Anderson, VC*
SP4 Paul F. Beeman; Leroy H. Bell, B.S.; David E. Hayes

Description

Studies are conducted on the infectivity of human and simian malarial parasites to various mosquito vectors of malaria. Special emphasis is placed on the role of genetic and environmental factors in mosquito susceptibility. Mosquito transmission of falciparum and vivax malaria is attempted. The results of these studies are applied to the development of test systems for the evaluation of antimalarial drugs.

Progress

1. Plasmodium falciparum in Aotus trivirgatus and mosquito infectivity of gametocytes

Since Geiman and Meagher (1967) reported that the night monkey Aotus trivirgatus, was susceptible to Plasmodium falciparum from man, numerous workers have infected this host by serial passage of parasites. Attempts to establish the sporogonous cycle of falciparum malaria in this host have been largely unsuccessful. Collins and Contacos (1968) recorded the infection of Anopheles freeborni mosquitoes from Aotus with the Malayan IV strain of P. falciparum and subsequent transmission to human volunteers by mosquito bites. Jeffery (1969) relates that a Cambodian strain of P. falciparum produced rare infections in A. balabacensis and A. freeborni. The Chamblee group was unable to effect monkey to monkey transmission by sporozoite passage (Sodeman et al. 1969). Baerg and Young (1969) were unable to infect Anopheles albimanus in 46 feeding trials on Panamanian Aotus infected with Malayan Camp and Uganda Palo Alto falciparum malarias. Ward and his colleagues at WRAIR were unable to infect Anopheles stephensi and A. balabacensis with the Camp/W strain of falciparum malaria (Hickman, 1969).

A comparison of the single successful experiment of Contacos and Collins (1968) with those of other workers revealed that the former group of workers used a parasite strain in the first monkey passage from man while other investigators used parasites in 5th or higher passage levels from chimpanzees or night monkeys (Ward, 1969). At that time Ward stressed that it was too early to ascertain whether gametocyte infectivity is a characteristic of certain parasite strains in Aotus or

an easily lost attribute following several blood transfers in heterologous host systems.

The present series of experiments were designed to test the hypothesis that there would be a stronger probability of establishing mosquito infections of falciparum malaria from Aotus with parasite isolates which had a recent history of mosquito transmission among human volunteers rather than with falciparum strains which had an extensive history of consecutive blood passage in subhuman primates. If this hypothesis were shown to be valid, then subsequent studies would be designed to study the pattern of mosquito infectivity, determine maximal gametocyte levels for donor monkeys and develop procedures for enhancing gametocyte production.

D.F. Clyde and V.C. McCarthy, University of Maryland School of Medicine, Baltimore, provided fresh isolates of Plasmodium falciparum infected blood of the Vietnam (Brai.), Philippines (Per.) and Vietnam (Smith) strains from male volunteers, inmates of the Maryland House of Correction, Jessup. On days when the appropriate parasitemia levels were observed, 10 cc. of heparinized blood was collected under sterile precautions and carried on wet ice to WRAIR for inoculation into Aotus. In most instances, less than 2 hours lapsed between the transfer of infected blood from man to monkey. Usually, 4-9 X 10^7 parasites were inoculated intraperitoneally into splenectomized monkeys. For transfer between monkeys to maintain the parasite strain or provide donors for mosquito infectivity studies, the inoculum contained 2.4 X 10^5 - 8.3 X 10^8 parasites.

Isolates of the Vietnam (Brai.) and Philippines (Per.) falciparum strains were inoculated into splenectomized Aotus monkeys. Both these isolates were from sporozoite-induced infections from volunteers at the Maryland House of Correction.

The details of the 4 blood transfers of the Brai. strain are summarized in Table 1. This strain appeared to be extremely virulent from the first passage level; 4/6 Aotus exposed developed parasitemia in excess of 10^6 parasites/mm^3 and died of malarial infections. The gametocytes appeared relatively immature during the first 2 passage levels and dissection of 22 lots of mosquito pools of Anopheles stephensi and A. quadrimaculatus disclosed no oocyst development.

Monkeys of passage levels 3 and 4 were given small doses of an immunosuppressant drug, Imuran, on the assumption that such treatment might prolong gametocyte survival until a degree of mature was achieved that would be suitable to infect anophelines. Small doses were administered 1 day prior to infection and 1-3 days subsequent to infection. It was observed that Aotus G-984, which received the above treatment, infected small numbers of Anopheles stephensi 12-16 days after the first appearance of gametocytes. The last 2 Aotus, G-992 and 228 died before mosquito feeds could be achieved.

The behavior of the first 3 passages of the Philippine, (Per.) strain are reviewed in Table 2. Three Aotus, 2 of which were splenectomized were inoculated with infected human blood. Although all monkeys became infected, the non-splenectomized G-967 only exhibited a transitory parasitemia for a single day. This strain was not as virulent as the Brai. strain and peak parasitemias (6.7-7.5 X 10^5 parasites/mm^3) only attained a level half that of the former strain. Although most gametocytes were immature during the first passage level, 1/32 pools fed contained infected anophelines. Aotus, H-233, infected with 3rd passage parasites was treated with Imuran to determine whether the observation with the Brai. strain could be repeated. Mosquito infections were found on 3/9 days that Anopheles stephensi cages were applied to Aotus H-233. The first mosquito infection occurred on day 31 of the infection or 17 days after gametocytes first appeared in the peripheral circulation.

Studies on the Brai. and Per. isolates indicated that treatment of monkeys with an immunosuppressant rendered them more suitable as gametocyte carriers. One alternate possibility would be that as a parasite strain became better adapted to a host following consecutive transfer, gametocytes would persist for a longer period of time, and thus attain sufficient maturity to be infectable to mosquitoes. Another possibility might be that inherent differences in Aotus monkeys, such as prior malarial exposure or length of time in a laboratory colony might alter the course of a subsequent malarial infection and the resultant production of gametocytes.

In order to support studies on the chemotherapy of malaria, three isolates of the Vietnam (Smith) strain were obtained from the University of Maryland School of Medicine between December 1970 and April 1971. The Smith strain, which was isolated at the end of 1968 from a patient who acquired the infection near the Cambodian border, was of special interest due to the resistance to chloroquine, pyrimethamine and chlorguanide (Clyde et al, 1971). Successful adaptation of this strain to Aotus would provide material for antimalarial drug evaluation that could be compared with studies in human volunteers.

The first 2 inoculations of Smith strain falciparum isolates did not establish patent parasitemias. On the 3rd attempt, 1 of the 2 Aotus (546) became patent on day 12. Since then, 9 blood transfers of the isolate have been made in splenectomized Aotus monkeys as indicated in Table 3. After the 1st passage, all animals developed patent infections. 7/12 Aotus were treated with the immunosuppressant, Imuran, for varying periods of time (10-40 days) at a dose of 5 mg/kg daily. Unlike the previously described experiment with the Per. strain, this treatment did not significantly change the course of parasitemia nor affect gametocyte development and maturation. Maximal levels of parasitemia in the Smith strain were similar to the Per. strain and only half those observed in the Brai. strain.

Two general patterns of parasitemia were observed in the Smith strain. The first was exemplified by Aotus 387 (Figure 1). This animal

attained a peak parasitemia of 200,000 parasites/mm^3, then gradually decreased to extremely low levels over the next 3 weeks. The immature gametocytes (stages I - IV) gradually increased and remained at levels between 1,000 - 10,000/mm^3 for a month. Mature gametocytes (stage V) were visible for a similar period. Aotus 976 was typical of the 2nd pattern in which the parasitemia attained an extremely high peak, over 350,000/mm^3 (Figure 2). Abundant immature gametocytes were present but mature gametocytes only appeared in low numbers prior to the death of the monkey from the malarial infection. This second pattern occurred with greater frequency at higher passage levels.

Gametocyte development was examined in detail in the studies of the Smith strain to determine whether there were any relationships between gametocyte production, maturation of gametocytes and levels of mosquito susceptibility to infection. In this study, gametocytes were examined daily on thin film smears prepared at 0730 hrs. The gametocytes were classified into stages of development as outlined by Field and Shute (1956) and emended by Hawking et al. (1971). Five developmental stages were recognized, i.e.

I Rounded forms, do not fill erythrocyte.

II Irregular shape (sometimes triangular), slightly elongated.

III Elongated, pointed or slightly rounded ends; erythrocyte slightly distorted.

IV Elongated, rounded ends, erythrocyte very distorted.

V Mature, sausage-shaped, rounded ends.

Unlike human falciparum infections, all developmental stages of the gametocyte of this species occur in the peripheral circulation of Aotus. Gametocytes appeared as early as the first day of a patent infection or as late as 13 days after the first appearance of ring-stage trophozoites. 75% of the animals had circulating gametocytes within the first 5 days of a patent infection. The general pattern of gametocyte development is summarized in Figures 3 and 4 which show this pattern in respect to time and level of mosquito infection in Aotus 388, 922 and 971. An analysis of these distributions and those observed in other Aotus listed in Table 3 shows that the very immature stages (I and II) peak earlier than stages III-V and disappear from circulation sooner. Sometimes stages III and IV reach peak numbers the same day as in Aotus 922 while in other animals the peak frequencies were separated by 2-4 days (Aotus 388 and 971).

The ability of gametocytes to infect anopheline mosquitoes was related both to the stage of the gametocyte, its maturity and frequency. Mosquito infections occurred 1-13 days after mature gametocytes first were detected. Peak mosquito infections appeared 3-9 days later. With

the exception of Aotus 593, all peak oocyst counts in anophelines occurred in feeds made 27-31 days after the animal was infected. In most instances, mosquitoes could be infected daily for a 13-23 day period after the first day gametocytes were infective in a monkey.

On several occasions, 2 or 3 species of Anopheles were simultaneously fed on the same monkey to compare the relative susceptibility of different species to infection. Anopheles stephensi and A. balabacensis showed similar levels of infection while A. quadrimaculatus was refractory to infection (Table 4).

Four attempts were made to transmit P. falciparum back to splenectomized monkeys through the bites of Aotus-infected mosquitoes or the inoculation of infected salivary glands. Each Aotus received a subcutaneous or intravenous inoculation of 20 heavily infected salivary glands or a feed of 50-200 infected anophelines. The first 2 experiments were conducted with anophelines infected with the Per. strain while the latter trials were conducted with the Smith strain. During a 50 day period, the Aotus were examined twice weekly to detect patent infection. In addition, a splenectomized chimpanzee, 578, served as a control to verify the infectivity of sporozoites of the Per. falciparum strain in the 2nd experiment. None of the exposed Aotus developed a patent infection. However, chimpanzee 578 became positive 13 days after exposure to infected Anopheles stephensi and attained a peak parasitemia of 320,000/mm^3 on day 21. Immature falciparum gametocytes were observed 2 days later, thus confirming the identity of the transmission.

The facility with which splenectomized Aotus monkeys can be infected with Asian and African isolates of falciparum malaria presents a contrast to the inability of this host to sustain high level infections with New World isolates of this parasite. The ready adaption of 3 new Asian isolates to Aotus affirms this premise. A possible explanation may be that an insufficient number of isolates from different geographical areas in the New World have been inoculated into Aotus. Young (1970) states that in the many attempts to establish P. falciparum of Panamanian origin in the local Aotus, none have become well established. The McClendon strain (isolated from a South Carolina patient in 1940) was not able to infect Aotus (Jeffery, 1969). However, the Central America Malaria Research Station (1970) reported infection of Aotus with falciparum strains from Honduras and El Salvador. These are apparently the only geographical areas from which falciparum isolates have been collected for inoculation into Aotus.

An alternate hypothesis is that falciparum malaria strains from the Western Hemisphere differ at an antigenic or enzymatic level from Old World parasite strains. The 400 or so years which have lapsed since the probable introduction of falciparum strains from Africa via the Spaniards and their slaves (Bruce-Chwatt, 1965); the geographic isolations of clones of malarial parasites in a new environment and the selection within a new set of anopheline vector species are all very

favorable attributes for rapid selection. Hence it would not be unexpected to find such a difference in susceptibility of Aotus to these isolates.

At the Teheran International Congresses on Tropical Medicine and Malaria the question of gametocyte infectivity was raised (Ward, 1969). Two alternate hypotheses were proposed. The first was that gametocyte infectivity to anophelines is a characteristic of certain parasite strains of P. falciparum in Aotus and could probably be observed more often if different parasite isolates were studied. A 2nd hypothesis was that gametocyte infectivity was an easily lost attribute following several consecutive blood transfers in heterologous host systems.

The recorded observations on 2 Vietnam isolates (Brai. and Smith) and 1 from the Philippines (Per.) indicated the gametocytes of an Oriental region falciparum strain can probably produce infective gametocytes in Aotus during the early passage history from man. In the above strains it occurred during the first 3 passage levels. The point of loss of gametocyte infectivity is substantiated by the history of the Smith strain (Table IV). In the latter passages, as more virulent clones of the parasite were selected, insufficient time was usually available for full maturation of gametocytes to stage V levels. Consequently, parasitemia attained such high levels (over 200,000/mm^3) that the Aotus died of acute malarial infection before mosquito exposure could be effected. With further blood passage, the Smith strain may lose the ability to develop even early stage gametocytes as has occurred in certain isolates of the Malayan Camp Strain.

The application of the stabilate principle as proposed by Cunningham and Lumsden (1965) to the low temperature preservation of malarial parasite isolates at passage levels which favor the production of moderate parasitemias at a non-lethal level and maturation of infective gametocytes should prevent the above cited problems. It is quite obvious that a stabilate of 5th passage Smith material will differ from material preserved at the 11th passage.

Immunosuppressant drugs were shown to exert an influence on gametocyte infectivity in the experiment with the Per. strain when parasite dosage and other factors were well controlled. In routine passages, such an effect was difficult to detect due to possible interaction between level of passage and drug dosage. As an example, Aotus 971 (Smith strain) exhibited the best gametocyte donor pattern observed to date despite the lack of Imuran treatment.

The pattern of mature gametocyte production in Aotus as related to mosquito infectivity showed a close parallel to the situation in non-immune humans. Pampana (1963) points out that during the primary attack, gametocytes do not appear before the 7th day of the parasitemia, and usually only are visible 10-12 days after the asexual parasites become patent. Similarly, falciparum gametocytes are not infective to mosquitoes until 2 days after they are observed in the peripheral

circulation of man (Jeffery and Eyles, 1955). Gametocytes remain infective for a period of several weeks in man. Boyd (1949) discusses a patient who produced infectious gametocytes 4 days after the first appearance of this stage, and served as an infectious donor to anophelines for a 19 day period. He points out that mosquitoes were only infected when gametocyte levels were between 40-400/mm^3. These observations closely parallel our results in Aotus. In the Smith strain, where gametocyte maturity was assayed daily, the first mosquito infections occurred at an average of 12.6 days after the infection became patent (range = 7-21 days). The duration of mosquito infectivity averaged 19 days (range = 13-23 days). Similar mature gametocyte levels are observed in Aotus.

The failure of Philippines (Per.) and Vietnam (Smith) strains to undergo sporogonous development in Anopheles quadrimaculatus parallels the experience of Clyde, et al. (1971). As A. quadrimaculatus is a North American vector, and A. stephensi and A. balabacensis Asian vectors, this is not unexpected. This observation has been noted numerous times during the study of falciparum malaria from different geographic areas and has epidemiological significance.

The fact that Contacos and Collins (1968) and ourselves were able to cyclically transmit falciparum infections from Aotus to humans and a chimpanzee respectively indicate that the sporogonous cycle of the parasite is compatible with that in anophelines infected on man. Since Sodeman, et al. (1969) have observed partial development of the exoerythrocytic schizont of P. falciparum in Aotus it is predicted that complete development of this parasite in the hepatic tissues will be demonstrated within the very near future.

2. Attempted immunization of rhesus monkeys against cynomolgi malaria with irradiated sporozoites

Irradiation of erythrocytic stages of human malarial parasites with gamma irradiation have produced avirulent strains that maintain their immunogenic properties. Protection against erythrocytic parasite challenge following various immunization schedules has been demonstrated against Plasmodium falciparum in Aotus trivirgatus. (Sadun et al. 1969). Sporozoite challenge has not been achieved with the Aotus - falciparum malaria system due to an inability of the Aotus liver to sustain complete development of the exoerythrocytic schizont of P. falciparum (Sodeman et al. 1969).

The successful immunization of rodents by irradiated P. berghei sporozoites against experimentally induced sporozoite infection poses the question as to whether or not comparable results may be observed with primates and their malarial parasites. At the present time, the P. cynomolgi - rhesus - anopheline model is the one most comparable to a human infection. The relatively large size of the host, its predicable.

This preliminary experiment was designed to determine whether or not immunization of rhesus monkeys by irradiated P. cynomolgi sporozoites would protect monkeys from sporozoite induced challenge.

Macaca mulatta monkeys weighing 2.3 - 2.8 kg were used. The isolate of P. cynomolgi bastianellii was received from P.G. Contacos in 1968 and has been maintained by mosquito transmission since that time. Anopheles stephensi (India strain) was utilized as the vector.

Fourteen days after an infective blood meal, mosquitoes with heavy salivary gland infections (mean level of infection = 3 + or 10^3 - 10^4 sporozoites) were irradiated at a dosage of 10 kilorads in a "Gammacell 220" cobalt irradiation source which produced essentially pure gamma radiation. The mosquitoes were exposed to the source in pint-sized cardboard containers covered with nylon mesh at a dose rate of approximately 8,700 rads per minute. As a control, mosquitoes of the same age which had received a non-infective blood meal were exposed to a similar radiation schedule.

To circumvent any possible immunogenic effect of mosquito body tissues or salivary gland cells, sporozoites were transferred to monkeys directly by mosquito bite rather than by dissection and trituration of infected salivary glands. Three monkeys (LO-46, LO-47 and LO-489) received irradiated sporozoites and two (LO-88 and LO-89) were bitten by non-infected mosquitoes. One hour after exposure the irradiated mosquitoes and their controls were allowed to feed upon the respective monkeys on days 0, 14 and 42 of the experiment. 25-30 A. stephensi were permitted to feed to repletion on each animal on a given date. If an insufficient number fed, additional mosquitoes were added to the feeding cages. On day 56, all monkeys were challenged with the bite of 15-20 infected anophelines. Blood films were examined from all animals biweekly from the commencement of the experiment until day 60; after which blood smears were made daily. At selected intervals, when gametocytemia was evident, cages of Anopheles stephensi were fed on the monkeys to evaluate gametocyte infectivity.

The results of the experiment are summarized in Table 5 and Figures 5 and 6. Prepatent periods were similar in both groups of rhesus monkeys. The slight delay in LO-46 is within the normal range of variation. The date of peak parasitemia was similar in monkeys receiving irradiated sporozoites. Rhesus LO-489 attained a peak parasitemia only 50% as high as the other animals. This is well with normal variation as levels as low as 50,000/mm^3 are observed in approximately 10% of normal rhesus exposed to P. cynomolgi challenge. Similarly, no appreciable differences were present in days of parasitemia in excess of 1000 parasites/mm^3. This latter attribute is a good indicator of an immune response. An inspection of Figures 5 and 6 reveals no significant differences in the pattern of parasitemia in sporozoite immunized animals as compared to normal animals. On the basis of these observations it is evident that this immunization schedule did not elicit a protective immunity against sporozoite challenge.

Feeding trials of Anopheles stephensi upon treated and control monkeys indicated that both groups were equally efficient as gametocyte carriers

(Table 6). Rhesus LO-46 was a particularly poor gametocyte donor for an unknown reason. This refractoriness has been observed at occasional intervals in other monkeys.

The selection of 10 kilorads as the minimal dose to inactivate P. cynomolgi bastianellii sporozoites was based upon the experience of Warren and Garnham (1970). They observed that X-irradiation of sporozoites of P. c. cynomolgi (M-strain) in Anopheles atroparvus at doses of 5 and 6.5 kilorads resulted in smaller exoerythrocytic schizonts than observed in controls but normal patent infections developed. At a dosage of 10 kilorads, there was no development of either exoerythrocytic schizonts nor a patent parasitemia. Studies with the langur strain of P. cynomolgi indicated a slightly different dose response to irradiation which was suggestive of a different susceptibility to radiation.

It is difficult to extrapolate from the studies of Nussenzweig et al. (1969), who worked with P. b. berghei (NK65 strain) and A/J mice, to the present primate model. Firstly, the rodent malarial parasites which are members of the subgenus Vinckeia have a primary exoerythrocytic cycle of 43-60 hours while primate malarial parasites of the subgenera Plasmodium and Laverania have exoerythrocytic cycles which range from 132 - 300 hours depending upon host and parasite species. This five-fold difference in time span may appreciably influence action of immunogenic materials upon exoerythrocytic parasite development. If the effect of protective immunity is directed towards the invading sporozoite, then this is immaterial.

Another factor in determining effective dosage is the relative difference between the size of a mouse (25 g) and a rhesus monkey (2500g)-a 100 fold difference. Based upon immunological investigations with plague and arboviruses it would be expected that a monkey would require an immunization dose approximately 10 times (one log) greater than required in a mouse (Cavanaugh, D.C. and Schneider I., personal communication). On the basis of the berghei model, which requires the use of 2.5×10^5 irradiated sporozoites to protect one mouse against a P. berghei sporozoite challenge, 2.5×10^6 irradiated sporozoites would be required for a single monkey. Assuming that sporozoites of P. cynomolgi from dissected salivary glands of Anopheles stephensi are as antigenically active as those of P. berghei, 100 heavily infected mosquitoes would have to be dissected for every booster for each rhesus monkey. If this system of feeding mosquitoes directly upon a monkey were to be used, 2,500 infected mosquitoes would be required for each feed.

Inspection of the immunization schedule of the present experiment indicates that each monkey received $7.5\text{-}9 \times 10^4$ irradiated sporozoites or approximately only 1% of those required by the above assumptions.

Over the past few years, the development of mass rearing procedures for Anopheles stephensi by Gerberg et al. (1968) and this laboratory have

facilitated the production of large numbers of mosquitoes with minimal effort and manpower. One individual can rear 100,000 adults weekly. However, the production of large quantities of infected mosquitoes and the extraction of sporozoites still offer problems. A single rhesus monkey can only serve as an efficient gametocyte carrier for a 4-6 day period (i.e., produce oocyst counts of 100 or more which produce 3 + salivary gland infections). Approximately 4,000 anophelines may be fed upon a monkey daily without deleterious effects upon the donor. Of the 4,000 mosquitoes, only 80-90% will have fed, and after a 14 incubation period, 1,500 surviving mosquitoes may be available for sporozoite isolation. It is evident that a large rhesus colony with a rapid turnover of animals is necessary to produce the infected donors.

The application of density gradient centrifugation has provided a new technique for the mass isolation of sporozoites from infected mosquitoes (Chen and Schneider, 1969). With this procedure it is possible to provide large quantities of extremely clean sporozoite suspensions for immunological and biochemical studies. It has been estimated that a technician can daily harvest 2 X 10^7 sporozoites with existing facilities. With some modification in the basic transferring procedures of infected Anopheles, the yield can be appreciably increased. It is not known whether sporozoites processed in such a manner differ immunogenically from material collected by standard procedures.

The fact that immunization with irradiated sporozoites against sporozoite infection was not achieved in this simian model was not unexpected. It is now technically feasible to produce the quantities of infected anophelines required for such studies, and optimistically, within the near future, it should be possible to produce large quantities of immunologically active sporozoites.

Conclusions and recommendations

1. Three new Oriental strains of falciparum malaria (Vietnam Brai.), Vietnam (Smith) and Philippines (Per) were successfully adapted to splenectomized Aotus trivirgatus monkeys. The Smith strain is now being utilized by the WRAIR program on malaria chemotherapy to collect data on a drug resistant parasite strain that could only previously be achieved in human volunteer studies.

2. An immunosuppressant drug, Imuran, was shown to influence gametocyte maturation and subsequent mosquito infectivity in the Per. strain. Mosquito susceptibility to P. falciparum in Aotus is apparently related to early passage history of a particular strain rather than to any inherent qualities of the strain. With increased blood passage, Aotus-adapted strains become more virulent and the infection is fatal before gametocyte maturity is achieved. It is recommended that parasite isolates be preserved as stabilates at low temperatures to provide passage material which favors the production of moderate parasitemias at a non-lethal level and gametocyte maturation.

3. In most respects, with the exception of immature forms in the peripheral circulation, the pattern of gametocyte maturation, infectivity to anophelines and duration of gametocyte infectiousness in Aotus parallels the situation in man. It is concluded that the Aotus system will provide the optimal model for studying human falciparum malaria upon the demonstration of a complete exoerythrocytic cycle in the host.

4. Immunization of rhesus monkeys with $7.5 - 9 \times 10^4$ irradiated sporozoites of Plasmodium cynomolgi did not protect monkeys from sporozoite challenge. It is estimated that 2.5×10^6 irradiated sporozoites will be required to immunize a single rhesus. This can be accomplished through refinements in procedures used for harvesting large quantities of sporozoites from infected mosquitoes.

TABLE 1

Infection of splenectomized Aotus trivirgatus with Plasmodium falciparum (Vietnam, Brei., strain)

Passage	Aotus no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm^3	Day of first gametocytes	No. of mosquito pools infected / No. of pools fed	Day of death
1[1]	G-359	10	24	1,662,000	17	0/8	24
2	G-396[2]	11	23	212,000	11	0/11	-
2	G-985	13	23	1,673,000	15	0/3	24
3	G-984[3]	7	7	100,170	3	2/11	-
3	G-992[4]	2	13	1,320,000	3	-	14
4	428[5]	5	14	1,140,000	0[6]	-	14

[1]One other Aotus inoculated did not exhibit patent parasitemia.

[2]Treated with quinine on day 23.

[3]Treated with Imuran, 2 mg/kg X 4 days.

[4]Treated with Imuran, 3.3mg/kg X 3 days.

[5]Treated with Imuran, 1 day at 5 mg/kg and 1 day at 2.5 mg/kg.

[6]Gametocytes not observed.

TABLE 2

Infection of splenectomized Aotus trivirgatus with Plasmodium falciparum (Philippine, Per strain)

Passage level	Aotus no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm^3	Day of first gametocytes	No. of mosquito pools infected / No. of pools fed	Day of death
1	G-388	14	26	752,400	21	0/8	27
1	G-067	17	27	166,650	24	1/24	-
1	G-967[1]	12	13	25	-	-	-
2	G-983	13	40	667,500	14	0/6	41
3	H-233	5	22	490,140	16	3/9	-

[1] Non-splenectomized

[2] Treated with Imuran, 5 mg/kg X 6 days and 2.5 mg/kg X 5 days.

TABLE 3

The course of infection of Plasmodium falciparum (Smith strain) in splenectomized Aotus trivirgatus and subsequent susceptibility of anopheline mosquitoes of falciparum gametocytes in this host

Passage	Aotus no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm^3	Day of first gametocytes	Day of first mature gametocytes	No. days 1000 mature gametocytes/mm^3	Day of first mosquito infection	Day of last mosquito infection	No. of mosquito pools infected / No. of pools fed
1	546[1]	12	14	36,570	14	22	0	-	-	0/10
2	383	2	20	22,750	14	19	5	20	39	12/18
3	700[2]	7	20	17,650	20	29	0	-	-	0/2
4	387[2]	4	14	203,075	4	13	17	18	39	9/29
5	484[2]	1	11	177,800	2	8	12	21	38	9/21
6	503	3	61	67,080	3	Not determined	Not determined	53	73	5/21
6	927[2]	7	20	299,720	7	1	Not determined	-	-	0/9
7	922[2]	2	19	102,110	15	17	20	38	51	15/26
7	978[2,3]	17	17	355,600	14	23	0	-	-	0/1
8	923[2]	9	49	142,430	23	31	1	-	-	0/5
9	983[4]	18	15	882,650	4	-	0	-	-	-
10	971	2	17	171,700	12	18	21	21	34	13/31
11	S-2[5]	7	10	377,190	8	-	0	-	-	-
		4								

[1] Aotus 546, which was infected at the same time did not develop a patent parasitemia

[2] Human administered 5 mg/kg daily for 10-40 days.

[3] Died day 24.

[4] Died day 18; mature gametocytes not observed.

[5] Died day 12; mature gametocytes not observed.

TABLE 4

Comparative susceptibility of Anopheles species to Smith and Per. strains of falciparum malaria in Aotus trivirgatus

Aotus no. & strain	Day of Feed	Mean no. malarial oocysts /midgut		
		A. stephensi	A. balaba-censis	A. quadri-maculatus
971 (Smith)	21	0.7	0.8	0
	24	1.6	10.8	0
	25	1.4	1.5	0
	26	6.3	1.9	0
254 (Per.)	40	20.7	-	0
	41	0.8	0.2	0
	55	1.8	-	0
	56	3.7	-	0

TABLE 5

The course of infection in rhesus monkeys immunized with Plasmodium cynomolgi irradiated sporozoites

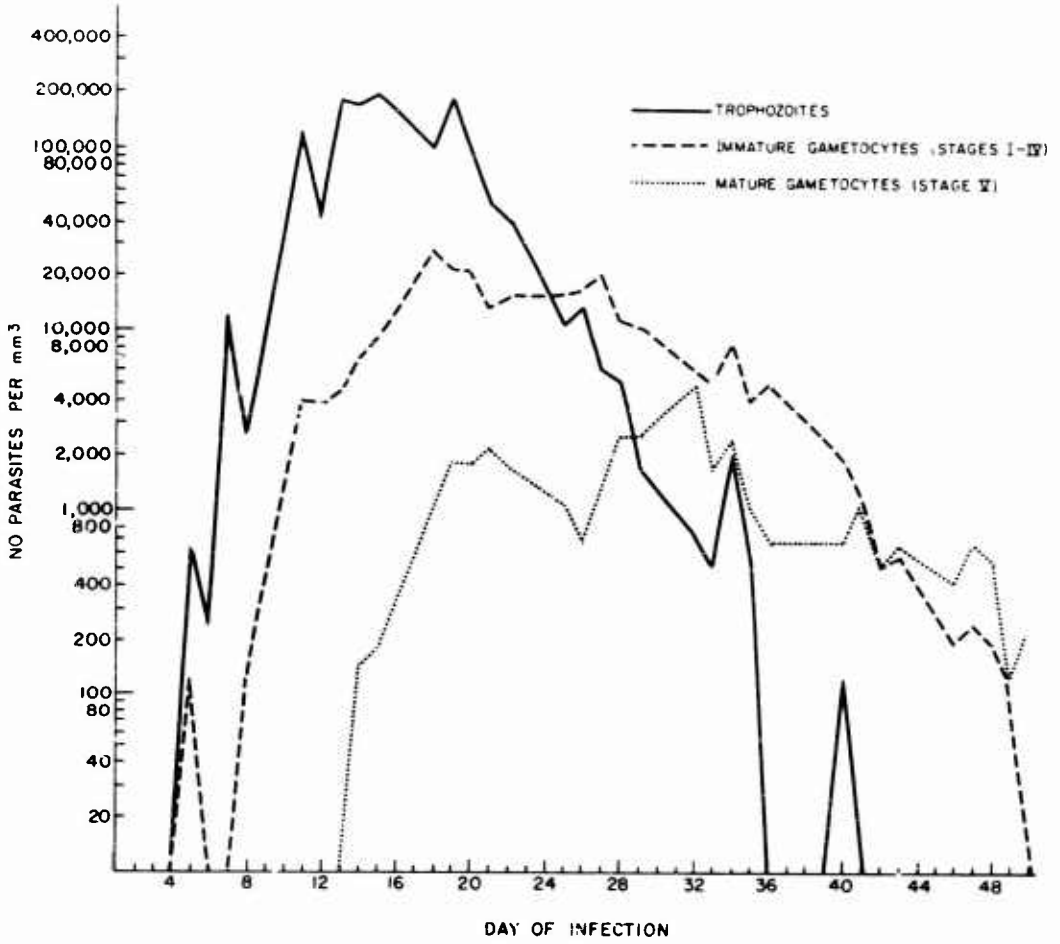
Group (Treatment)	Animal number	Prepatent period (days)	Day of peak parasitemia	Maximum no. parasites/mm^3	Days parasitemia 1000/mm^3
Irradiated mosquitoes (Normal)	LO-88	9	16	339,000	24
	LO-89	9	17	334,800	22
Irradiated mosquitoes (Sporozoites)	LO-46	12	17	361,200	19
	LO-47	8	11	200,000	21
	LO-489	9	16	158,400	16

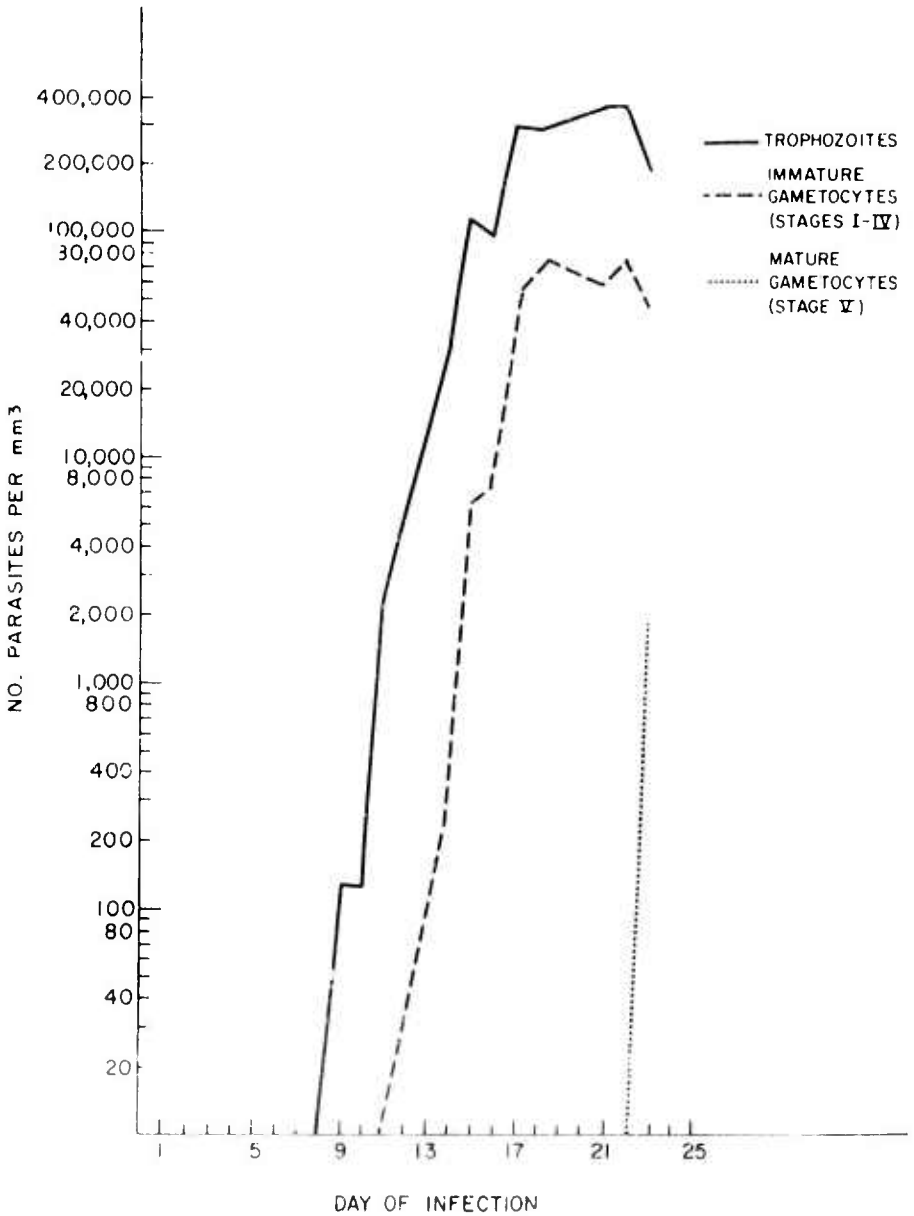
TABLE 6

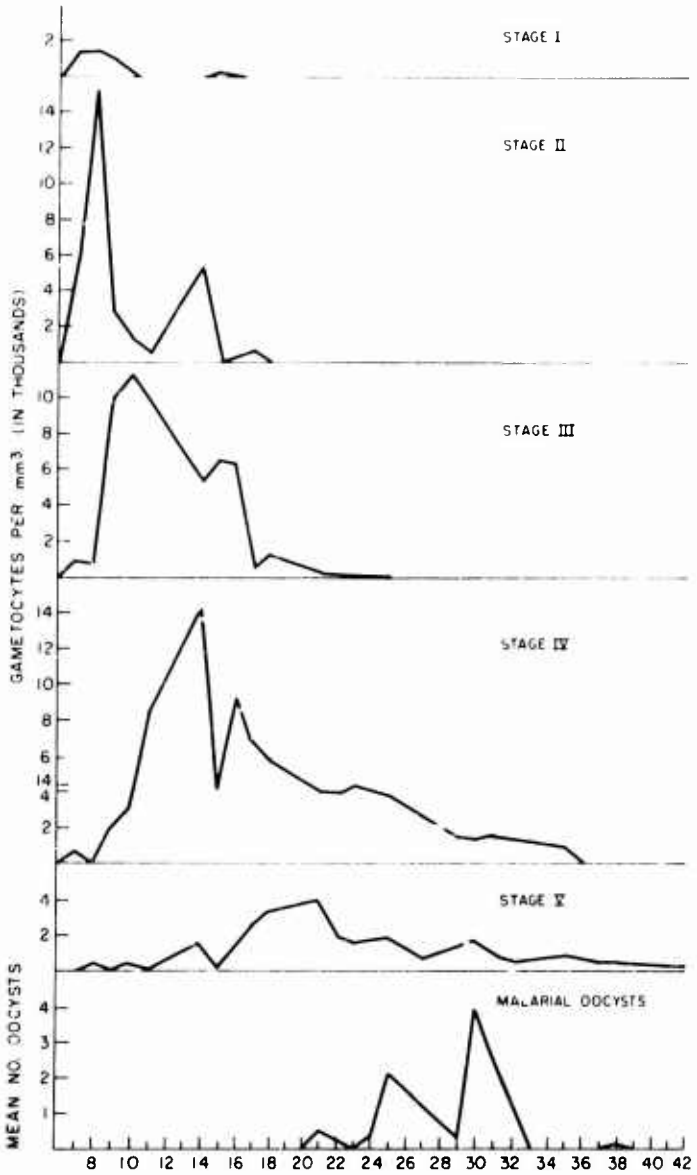
Susceptibility of Anopheles stephensi to Plasmodium cynomolgi bastianellii gametocytes from immunized rhesus monkeys

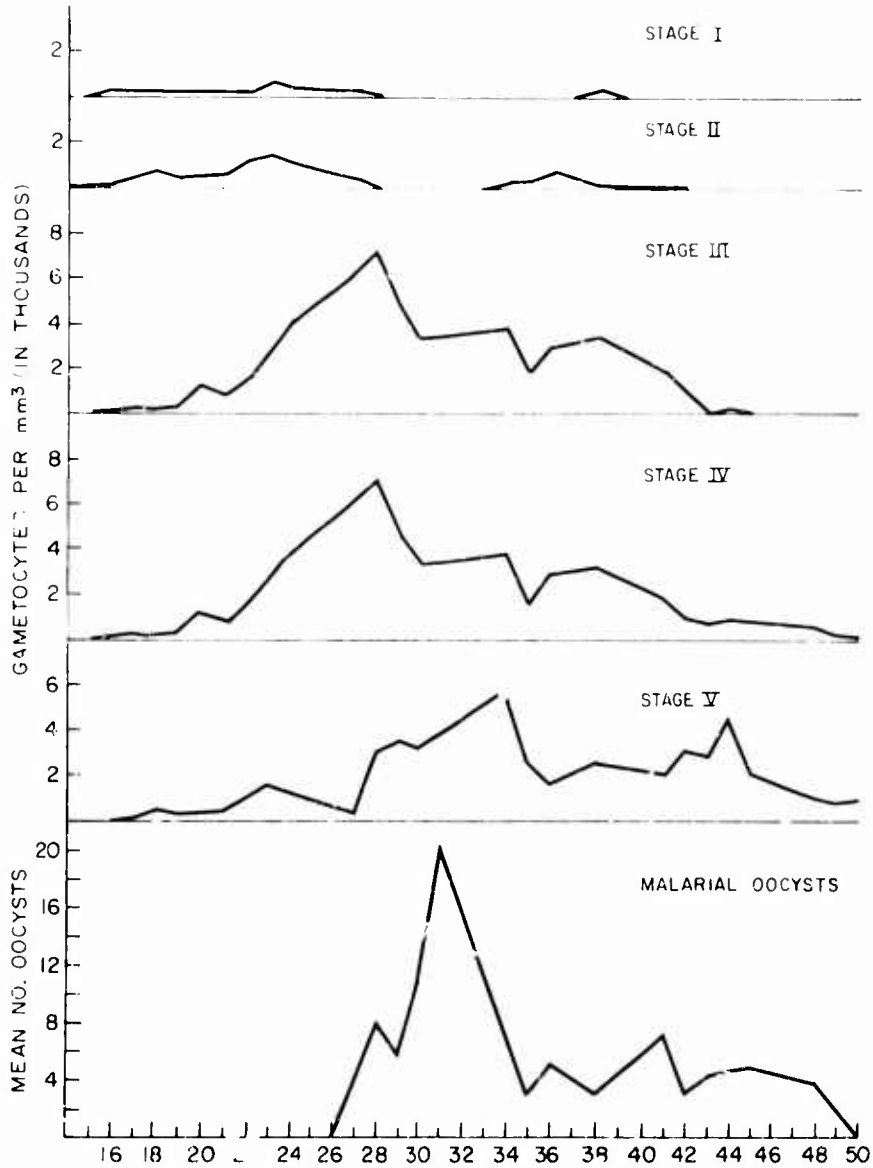
Group (Treatment	Animal no.	Peak mean no oocysts/ mosquito midgut	Days after challenge
Irradiated mosquitoes (Normal)	LO-88	106.1	16
	LO 89	115.8	17
Irradiated Mosquitoes (Sporozoites)	LO-46	1.6	19
	LO-47	266.5	6
	LO-489	102.8	26

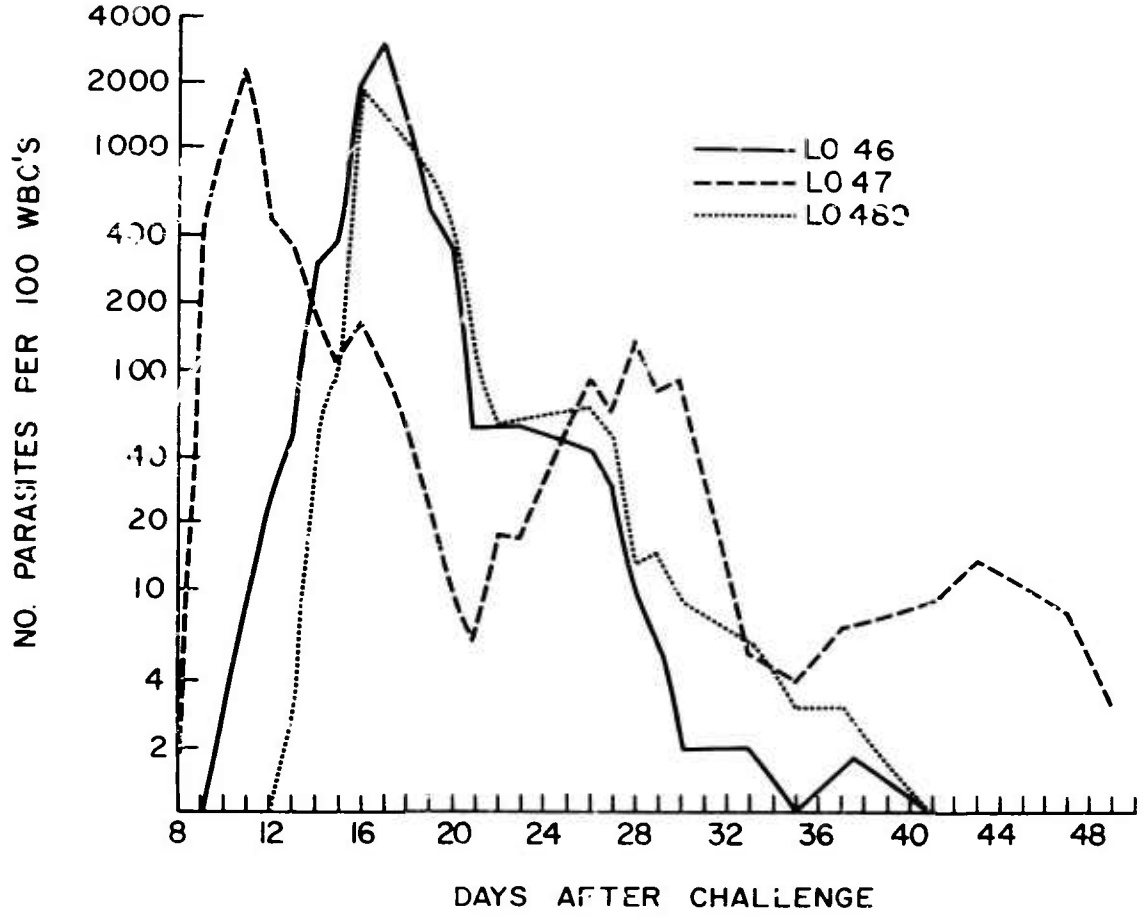
FIGURES

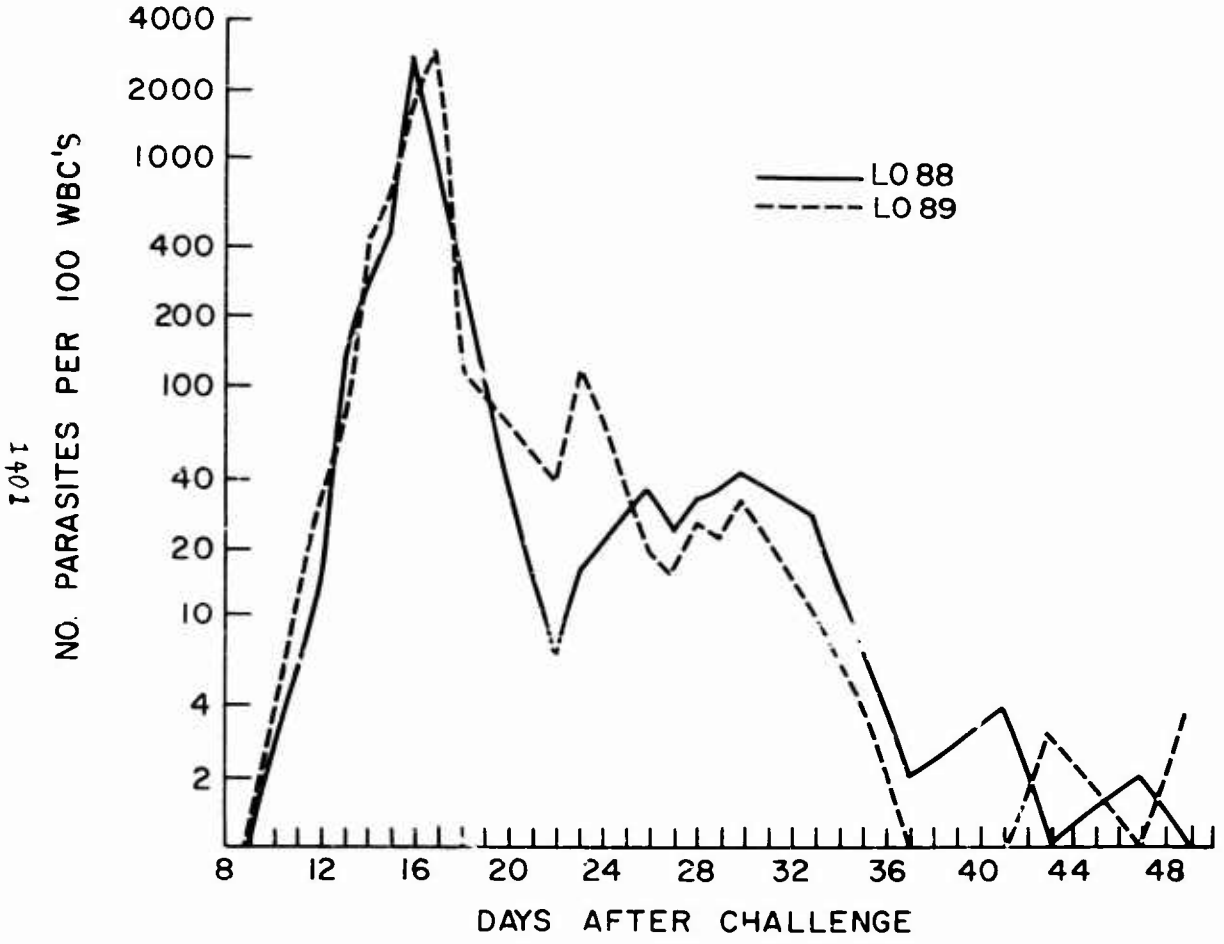

TROPHOZOITES
IMMATURE GAMETOCYTES (STAGES I-IV)
MATURE GAMETOCYTES (STAGE V)
NO PARASITES PER mm3
400,000
200,000
100,000
80,000
40,000
20,000
10,000
8,000
4,000
2,000
1,000
800
400
200
100
80
40
20
4
8
12
16
20
24
28
32
36
40
44
48
DAY OF INFECTION


400,000
200,000
100,000
30,000
40,000
20,000
10,000
8,000
4,000
2,000
1,000
800
400
200
100
80
40
20
NO. PARASITES PER mm3
1
5
9
13
17
21
25
DAY OF INFECTION
TROPHOZOITES
IMMATURE GAMETOCYTES (STAGES I-IV)
MATURE GAMETOCYTES (STAGE V)


STAGE I
STAGE II
STAGE III
STAGE IV
STAGE V
MALARIAL OOCYSTS
GAMETOCYTES PER mm3 (IN THOUSANDS)
MEAN NO. OOCYSTS
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
DAY OF INFECTION


STAGE I
STAGE II
STAGE III
STAGE IV
STAGE V
MALARIAL OOCYSTS
GAMETOCYTES PER mm3 (IN THOUSANDS)
MEAN NO. OOCYSTS
DAY OF INFECTION


4000
2000
1000
400
200
100
40
20
10
4
2
NO. PARASITES PER 100 WBC'S
8
12
16
20
24
28
32
36
40
44
48
DAYS AFTER CHALLENGE
LO 46
LO 47
LO 48?


4000
2000
1000
400
200
100
40
20
10
4
2
NO. PARASITES PER 100 WBC'S
LO 88
LO 89
8
12
16
20
24
28
32
36
40
44
48
DAYS AFTER CHALLENGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 124 Biological studies of mosquito malaria infection and transmission

Literature Cited

References:

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Publications:

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6515	72 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
71 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	63713A	3A663713D829	00	125
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG114(f)			

11. TITLE (Provide with Security Classification Code)

(U) Taxonomy and Ecology of Disease Bearing Mosquitoes of Southeast Asia (09)

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: Not Applicable 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: 72	1	35
FISCAL YEAR CURRENT: 73	1	35

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

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

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Div of CD and I
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Eldridge, LTC B. F.
TELEPHONE: 202 - 576-3719
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Reinert, MAJ J.F.
NAME: Harrison, CPT B.A. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Anopheles; (U) Aedes; (U) Mosquitoes; (U) Malaria; (U) Arboviruses; (U) Disease Vectors

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

23. (U) To produce keys, guides, and other guides for identification of mosquitoes which are vectors of diseases of military importance. Emphasis is on vectors of malaria and arbovirus diseases such as dengue. Also, to obtain data on the ecology, biology, and disease transmission potential of these mosquitoes.

24. (U) Mosquitoes are collected by military and civilian cooperating agencies and are forwarded to a joint WRAIR-Smithsonian Institution team for identification. This team intensively studies these collections, as well as collections in established museums, and publishes keys, guides, and other identification aids which are returned to SE Asia for use by entomologists engaged in control and survey operations. Team also accumulates and makes available disease transmission and biological data.

25. (U) 71 07 - 72 06. Progress continues on the revision of the subgenus Anopheles of Thailand, with 70% of the specimens present in the U.S. National Museum collection identified and integrated into the study collection. Thirteen of 27 keys are complete, with the remaining 14 needing only final review and editing. Eighty-five illustrations have been completed. Revisions of the subgenera Aedimorphus and Ayurakitia of the genus Aedes are complete. Eggs of the subgenera Aedimorphus and Diceromyia have been described. A revision of the subgenus Aedimorphus of the Oriental, Australian, and Pacific regions, with a description of the geographical distribution of the world species will be finished soon. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria investigations

Work Unit 125 Taxonomy and ecology of disease bearing mosquitoes of Southeast Asia

Investigators
Principal: LTC Bruce F. Eldridge, MSC
Associate: MAJ John F. Reinert, MSC; CPT Bruce A. Harrison, MSC; Ronald A. Ward, Ph.D.; John E. Scanlon, Ph.D*

Description

Mosquitoes are collected in Southeast Asia by cooperating military organizations and other groups. Other supplementary materials are obtained from existing collections in museums and other institutions. After study taxonomic revisions and descriptions are prepared for all of the mosquitoes of Southeast Asia, with emphasis on the species of medical importance. Sections of the work are published as completed, and keys of value to military entomologists are prepared as required. The eventual aim of the project is the publication of a series of monographs completely describing the mosquitoes of the area. In addition, collection and ecological data are recorded later for collation with published data on the ecology of the various species. Short term field studies are conducted in endemic areas for vector incrimination and collection of specimens for laboratory analysis. Laboratory crosses are made among members of wide-ranging species or related species to detect the presence of mating barriers which might affect the specific status of various taxa. Studies under this work unit are performed in conjunction with the Smithsonian Institution under U.S. Army Medical Research and Development Command Contract MD 2672. This report covers the in-house portion of the work only.

Progress

1. Anopheles of Thailand

The main effort since the last reporting period has been in the revision of the subgenus Anopheles of Thailand. Approximately 70% of the Anopheles specimens accrued at the Smithsonian Institution in the SEAMP collection have been identified and integrated into the collection. Thirteen of the 27 keys needed to complete the monograph of this subgenus have been finished; the remaining 14 need only a small amount of review and editing. Eighty-five plates of illustrations are completed for this group. Thirty-three of these have been thoroughly checked for errors and have been returned to the artists for correction.

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*University of Texas, School of Public Health, Houston

2. Studies of the genus Aedes

The revision of the subgenus Aedimorphus of Southeast Asia has been completed and the manuscript submitted for publication. A revision of the subgenus Ayurakitia of Thailand has also been completed. A description of the eggs of the subgenera Diceromyia and Aedimorphus is nearing completion. The revision of the subgenus Aedimorphus in the Oriental, Australian, and Pacific Island regions is nearing completion, plus a study of the geographic distribution of all species of the subgenus on a world-wide basis. A study of the female genitalia of the entire genus has been initiated.

Conclusions and Recommendations

Significant progress has been made toward the revision of the genera Anopheles and Aedes of Southeast Asia. Studies of several subgenera of Aedes have been completed, including comprehensive reviews of various aspects of their biology and distribution from a world-wide basis. Additional research should be done to complete the revision of the subgenus Anopheles.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria investigations

Work Unit 125 Taxonomy and ecology of disease bearing mosquitoes of Southeast Asia

Literature Cited

References: None

Publications:

1. Harrison, B.A., P. Boonyakanist and K. Mongkolpanya: Biological observations on *Aedes seatoi* Huang in Thailand with notes on rural *Aedes aegypti* (L.) and other *Stegomyia* populations. J. Med. Entomol. 9: 1-6, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6516	72 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. DOWN INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	126
b. CONTRIBUTING				
c. [redacted]	CDOG114(f)			

11. TITLE (Precede with Security Classification Code)

(U) In Vitro Cultivation of Mosquito Tissues and Malarial Parasites (09)

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. DAT. I/EFFECTIVE: Not Applicable — 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 72	2	30
	CURRENT 73	2	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D.C. 20012

ADDRESS:

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

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of CD and I

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic institution)
NAME: Schneider, I. Ph.D.
TELEPHONE: 202 - 576-3049
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code) (U) Aedes; (U) Anopheles; (U) Culex (U) Mosquitoes; (U) Malaria; (U) Tissue Culture; (U) Immunology

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 reliable in vitro procedures by which large quantities of relatively pure malarial sporozoites can be produced for malaria vaccine development studies. Also, to develop mosquito tissue culture systems for studies on pathogen invasion and growth dynamics in invertebrate tissue.

24. (U) Development of culture media which will support growth of invertebrate stages of malaria parasites. Development of various techniques for the isolation and purification of individual stages of parasite. Evaluation of mosquito life cycle stages for suitability for establishing primary cultures.

25. (U) 71 07 - 72 06. Methods have been perfected which virtually assure the establishment of cell lines from the embryonic and early larval stages of various mosquito species. Diploid cell lines from Aedes taeniorhynchus and Culex salinarius were established during the past year and an incipient line of Culex restuans initiated. Plasmodium cynomolgi oocysts responded more favorably, by growth and/or differentiation, when cultured together with Anopheles stephensi cells than in medium alone. Primary cultures were slightly more effective in this respect than were cells from an established line. Attempts are continuing the separate P. cynomolgi sporozoites from homogenized mosquitoes and gametocyte-infected blood cells from the other erythrocytic stages on density gradients. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*Available to contractors upon requestor's approval

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

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 126 In vitro cultivation of mosquito tissue and malarial parasites

Investigators
Principal: Imogene Schneider, Ph.D.
Associate: CPT Jay Abercrombie, MSC; SP4 David Spooner

Description

This investigation involves three major areas: (1) to design a culture system which will support the growth and development of the insect cycle of malaria parasites with the eventual aim of collecting large numbers of sporozoites free of mosquito tissue, (2) the use of density gradients to isolate the sporozoite stage from mosquitoes and the gametocyte stage from the other blood forms and (3) the development of primary and established cell lines from a selected number of mosquito species. The sporozoites obtained directly or indirectly through the first two approaches are to be used as the source of malarial antigen for immunological studies. The mosquito cell lines are to be utilized in both malaria and arbovirus research.

Progress

1. Cultivation of malaria parasites and mosquito tissues

The purpose of this study was to compare the development of Plasmodium cynomolgi in primary cultures versus an established cell line of the anopheline host as well as to determine whether cultures initiated from adult tissues elicited a more favorable response on the part of the parasites than did those from immature stages.

P. cynomolgi bastianelli was maintained by both sporozoite and blood transfer in rhesus (Macaca mulatta) monkeys. Adult female Anopheles stephensi (India strain) served as the invertebrate hosts.

The cell line of A. stephensi was initiated in 1968 and has since been maintained in continuous culture by weekly passage. Primary cultures of A. stephensi were initiated from trypsinized fragments of neonate larvae or adult ovaries. The cultures were allowed to develop to the point of active cell migration and division, usually 4-7 days, before the parasites were introduced.

Since it was not technically feasible to dissect out oocysts less than 20μ in diameter, entire midguts containing the oocysts were placed in culture until the latter were 6 days old. From day 9 on, the oocysts could be excised without any detectable extraneous material. Some midgut

tissue was deliberately left attached to the 6 and 8 day oocysts to avoid dissection injury. A single midgut or from 5-15 oocysts were placed in each culture.

Separation of erythrocyte from leukocytes was readily achieved by passing the blood through packed, powdered filter paper columns. However, attempts to separate the various blood forms of P. cynomolgi in different fractions of a serum albumin gradient were unsuccessful. The majority of gametocyte-infected cells were found in the two lightest fractions but so were the majority of schizonts.

Although exflagellation may be seen within 10-15 minutes after freshly drawn blood is placed in culture, this process was not observed after aliquots of cells from the lightest fractions of the gradient were placed in vitro with medium alone or with A. stephensi cell line. After 24 and 48 hours, cells from both series were fixed with methanol and stained with Giemsa. There was no evidence of zygote formation or ookinete development in any of the 14 preparations examined.

Four day old oocysts showed the least favorable response to in vitro culture. Such oocysts survived less than 24 hours when placed in medium alone and showed degenerative changes in both primary cultures and in the established cell line within 48-72 hours. Neither growth nor any visible signs of internal development took place. However, this test may not have been too valid as the inclusion of the entire midgut in a culture necessitated using fungazone, in addition to penicillin and streptomycin, to control contaminants. There is evidence that some insect cells are markedly sensitive to this antibiotic and it may have had an adverse effect, visually undetectable, on the midguts which in turn affected the oocysts.

Based on measurements of 55 individual oocysts, the diameter of 6 day oocysts when placed in culture averaged 18μ. Although a few oocysts showed no evidence of growth the majority of those cultured in the presence of mosquito cells increased in size. With a period of 72 hours, these increases ranged from a modest 3-4μ to a fairly substantial 13μ. Further growth did not take place even though some of the oocysts showed no signs of deterioration for as long as a week thereafter. With the exception of one culture, there was no evidence that the oocysts responded more favorably to the cells of a primary culture than to those of the established line. This culture consisted of 12 oocysts and numerous cell colonies from a larval explant. Cleavage of the cytoplasm took place in all but one of the oocysts and after 72 hours their appearance was similar to that of an 8 day oocyst although smaller than normal. At this point the medium was renewed but rather than stimulating further growth it had quite the reverse effect and the culture was discarded.

The growth of 8 day oocysts was quite limited, usually to 5μ or less. However, cytoplasmic cleavage took place in most of the oocysts including those in medium alone. If the oocysts were cultured in the

presence of A. stephensi cells, cleavage of the cytoplasm was followed by the appearance of immature sporozoites. Most of these did not attain a mature shape or size but instead were foreshortened and resembled broken signets. The oocysts did not rupture spontaneously but if external pressure was applied the liberated sporozoites made active flexing movements.

By day 9, immature sporozoites had already budded from the sporoblast cores and required only an additional 24-48 hours to mature. Freed sporozoites were present in almost all of the cultures containing cells, usually within 24 hours. Approximately 40 percent of the oocysts cultured with the cell line did not rupture whereas the figure for both types of primary cultures was about 25 percent.

On day 10, most oocysts contained virtually mature sporozoites and needed less than 24 hours to rupture. About 30 percent of the oocysts did not rupture in cultures containing only medium as compared to less than 5 percent in those containing cells. There was no difference in this percentage between the established line and the primary cultures.

2a. Isolation of the gametocyte stage from the other blood forms and from uninfected erythrocytes

Infected, heparinized or defibrinated blood was layered on the bottom of discontinuous bovine serum albumin gradients with specific gravities ranging between 1.068 and 1.108 gm/cm^3. Before use, the albumin was routinely dialized in the cold and the osmolarity adjusted to approximately 290 mOsm/l with appropriate amounts of salts and dextrose. The gradients were spun at 39,000 rpm for 60 minutes in a Spinco centrifuge with a SW-50.1 head. Since most of the gametocyte-infected cells were found in the uppermost fractions, as were the schizonts, continuous linear gradients were prepared using the same densities at the top and bottom. Three bands were found near the top of the gradient (at about 1.072-1.076) but the separation between them was not sufficient to withdraw each fraction without some mixing taking place. Increasing the differences in the densities of the lightest and heaviest fractions may resolve this problem but even so the outlook is not too encouraging in view of the results obtained when crude fractions were placed in culture (see preceeding section).

2b. Isolation of malaria sporozoites on density gradients

Attempts to isolate P. cynomolgi sporozoites from homogenized A. stephensi on density gradients have met with only limited success. The main obstacle seems to be the osmotic fragility of the parasites. This is readily demonstrated by placing macerated, infected glands in sitting drops containing serum albumin-Fraction V and Renografin (the mixture found most effective in isolating infectious P. gallinaceum sporozoites from Aedes aegypti) in the percentages used for the top and bottom fractions of the gradient. At the higher density, the sporozoites lose their motility and become very fuzzy in outline within 30 minutes of being exposed to the mixture at 4°C. In the lighter fraction, the

sporozoites appear normal for approximately two hours. If lower percentages of the compounds are used, the densities are not optimal and isopycnic gradient centrifugation is not achieved. Since the separation is dependent upon both the relative densities and sedimentation rates, the sporozoites do not peak in any one fraction or cluster of fractions but are instead found throughout much of the gradient. Increasing the speed of centrifugation as well as the densities of the various fractions while decreasing the length of time for a gradient run may result in tighter banding.

Attempts to use other materials to form the gradients (e.g., sucrose, Ficoll and Plasdone) have been even less successful. The principal drawback in using sucrose is its high osmotic pressure. The latter two compounds proved unacceptable due to their high viscosities at the appropriate densities.

3. Establishment of cell lines from various mosquito species and other dipterans

It is now possible to establish on a routine basis cell lines from many mosquito species, using the neonate larval stage as the source of cells. For two genera, Aedes and Culex, the interval between initiating the primary culture and the subsequent transition to subculturing now takes as little as three days. For other genera, such as Anopheles, this interval may require 10-21 days. This difference is primarily a reflection of the relative merits of the culture media employed rather than any any inherent differences in the ability of the cells to adapt to in vitro conditions.

During the past year, cell lines have been established from Aedes taeniorhynchus and Culex salinarius. One incipient cell line from Culex restuans has also been initiated. In each instance, the cells grow in a monolayer, reach a logarithmic growth phase between days 3 and 4, and are predominently diploid.

To determine whether the technique used for mosquito species could be applied to other dipterans, embryonic and larval fragments of Drosophila melanogaster and Glossina austini and/or G. morsitans, respectively, were subjected to treatment with trypsin and placed in culture. The former species was chosen because of its almost unique status as the organism of choice in studying many aspects of developmental biology and genetics. The latter two species because of their usefulness as models for the transmission of African trypanosomes under laboratory conditions.

So far, it has not been possible to maintain Glossina cells in culture for more than two months and all attempts to subculture the cells have been unsuccessful. In contrast, three diploid lines of D. melanogaster were established with the length of time between primary culture and subculture varying from eight months for the first line to three weeks for the third. In response to requests from other investigators,

some 55 dipteran cell cultures were shipped this past year to many sections of the U.S. as well as to Canada, Brazil, England, Germany, India and New Zealand. The requests were fairly evenly divided between the mosquito and Drosophila cell lines.

Conclusions and recommendations

1. P. cynomolgi cocysts responded more favorably, by growth and/or differentiation, when cultured together with A. stephensi cells than in medium alone. Although primary cultures from adult ovaries did not appear to serve as a better supportive system than those from an immature stage, this may have been a reflection of their relative growth rates since explants of the latter invariably grew much faster than those of the former. Emphasis should be placed on minimizing this difference by concentrating on the cultivation of cells from various adult tissues or organs, particularly from the midgut. The use of axenically reared anophelines would reduce or eliminate the need for antibiotics.

2. Efforts to separate blood cells containing gametocytes from those containing schizonts were only partially successful. More resolution might be obtained by altering the relative densities in the gradient or by employing two successive gradients.

3. The isolation of P. cynomolgi sporozoites from homogenates of infected A. stephensi mosquitoes on density gradients has not been fully realized. The use of ultracentrifugation should be explored and further attempts made to find a better supportive medium for the gradient.

4. Methods have now been perfected which virtually assure the establishment of cell lines from the embryonic and larval stages of various mosquito species. Efforts should be directed towards obtaining equally successful cell cultures from the adult stages.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 126 In vitro cultivation of mosquito tissue and malarial parasites

Literature Cited

References: None

Publications:

1. Schneider, I.: Cultivation of Dipteran Cells in vitro. Curr. Topics Microbiol. Immunol. 55:1-12, 1971.

2. Schneider, I.: Cell lines derived from late embryonic stages of Drosophila melanogaster. J. Embryol. Exp. Morph. 27:353-365, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	127
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (F)			

11. TITLE (Precede with Security Classification Code)

(U) Test Systems for Plasmodium falciparum (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR CURRENT 73	2	70

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunity; (U) Chloroquine; (U) Gamma Globulin; (U) Isotope; (U) Susceptibility; (U) Owl Monkey

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

23(U) Study susceptibility of chimpanzees and other primates to infections of human malaria. Study the characteristics of drug resistant strains, provide high density of parasites for morphological and biochemical studies. Conduct physiological and pathological studies of malaria and provide test animals for chemotherapeutic and immunological investigations.

24(U) Infect splenectomized, drug treated chimpanzees and other primates with plasmodia of human origin. Observe the extent and duration of parasitemias, study and response of different strains to chemotherapy, study susceptibility to reinfection with homologous and heterologous strains.

25(U) 71 07 - 72 06 Electron microscopy of erythrocytes infected with Plasmodium falciparum revealed electron dense, cone-shaped excrescences (about 40 millimicrons high and 100 millimicrons wide) along the plasma membrane. These excrescences formed focal junctions with the plasma membrane of reticular cells of the spleen. The resulting complexes appear to be responsible for sequestering erythrocytes infected with P. falciparum in the host's spleen. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* 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 58 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 127, Test Systems for Plasmodium falciparum

Investigators.

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For many years the host specificity of Plasmodium falciparum served as a barrier to laboratory experimentation involving this parasite. Monkeys (Alouatta sp.) were successfully infected with P. falciparum in 1934, but this human parasite has been transmitted and maintained in sub-human primates only recently. Splenectomized chimpanzees (Pan satyrus) and gibbons (Hylobates lar) are susceptible to both blood and sporozoite induced infections of P. falciparum. Several other old world species have been designated as potential hosts for this parasite. In addition, the following new world species of primates have been used as experimental hosts for P. falciparum: the owl monkey (Aotus trivirgatus), the marmoset (Saguinus geoffroyi), the white faced Capuchin (Cebus capucinus), and the squirrel monkey (Saimiri sciureus).

Two reviews of human malaria in sub-human primates have recently been published. The owl monkey appears to be the most acceptable model for the study of falciparum malaria. In contrast to many of the other species susceptible to P. falciparum, no natural malaria infections have been reported in owl monkeys, thus eliminating the need for antimalarial treatment prior to their use. Since the nonsplenectomized owl monkey is also highly susceptible to a number of strains of P. falciparum, there is usually no need for surgical procedures or immunosuppressive treatments to increase their susceptibility.

In the experiments reported here parasitologic, hematologic and serum biochemical determinations were conducted in owl monkeys infected with P. falciparum in an attempt to better define the sequence of pathologic changes associated with this infection in the monkey and to compare these findngs with those described for falciparum malaria in human patients.

Experimental Animals

The source, care and maintenance of Aotus monkeys used in our laboratory has been reported.

Parasites and Inoculation

The Camp. strain of *Plasmodium falciparum* was used for all experiments and its history in man and subhuman primates since isolation has been described. Parasite densities were calculated from erythrocyte counts and the percentage of parasitized erythrocytes observed on Giemsa stained blood smears. Parasitized blood was diluted with sterile physiologic saline and injected into a superficial vein in the back of the leg. Parasitized blood was shell frozen in 5% glycerol and kept at -70 C.

Sample Collection

Blood samples were obtained from the femoral vein and mixed with EDTA for hematologic procedures which were completed on the same day. Serum for biochemical determinations was collected from clotted blood and frozen at -70 C within 2 hours after the blood samples were taken. Usually parasitemias were monitored daily by examining thick and thin blood smears made from the ear of each experimental animal.

Experimental Design

Experiment I compared values obtained from non-infected monkeys with those of monkeys heavily infected with *P. falciparum*. Animals which were inoculated with relatively large numbers of parasitized cells ($1\text{-}5 \times 10^8$) were used to maintain the *P. falciparum* parasites and to provide material for antigen preparations. Samples of blood were taken from these monkeys for hematologic and biochemical determinations before necropsy, and impression smears were made from cut sections of brain, heart, lungs, liver, spleen, kidneys and adrenal glands during necropsy. Portions of organs and sections of gut were also preserved in buffered 10% formalin for histopathology.

Experiments II and III were designed to study serial samples of blood and serum from infected monkeys to relate the observed changes in Experiment I with levels of parasitemia. Relatively small inocula (1.5×10^4) were used in these two experiments in order to extend the period of observation. One ml of blood was obtained from each animal every third day in the second experiment, while weekly bleedings of 2 ml were obtained from monkeys in the third experiment. Control animals were injected with saline in both experiments and were bled at the same intervals. Some monkeys were killed early in the course of

infection while others were not killed until heavy parasitemias had developed. In experiments I and II moribund animals were bled until death. Necropsy in all monkeys of experiment III was done after the animals were given pentobarbital anesthesia. Impression smears and tissue sections were taken as in the first experiment and portions of the various organs were also frozen for histochemical studies.

Hematologic and Biochemical Techniques

Standard hematologic methods were used and have been reported. Serum glutamic pyruvic transaminase (SGPT) glutamic oxaloacetic transaminase (SGOT), glucose, and total protein levels were determined by ultra micro methods. Uric acid determinations were performed by an ultramicro modification of Carraway's method. Levels of urea nitrogen were determined by a modified urease method. Serum electrophoresis on cellulose acetate strips were performed by the Beckman Microzone technique using Beckman B-2 buffer.

Virulence

The results of P. falciparum infections initiated with different numbers of parasitized RBC's are summarized in Table 1. Each of the 68 animals receiving at least 1 X 10^8 parasitized cells developed a patent infection in a short period and most developed high parasitemias. Three monkeys in this group did not have high parasitemias and survived. One of these animals died 60 days after challenge, apparently from causes other than malaria while the remaining two survivors are still in our colony more than a year after challenge. All the monkeys in group II and 10 of 12 monkeys in group III had patent infections. The prepatent period and survival time was usually inversely proportional to the size of the inoculum although the duration of patency was generally similar for all animals in which the infection was lethal. Infections in 8 of 11 monkeys inoculated with glycerolized blood stored at -70 C became patent and two of these monkeys survived after relatively long periods.

Clinical Course of Infection

The clinical course of P. falciparum infection in Aotus monkeys as previously reported in an earlier series of animals generally applies to our experience. The monkeys remained alert and consumed food and water until the final days of infection. The first manifestations of the disease were apparently associated with anemia and did not occur until

TABLE 1

TABLE 1

Virulence of Plasmodium falciparum *(Camp strain) in owl monkeys (*Aotus trivirgatus*)*

Group	Inoculum size (parasitized RBC's)	No. animals inoculated	No. patent / No. survivors	Prepatent period* (days)	Maximum parasitemia* (%)	Survival time* (days)
I	$1\text{-}5 \times 10^8$	68	68/3	1.8(1-4)†	45(13–78)†	7.2(4–18)†
				2.7(2-3)‡	<1 ‡	(60–>300)‡
II	1×10^6	2	2/0	2.5(2-3)	29(25–34)	8.0
III	1.5×10^4	12	10/0	7.8(6-11)	47(15–74)	14.6(10–18)
IV	Unknown (frozen)	11	8/2	5.6(4–8)†	44(14–85)†	13.1(10–17)†
				7.0(6-8)‡	13(13–14)‡	(34–>230)‡

* Mean (range).
† Fatal infections.
‡ Survivors.

relatively large numbers of erythrocytes were parasitized. The extremities of the infected animals became cold, mucous membranes paled and the monkeys tired easily. Usually a hunched back, head down attitude was assumed. The monkeys were severely depressed and became progressively weaker. Finally they became recumbent, usually lying on one side before they died. Epistaxis was relatively common and occult blood was detected in stools in the latter stages of infection. Prolonged bleeding from lancet or needle punctures of the ear or femoral vein were also common findings. Hemoglobinuria was rarely observed.

Experiment I

Hematologic values from terminal bleedings of P. falciparum infected monkeys as well as normal values for uninfected monkeys are shown in Table 2. Erythrocyte and hematocrit values decreased to low levels in most infected animals, although relatively high values were recorded in some of them. The higher values may possibly have been due to hemo-concentration in the venous circulation. The numbers of reticulocytes appeared to be reduced in the infected animals. Levels of platelets in the blood of infected monkeys were greatly reduced when compared to normal values. While the average level of leukocytes was similar for the two groups of monkeys, the counts from infected monkeys were more varied and their blood showed a greater percentage of mononuclear lymphoid type leukocytes. The relatively high percentage of eosinophils found in normal monkeys was reduced in the infected animals.

The results of serum biochemical observations are shown in Table 3. The relatively high SGOT and SGPT values observed in normal animals increased following infection. SGPT values increased proportionately more than those of SGOT. Urea nitrogen levels increased approximately four-fold over those of normal animals. Most infected monkeys did not have increased levels of uric acid, though the mean value was elevated by very high determinations in a few animals. A decrease in total serum protein levels in the infected animals was accounted for by a corresponding reduction in both albumin and gamma globulin fractions while levels of alpha and beta globulins were similar in both groups of monkeys.

Many aggregates of parasitized erythrocytes were found in impression smears made from organs of the infected monkeys (Figs. 1 and 2). The erythrocytes composing these aggregates contained primarily mature trophozoites and schizonts. Although these aggregates were usually found in all organs examined, they were most often seen in heart and liver smears. Blood smears obtained

TABLE 2

TABLE 2

Hematologic values from malaria-free and Plasmodium falciparum*-infected owl monkeys*

Parameter	Normal values				Infected values			
	No. animals	Mean	(SD)	Range	No. animals	Mean	(SD)	Range
RBC's $\times 10^6/mm^3$	157	5.17	(.84)	3.50–7.74	44	3.08	(1.21)	0.87–5.57
Hematocrit %	"	42	(5)	31–56	"	24	(8)	6–39
Reticulocytes %	78	2.4	(1.7)	0.1–10.6	21	1.0	(1.4)	0.1–6.6
Platelets $\times 10^3/mm^3$	63	397	(109)	204–734	21	52	(43)	10–152
WBC's $\times 10^3/mm^3$	157	12.7	(4.7)	3.2–28.5	44	13.1	(8.9)	2.7–43.6
Differential %								
Neutrophils	157	55.4	(7.6)	13–91	32	37.4	(16.7)	4–73
Lymphs and monos	"	35.5	(18.3)	5–80	"	60.0	(16.3)	27–92
Eosinophils	"	9.5	(9.2)	0–37	"	2.3	(2.2)	0–8

TABLE 3

TABLE 3

Serum biochemical components in malaria-free and Plasmodium falciparum*-infected owl monkeys*

Parameter	Normal values				Infected values			
	No. animals	Mean	(SD)	Range	No. animals	Mean	(SD)	Range
Transaminase*								
SGOT	75	153	(71)	49–323	16	280	(112)	116–575
SGPT	75	47	(23)	21–121	16	124	(73)	48–275
Urea nitrogen (mg/100 ml)	56	14	(3)	7–26	28	59	(37)	15–170
Uric acid (mg/100 ml)	56	0.5	(0.4)	0–2	28	1.4	(1.1)	0–5
Total protein (g/100 ml)	75	7.0	(1.2)	4.9–10.2	22	6.3	(1.0)	4.7–8.7
Albumin (g/100 ml)	75	2.7	(0.7)	1.2–4.2	16	2.2	(0.4)	1.6–3.3
Alpha-1 (g/100 ml)	75	0.3	(0.1)	0.2–0.5	16	0.4	(0.1)	0.3–0.6
Alpha-2 (g/100 ml)	75	1.3	(0.3)	0.7–2.4	16	1.2	(0.3)	0.7–1.7
Beta (g/100 ml)	75	1.0	(0.2)	0.6–1.5	16	1.1	(0.3)	0.8–2.0
Gamma (g/100 ml)	75	1.8	(0.5)	1.0–2.9	16	1.3	(0.3)	0.9–1.8

* Reitman-Frankel Units.

Figure 1

FIGURE 1. Impression smear from heart muscle of infected owl monkey showing an aggregate of erythrocytes parasitized by large trophozoites and schizonts.

FIGURE 2. Impression smear from liver of an infected owl monkey showing many aggregates of infected RBC's.

Figure 2

TABLE 4

TABLE 4

Course of infection in owl monkeys inoculated with 1.2 × 10⁴ Plasmodium falciparum*-infected erythrocytes*

Animal no.	Prepatent period (days)	Day of death	Parasitemia at death (%)	Terminal clinical observations
		EXPERIMENT II		
613	6	10	46	Epistaxis, weakness
614	6	11	15	Bloody stool, coma
616	9	18	33	Moderate weakness
617	9	16	38	Moderate weakness
618	–	10	sc*	Apparent good health
619	–	16	sc	Ruffled fur, thin, but alert and eating
620	–	18	sc	Ruffled fur, thin, listless
		EXPERIMENT III		
947	6	10	18	Alert, killed for early tissue specimens
948	6	10	5	Alert, killed for early tissue specimens
949	9	17	69	Extreme weakness
950	6	13	68	Epistaxis, found dead
951	10	15	36	Moderate weakness
956	10	17	74	Moribund
963	–	15	sc	Apparent good health
964	–	10	sc	Apparent good health
965	–	17	sc	Apparent good health

* Saline control.

Figure 3a-c

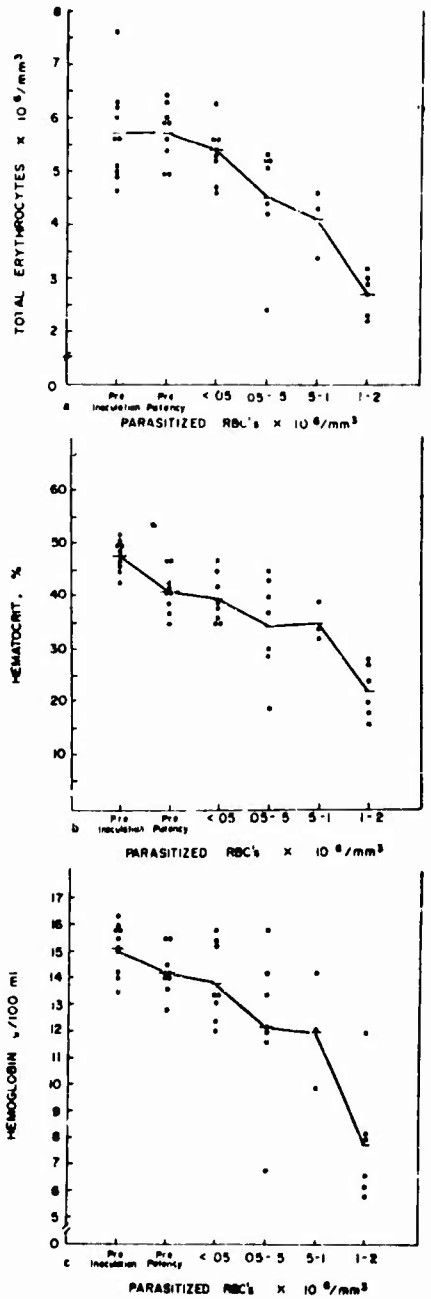

FIGURE 3. a-c. Hematologic changes in infected owl monkeys. Individual animals in Experiment II (●) and Experiment III (○).

Figure 3d-g

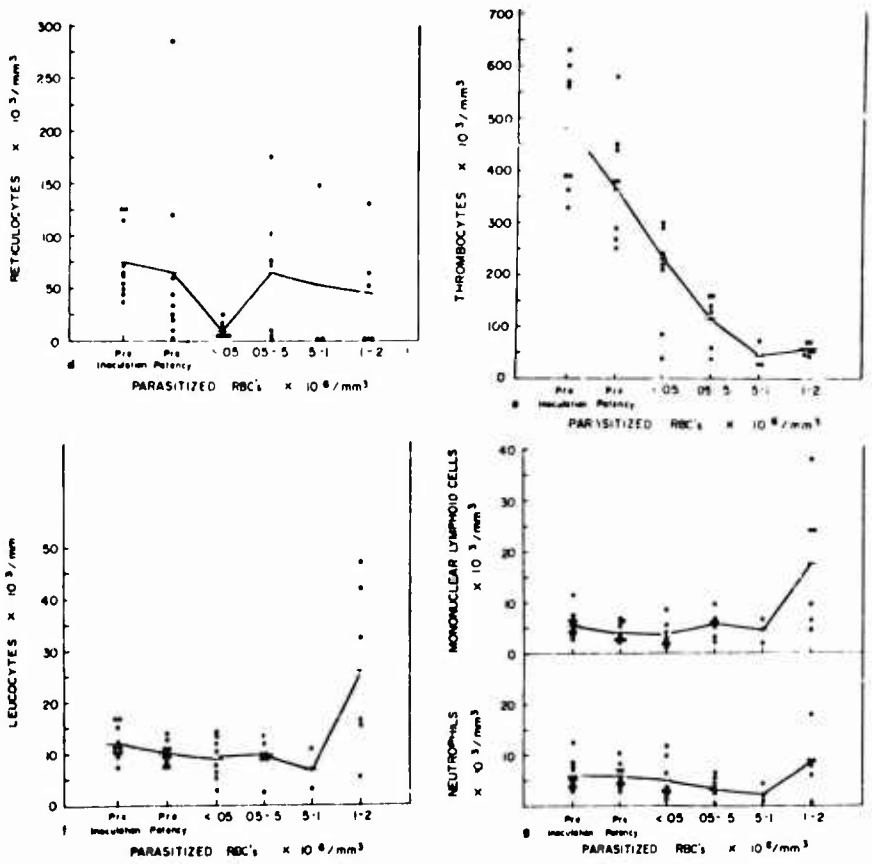

FIGURE 3, continued. d-g Hematologic changes in infected owl monkeys. Individual animals in Experiment II (●) and Experiment III (○).

Figure 4a-d

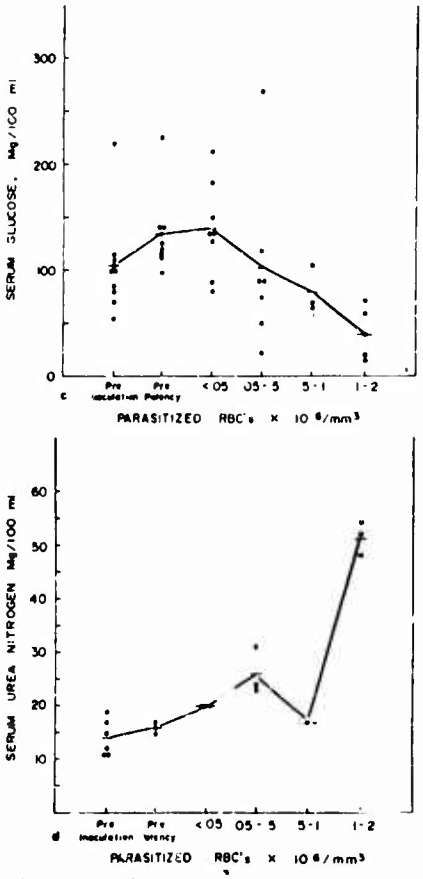

FIGURE 4. a-d. Serum biochemical changes in owl monkeys infected with *Plasmodium falciparum*. Individual monkeys in Experiment II (●) and Experiment III (○).

Figure 5a-b

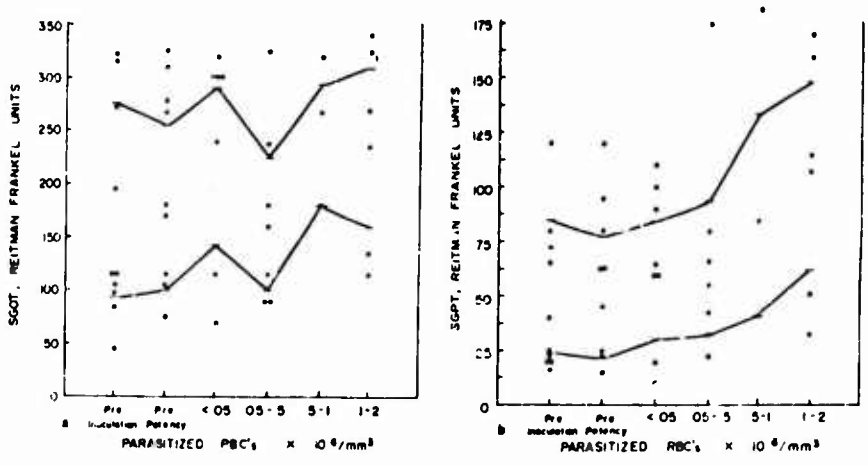
SGOT, REITMAN FRANKEL UNITS
350
300
250
200
150
100
50
0
Pre Incubation
Pre Potency
< 05
05 - 5
5 - 1
1 - 2
a
PARASITIZED PBC's x 10 6/mm3
SGPT, REITMAN FRANKEL UNITS
175
150
125
100
75
50
25
0
Pre Incubation
Pre Potency
< 05
05 - 5
5 - 1
1 - 2
b
PARASITIZED RBC's x 10 6/mm3

Figure 5c

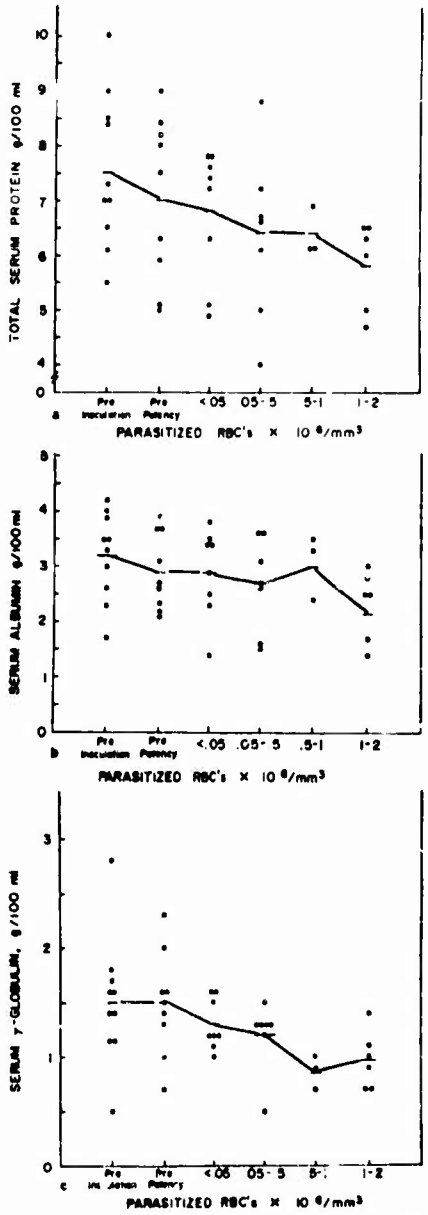

FIGURE 5. a–c. Serum protein changes in owl monkeys infected with *P. falciparum*. Individual monkeys in Experiment II (●) and Experiment III (○).

by ear puncture rarely showed aggregations of cells but RBC aggregates were commonly seen in hematologic procedures on blood obtained from the femoral vein.

Experiments II and III

Two separate experiments were performed in an attempt to relate the observed changes to levels of parasitemia. Monkeys used in these experiments had been observed in our animal quarters for at least one month and appeared to be in excellent condition at the beginning of each experiment. A summary of the course of infection in these animals is presented in Table 4.

Red blood cell parameters (erythrocyte count, hematocrit and hemoglobin values) progressively decreased as the numbers of parasitized cells increased (Fig. 3a, b, c). The rapid decreased noted in one monkey (614) was incomensurate with the level of parasitemia. Levels of reticulocytes appeared to decrease in most monkeys early in the infection and in some animals remained low throughout. A small subsequent increase was evident in a few monkeys, although their numbers did not usually surpass pre-infection levels (Fig. 3d). There was a precipitous drop in the numbers of circulating platelets which began in some animals during the prepatent periods (Fig. 3e). Leukocyte counts in femoral blood appeared to be somewhat lower during the early course of infection although some animals (616, 619, 956) which survived the longest period developed a leukocytosis late in the infection (Fig. 3f). This increase in the level of leukocytes was primarily due to an increase in the numbers of lymphocytes and monocytes (Fig. 3g). Many macrophages containing phagocytized malaria parasites and pigment were especially prominent in blood smears from monkeys surviving for longer periods.

Pre-infection values of both SGOT and SGPT of serum from monkeys in Experiment II were found to be considerably higher than those of animals in Experiment III (Figs. 4a and b). In each experiment the moderate rises in activity which were found with both enzymes did not appear until relatively high levels of parasitemia had developed. Glucose values tended to rise early in the course of the infection but decreased markedly as high levels of parasitemia were reached (Fig. 4c). On the other hand, urea nitrogen levels rose when the level of parasitemia was relatively low and increased about three-fold in animals with high levels of parasitemia (Fig. 4d). Total serum proteins decreased gradually as the level of parasitemia increased (Fig. 5a). This decrease was apparent in the albumin fraction in 8 of 9 monkeys (Fig. 5b). In 6 of 9 animals, the level of gamma globulin also decreased (Fig. 5c).

Three monkeys (948, 949, 951) developed small increases in gamma globulin concentrations. No consistent detectable changes occurred in the alpha or beta globulin levels. Double albumin components which were previously reported were found in electrophoretic patterns of monkeys in these experiments.

Controls

Erythrocyte parameters were not appreciably decreased as a result of bleeding the saline injected control monkeys which also did not develop a reticulocytosis. Platelet levels remained within normal range. Leukocyte levels for control animals in Experiment II ranged from 8.2 to 16.5 thousand per mm^3 before injection and increased gradually to a range of 15.3 to 31.0 thousand/mm^3 at the time of necropsy. Control monkeys in Experiment III had relatively stable levels of leukocytes which did not rise over pre-infection values. Differential counts in control monkeys did not change consistently in either experiment, although relatively high percentages of eosinophils (9-21%) were maintained throughout the experiment in control monkeys in Experiment III.

SGOT and SGPT levels were generally stable in 4 of the 6 control animals. Two monkeys in Experiment II (619 and 620) had high levels of these enzymes in pre-infection serum samples and gradually decreasing levels in samples taken as the experiment progressed. Glucose values remained within normal limits throughout the experiments. Urea nitrogen levels were determined on control animals in Experiment III and no elevation over pre-infection values was observed. Four of the 6 control monkeys developed a mild decrease in total serum protein as compared with that observed in the infected animals. Usually this loss of total protein reflected a decrease seen in all fractions.

The Camp. strain of P. falciparum usually produced a predictable course of infection in the owl monkey. Prepatent periods for inocula of similar size were relatively reproducible and high levels of parasitemia and death usually followed. The basis for the resistance seen in the few surviving monkeys is not understood at this time. Since the exact area in which our animals were captured is not known, different subspecies may have been included in our study and may have been a factor in the observed resistance. Evidence also exists that Aotus monkeys with microfilaremia may be resistant to falciparum malaria. These monkeys also develop an immunity to the homologous strain of P. falciparum after drug therapy and immunization with killed or irradiated parasites.

The progressive anemia accompanying the infection appeared to be consistent with the level of parasitemia. Extremely low erythrocyte parameters in one infected monkey (No. 614) were presumably due to hemorrhage into an ulcerated cecum. No pronounced response to the anemia was evident and there was no significant rise in levels of reticulocytes during the course of infection. This observation coincides with previous reports of a limited response by man to the anemia associated with falciparum malaria.

A marked leukocyte response was also not evident except in the later stages of the infection in some monkeys. This response was characterized by the proliferation of cells of the monocytic and lymphoid series and corresponds to the description by Taliaferro of leukocytes of South American primates infected with P. brasilianum.

The cause of the progressive thrombocytopenia is not clear. Bone marrow examination did not reveal a depletion of megacaryocytes or any obvious changes in their histology. Therefore, it seems less likely that a decrease in the production of platelets played a major part in the thrombocytopenia. The depletion of coagulation factors accompanying the thrombocytopenia as reported by O'Leary et al. strongly indicates a condition comparable to intravascular coagulation. Voller has previously suggested that the thrombocytopenia may be the result of disseminated intravascular coagulation and may be similar to that observed in man. Whether or not the aggregation of parasitized erythrocytes, a common finding in both Aotus monkeys and in man with falciparum malaria, plays a role in the thrombocytopenia and depletion of other clotting factors can only be speculated upon at this time. Further examination of the aggregates for products resulting from intravascular coagulation is needed since results pertaining to this subject are inconclusive. It appears, however, that the aggregations of parasitized cells are a more consistent and pronounced finding in Aotus than in man and that the accompanying depletion of platelets and other coagulation factors is also much more severe than that reported for man. The higher levels of parasitemia which usually develop in Aotus monkeys may account for the accentuation of the above processes. RBC's parasitized by large trophozoites or schizonts were involved in these aggregates. Changes in the RBC membranes have been reported in cells composing the aggregates and take the form of electron dense areas in the RBC membrane. Miller has described the sequestration of these cells primarily in the heart in two Aotus monkeys early in the course of infection and packing of the coronary capillaries by parasitized cells has been reported in man with falciparum malaria. As the level of parasitemia increased,

these aggregates were found throughout the vasculature in greater numbers. On impression smears the blood of the heart and liver showed the greatest number of aggregates with the brain having the fewest. The significance of these aggregates is not yet fully understood since tissue surrounding what appeared to be blocked vessels showed little evidence of necrosis and the clinical course of infection was not characterized by episodes typical of occlusive thrombosis.

Serum urea nitrogen levels progressively increased as the parasitemia increased and correlated with the histologic changes observed in the kidneys of the infected animals. Elevated levels of urea nitrogen associated with renal changes have been reported for man with falciparum malaria. Dehydration and increased protein catabolism may have also contributed to the elevated urea nitrogen levels.

Serum transaminase levels in malaria-free Aotus monkeys were relatively high when compared to those of old world monkeys or man as have been previously reported. These increases may in part be caused by unapparent infections, stress of captivity or genetic differences. The disparity between pre-infection levels of SGOT and SGPT from monkeys in Experiments II and III indicates that some factor not involved with the malaria infection was responsible for some of the observed pathology in these monkeys. Moderate rises in both enzymes usually occurred in infected animals when parasitemia levels were relatively high and confirms a previous report. Corresponding elevations of transaminase activity in man and chimpanzees infected with falciparum malaria have been found.

A decrease in total serum protein due to a reduction in albumin and gamma globulin was observed in most of our infected animals. Decreases in total serum proteins due primarily to a loss of albumin are a common finding in man where usually an increase in gamma globulin is reported. The relatively short duration and serverity of P. falciparum infections in Aotus monkeys may preclude the production of increased levels of gamma globulin. Both increased serum protein catabolism and decreased albumin synthesis due to liver dysfunction may be involved.

Although there appeared to be an initial rise in glucose levels early in the infection, a marked decrease was observed as high parasitemia levels were reached. Disturbances of the glycogenetic function of the liver and increased metabolism of available glucose by the multiplying parasites may have been causative factors in the observed hypoglycemia which has been a common finding for man and other species with malarial infections.

Serum chemical changes found in the infected Aotus, especially those which occur late in the course of infection when high levels of parasitemia are present must be interpreted cautiously. While effects of substances of malarial parasite origin on the test systems are relatively unknown, trypanosomes have been shown to produce materials which give high levels of transaminase activity. Erythrocytes are known to contain substances which cause high levels of activity in tests for uric acid and the widespread destruction of erythrocytes associated with the malaria infection may play a role in the elevated findings in this test. The high susceptibility of laboratory kept Aotus monkeys to viral and bacterial infections may also be reflected in altered levels of serum chemical components as well as in the histology of the affected organs. Generally, however, it appeared that a good correlation existed between serum chemical changes and the histology of the various organ systems of animals in these studies.

The experimental infections of Aotus monkeys with the Camp. strain of P. falciparum resemble fulminating infections in man in many respects which often are accentuated in the monkey model. Despite the problems encountered with Aotus monkeys, we believe that this model system is a valuable tool for the study of falciparum malaria and will become increasingly important as improved methods of laboratory maintenance of these monkeys are developed.

2. Junctional apparatus in erythrocytes infected with malarial parasites.

Erythrocytes infected by most species of malarial parasites can be observed in the peripheral blood. However, few erythrocytes containing parasites appear in the peripheral blood of man or experimental animals infected with Plasmodium falciparum and P. coatneyi. It was assumed that erythrocytes infected by P. falciparum and P. coatneyi sequester in the capillaries of the deep organs, including the spleen, heart, liver and brain.

In order to investigate this phenomenon, electron microscopy of the spleen of Aotus monkeys infected by P. falciparum was undertaken. The erythrocytes infected by the parasite showed minute surface excrescences, which are often located near the reticular cells of the spleen. This communication describes the structure of the excrescences and the role of this structure in the sequestration of the infected erythrocytes.

Two night monkeys (Aotus trivirgatus) were used in this experiment. Each monkey received an inoculum of 2×10^6 erythrocytes infected by P. falciparum (Malayan Camp strain).

Figures 6, 7 and 8

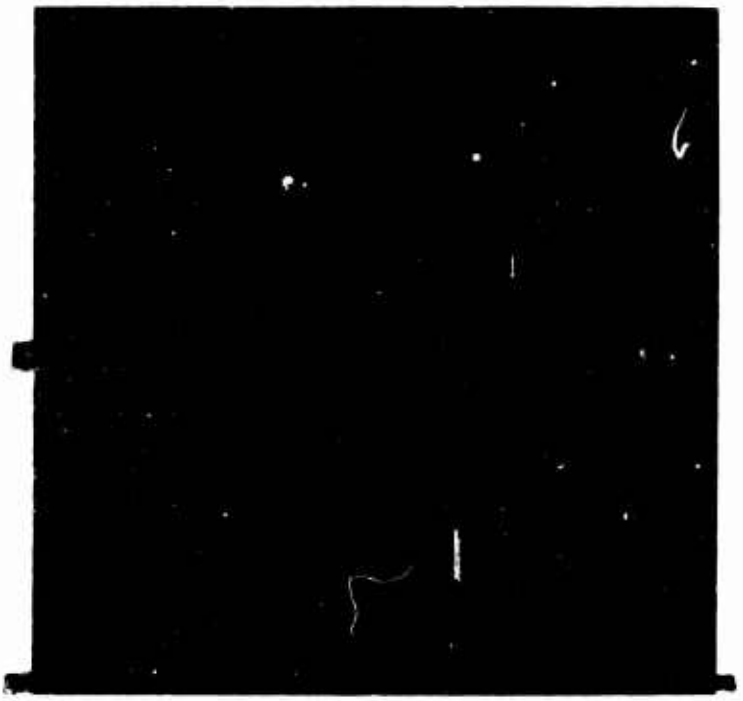

Fig. 6 Portion of a parasitized erythrocyte (*E*); parasite not included in micrograph. Note three cone-shaped excrescences in close contact with plasma membrane of reticular cell (*R*) of spleen. Two of these structures (arrows) appear to have fused with the splenic cell. × 72000

Fig. 7. Two excrescences along erythrocyte (*E*) plasma membrane at higher magnification. A short linear segment (arrow) bridges one excrescence and plasma membrane of reticular cell (*R*). The other excrescence-reticular cell complex shows a narrow gap. Portions of plasma membrane apposed to excrescence appear to be thickened due to slight increase in electron density in subjacent cytoplasm. × 146000

Fig. 8. Focal electron-dense zone (arrow) subjacent to erythrocyte (*E*) plasma membrane apposes another electron-dense zone in reticular cell (*R*). Unlike the excrescence, the electron dense portion of the erythrocyte does not protrude. × 128000

Seven days after inoculation, the spleen was removed and was fixed for one hour in 1% glutaraldehyde solution buffered with 0.05M PO_4 at pH 7.3, containing 4% sucrose. After fixation, small pieces of the spleen were washed in phosphate buffer solution andpostfixed for one hour in 1% osmium tetroxide. They were then dehydrated in an ascending series of ethanol and propylene oxides, and were embedded in Epon 812. Sections were cut with a Porter-Blum MT-2 ultramicrotome using a Dupont diamond knife. They were mounted on 300 copper grids and stained with 1% uranyl acetate and lead citrate. These sections were examined with a Siemens Elmiskop 101 electron microscope.

The sinuses and cords of the splenic red pulp are packed with erythrocytes infected by various forms of P. falciparum. The parasitized erythrocytes are misshapen and show an irregular contour with protrusions and invaginations. Along the plasma membrane of the erythrocytes infected by P. falciparum are several minute excrescences which are more electron-dense than the rest of the erythrocyte cytoplasm (Fig. 6). The number of the excrescences present in a parasitized erythrocyte appears to be related to the developmental stage of the parasite within the erythrocyte. In an erythrocyte infected by a late stage of schizont which occupies more than two-thirds of the host cell, about 50 excrescences are seen in a single section. On the other hand, the host cell with a small uninucleate trophozoite shows only 5 to 6 excrescences along the plasma membrane. Therefore, it appears that the larger the size of the parasite, the more excrescences are present. The non-infected erythrocytes do not show this structure.

Each excrescence is cone-shaped and measures 30-40 mu in height and 90-100 mu in width. The base is not sharply demarcated, but gradually merges into the erythrocyte cytoplasm (Figs. 7 and8). The excrescence is covered by the plasma membrane of the erythrocyte which demonstrates that it is not an extracellular deposit over the erythrocyte membrane. The appearance of the excrescence has a superficial resemblance to a budding virus such as Rous sarcoma virus and avian myeloblastosis virus. However, unlike viruses, the excrescence does not possess a nucleoid, nor does it bud from the erythrocyte.

These excrescences may contact the plasma membrane of the sinal and/or cordal reticular cells of the spleen. The plasma membrane of the splenic cells apposed to the excrescence appears to be slightly thickened due to an increase in density of the cytoplasm (Fig. 3). The tip of the excrescence sometimes touches the plasma membrane of the reticular cells and the two plasma membranes appear to fuse (Fig. 2). In other instances, a short electron-dense linear segment bridges two closely apposed points (Fig. 3). On occasion, a gap of 90 A is observed between the

thickened plasma membrane and the excrescence (Fig. 3). In addition to these excrescences, focal electron-dense zones without excrescences are noted along the plasma membrane of the erythrocytes. The plasma membrane of the reticular cells close to the electron-dense zone of the erythrocyte is also electron-opaque.

The excrescence-reticular cell complex resembles the focal tight junction described in the mesenchymal cells of chicken embryo by Trelstad et al., who suggested these junctions are a primitive type of cell adhesion. However, contrary to the focal tight junction, which is formed by a pair of enantiomorphic electron dense zones, the excrescence of the parasitized erythrocytes is more electron-dense than the apposed cytoplasm of the reticular cells. The excrescence-reticular cell complex thus, is similar to hemidesmosomes.

From their structure and relationships, it is apparent that the excrescences observed in the parasitized erythrocytes form junctions with the reticular cells in the spleen. Although primitive, excrescence might be responsible for the adhesion of the erythrocytes infected with P. falciparum to reticular cells, leading to sequestration within the spleen. This supposition is supported by the observation that large schizonts sequester in the deep organs to a far greater extent that the uninucleate trophozoites; the host cell of the schizonts have correspondingly more excrescences than the host cells of the trophozoites.

A similar structure has been reported in the erythrocyte membrane infected by P. coatneyi and in those infected by P. falciparum. The former investigators suggested that it may be the site of erythrocyte agglutination, which produces clumping of the infected erythrocytes, whereas the latter workers suggested that these excrescences form adhesions with the endothelial cells of capillaries and these lead to the sequestration of the parasitized erythrocytes. It is possible that the excrescences formed in the parasitized erythrocyte have adhesive affinity for certain cells such as the reticular cells, endothelial cells, and erythrocytes with which the excrescences of the parasitized erythrocytes come into proximity.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 127 Test systems for Plasmodium falciparum

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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
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(U) Natural and Acquired Immunity in Rodent Malaria (09)

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19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

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Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Rodents; (U) Susceptibility; (U) Immunity; (U) Plasmodium; (U) Irradiate

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

23(U) To evaluate the role of humoral and cellular factors in determining susceptibility of hosts to malaria, for the maintenance of the complete life cycle of malaria in the laboratory, to find a laboratory animal suitable for the production of large amounts of infected blood for immunological and biochemical studies.

24(U) To test a variety of rodent species for natural susceptibility to P. berghei. Attempt to increase susceptibility by splenectomy and chemical treatment. Standardize the course of infections quantitatavely. Evaluate the mechanism of antibody action on host and parasite, and characterize antibodies responsible for these activities. Study the effects of antibody on the parasite and on the host.

25(U) 71 07 - 72 06 The serum complement requirement for antibody mediated passive immunity to Plasmodium berghei malaria in rats was studied. An anticomplementary factor from Cobra venom was used to destroy the third component of rat complement in vivo. Little or no direct effect of Cobra venom factor on parasitemia was observed. Immune serum administered at the time of infection inhibited development of parasitemia. Depression of the third component of rat complement levels by Cobra venom factor did not diminish this antibody mediated protection. These results indicate that immune effector mechanisms in P. berghei malaria in the rat include a protective pathway which is antibody mediated but third component of rat complement independent. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*Available to contractors upon originator's approval

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 128, Natural and Acquired Immunity in Rodent Malaria

Investigators.

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1. Antibody mediated immunity to Plasmodium berghei independent of the third component of complement.

Antibody has been shown to be an important determinant of immunity to Plasmodium berghei infections in rats. To date, no information has been obtained which bears on the possible participation of complement components in this reaction. An anticomplementary factor (CVF) derived from cobra venom, first used successfully in vivo by Nelson, provides an approach through which information relevant to this possibility can be obtained. This factor, which destroys the third component of complement (C3), has been used extensively to investigate immunopathologic mechanisms.

It was considered that administration of CVF to rats infected with P. berghei but protected by passively supplied antibody would allow a determination of whether or not C3 is required for the antibody mediated protection. The present study demonstrates a protective pathway which is antibody mediated but C3 independent.

A derivative of the NYU-2 isolate of P. berghei was used throughout. Forty to ninety gram inbred Fischer rats were used for the experiments and for maintenance of the parasite. Methods of parasite maintenance have been previously described. Hyperimmune serum (IS) was prepared by infection of one month old rats with 1×10^6 P. berghei parasitized erythrocytes followed by three additional injections of parasitized cells over a period of six months. A total of $3 \times 10^{-}$ parasitized cells and 1×10^9 erythrocytes per animal was administered. Control serum (NS) was obtained by parallel injections of uninfected red cells at the time of injection of the immunized

rats. All animals were bled one week after the last injection, the serum collected, pooled and stored at -20 C.

Experimental animals were infected with 2.5 X 10^6 parasitized erythrocytes in 0.05 M sodium phosphate, 0.01 M NaCl at pH 7.0 with 5% control rat serum per 100 gm body weight.

CVF was prepared from the crude venom of Naja haje as previously described by Shin et al. Parasitemia was monitored by examinations of thin blood films stained with Giemsa's stain; unless otherwise stated, fifty oil immersion fields were studied before a slide was considered negative, and 200 erythrocytes were counted for a quantitative estimate of the percentage of erythrocytes parasitized. Activity of C3 was estimated by a hemolytic assay using purified guinea pig components as described by Shin and Mayer. Statistical analyses were by the non-parametric Mann-Whitney U test; the 95% confidence level was chosen.

In vivo decomplementation by CVF was first explored. Whereas serum C3 levels varied but little during the observation period in control animals, experimental rats had no detectable C3 2.5 hours after injection of CVF and this depression was maintained during the 100 hours of sample collection; only a slight tendency towards a return to normal was noted and this was only apparent in the 75 and 100 hour samples. Experiments with durations of up to 4 days can therefore be performed with confidence that C3 levels will remain depressed.

Studies were then initiated to determine whether or not CVF can influence malaria due to P. berghei. Since the effect of CVF is known to be transient, it was important to design experiments in which possible effects would be expressed shortly after CVF treatment. Two kinds of effects of CVF might be anticipated; (a) a direct influence on parasites which might be detected by altered parasitemias when CVF is given at the time of infection and (b) an interference with complement interaction with parasite-antibody complexes. It was considered that the probability of detection of this latter event could be increased by passive administration of antibody simultaneously with CVF and the infectious inoculum. An experiment was therefore conducted in which animals were injected intravenously with mixtures of parasitized erythrocytes, either CVF or saline, and IS or NS in the 4 possible combinations. The animals were examined for parasitemia over the next 26 days, during the first eleven days of which daily examinations were performed.

All NS treated animals were patent on the day after infection whether or not they received CVF. In contrast, all 16 rats receiving IS remained negative for parasites for five days and not until the ninth day were all positive. A slight contraction of the prepatent period by CVF in the IS treated groups is, however, suggested. The subsequent course of parasitemia also indicated a profound effect of IS but only marginal effects of CVF within serum groups.

Statistical comparisons between parasitemias in NS groups receiving either CVF or saline as observed on each of 17 days revealed one isolated significant difference (higher parasitemia in the CVF group) and this occurred on the fourth day after infection. The comparisons in levels of parasitemia in the IS groups revealed a significant difference in CVF vs saline categories only on day 7 (Table I).

In order to study the system for complement dependence in more detail, it was necessary to obtain evidence of decomplementation prior to introduction of the parasite and to then examine the animals for (a) persistent low activity of C3 and (b) parasitemia. This was achieved by an additional experiment in which 33 μ gm of CVF and 0.66 ml of IS or NS per 100 gm body weight were injected before infection with the standard inoculum of P. berghei.

Table IIa demonstrates that C3 activity was reduced to undetectable levels in the CVF treated animals and that no restoration of activity occurred over the next 18-20 hours. Nevertheless, there was an easily detectable effect of the immune serum on parasitemia during the same interval. Subsequent data on parasitemia are presented in Table IIb, in which it can be seen that depressed parasitemia persisted in both IS treated groups. Later bleedings were less informative due to death of a number of the animals.

These studies demonstrate that the third component of complement is not required for expression of the protective effect of immune serum in the P. berghei-rat system (Tables I and II). Although it is conceivable that amounts of C3 too small to be measured in the tests we employed could have persisted and participated in the observed immune effects, this possibility seems unlikely. Evidence for a minor enhancement of parasitemia by CVF in both the NS and IS treated rats was obtained but is less than compelling. Even if further studies should indicate that an effect does occur, it is evidently a minor one. It should be pointed out that these experiments were performed in the presence of a relatively large amount of antibody, and that

Table I

Development of Latent *P. berghei* Parasitemia After Treatment with Immune Serum and Cobra Venom Factor

Immune Serum	Venom Factor	No. Animals* Patent on Day				
		1-5	6	7	8	9-11
+	-	0	2	0	3	8
-	-	8	8	8	8	8
-	+	8	8	8	8	8
+	+	0	1	4	7	8

Table IIa

Effect of Preinfection Decomplementation on Expression of Antibody Mediated Immunity to P. berghei; C3 Levels and 18-20 hour Patency

Immune Serum	Venom Factor	Serum C3 Activity[1,2] 2-5 Hr Before Infection	Serum C3 Activity[1,2] 18-20 Hr After Infection	No. Animals Patent[2,3] 18-20 Hr After Infection
+	-	57 (48-58)	59 (54-61)	1
-	-	55 (53-63)	60 (59-66)	5
-	+	0 (0 - 1)	1 (0 - 1)	5
+	+	1 (0 - 1)	1 (0 - 1)	2

[1]Per cent lysis, median (range). Serum dilution 1:3000.

[2]Five animals per group.

[3]Smears were exhaustively studied (>150 microscopic fields) before being declared negative.

Table 13b

Effect of Preinfection Decomplementation or Expression of Antibody Mediated Immunity to P. berghei; Peripheral Parasitemia on the Fourth, Seventh and Eleventh Day After Infection

Immune Serum	Venom Factor	Per Cent Erythrocytes Parasitized[1] 4 Days	7 Days	11 Days
+	-	+[2]	7.5 (5.5-10.0)	17.0 (9.5-27.5)
-	-	6.0 (3.0-7.5)	18.8 (10.5-21.0)[3]	36.0 (20.5-43.5)[3]
-	+	6.0 (1.5-7.0)	16.0 (9.5-20.5)	40.5 (24.5-52.5)
+	+	+[2]	9.5 (6.5-14.0)	26.5 (5.5-33.0)

[1]Median (Range); 5 animals per group.

[2]All animals positive, but parasites too scarce for quantitative estimates.

[3]One animal dead in this group.

if both complement dependent and independent reactions occur, the former could be masked by the latter. To resolve this question, it will be necessary to perform experiments with limiting amounts of antibody so that partial protective effects can be observed.

These findings are in agreement with those of Cohen and Butcher who used in vitro assay of antiparasitic activity involving P. knowlesi and homologous antibody. These workers obtained two kinds of evidence for a complement pathway; (a) fresh serum is not required and (b) the pepsin digest of immune IgG ($F(Ab')_2$) is active. Although whole serum was used to induce the protective effects observed in this report, evidence for involvement of antibody in the reaction has been obtained previously.

The results suggest that the antiparasitic effect of antibody on P. berghei may be analogous to a type of viral neutralization as has been previously suggested in that complement appears to play a minor role in these reactions. If either a cytotoxic reaction or opsinization were involved, the participation of C3 might be expected. It can be speculated that once "neutralized," malaria parasites are handled as inert debris, and phagocytized by the mononuclear phagocytes which act as scavengers. Alternatively, specific immune phagocytosis may occur by a complement independent pathway. Obviously multiple mechanisms may obtain and much more work will be required to elucidate the cellular and molecular mechanisms of the expression of humoral immunity in malaria.

2. Requirement of induction of immunity to Plasmodium berghei malaria by irradiated parasitized erythrocytes.

Artificial immunization of mice against Plasmodium berghei infections has proven to be a useful model in the study of immunity to malaria. Protection from this infection has been demonstrated in mice allowed to recover with the aid of chemotherapy, in animals administered irradiated parasitized erythrocytes, and with parasites attenuated in a tissue culture system. More recently, reports of protection induced by parasite fractions have appeared in the literature. Similar procedures have been used to immunize rats against the parasite. No direct comparisons of the immunogenic efficacy of intact versus disrupted parasitized erythrocytes have been reported. These experiments were designed to make such comparisons and to explore other characteristics of the host-parasite system which are required for an optimal immune response.

Parasite

The NYU-2 strain of Plasmodium berghei was used in all the studies reported herein. Procedures for maintenance of the parasites have been previously described.

All experiments were conducted using random bred albino mice reared at the Walter Reed Army Institute of Research (Wrm:(ICR)BR).

Immunization

Immunization of animals was achieved by the injection of parasitized erythrocytes which has been irradiated using a CO-60 source as previously reported. The immunizing schedule involved five injections over a two and one-half week period. Unless otherwise noted, the total dose was 1×10^9 parasitized erythrocytes. In some of the experimental groups, the inocula were disrupted either in a pressure cell or by freeze-thawing by immersion in a CO_2-ethanol bath with subsequent thawing: a total of three freeze-thaw cycles was employed. Morphological observations on these preparations were made after concentration of the residue by centrifugation at 27,000 x g and staining with Geimsa's stain.

In experiments involving the use of sulfadiazine, the animals were injected with 0.2 ml of a 10 mg/ml solution intraperitoneally on the day prior to exposure to parasites and then daily until one week after the last exposure; challenge was deferred until one week after the last dose of drug.

Assay of Immunity

Groups of animals were treated for immunity to Plasmodium berghei by intraperitoneal challenge with 2×10^4 parasitized erythrocytes. Significant prolongation of survival time beyond that of control animals was taken as evidence of immunity. The percentage of animals surviving for 30 days was another useful index of protection; animals alive at that time usually survived.

Assays of Protein Synthesis In vitro

The protein synthetic capacity of parasitized erythrocytes was assayed by the incorporation of C-14 isoleucine (301 mc/mM) into trichloracetic acid (TCA) insoluble material. Erythrocytes collected in heparin from heavily parasitized mice were washed

and suspended at a concentration of 2.5 X 10^7/ml in Hank's buffered salt solution with added glucose (1.75 mg/ml), potassium penicillin-G (500 µ/ml) and streptomycin sulfate (500 µgm/ml). C-14 isoleucine was added to a concentration of 0.2-0.3 µc/ml at 37 C in a metabolic water bath (Dubnoff) shaken at 60-100 cycles/min. One half ml samples were removed to 5 ml cold 0.01 M isoleucine in 0.15 M NaCl at intervals thereafter. The cells were sedimented at 800 X g, washed in an additional 5 ml isoleucine and lysed with 2.5 ml H_2O. The solution was brought to 10% TCA, the resulting precipitate sedimented at 800 x g, washed twice in 5 ml 10% TCA and dissolved in 1.0 ml of a quaternary ammonium base solvent, NCS. The preparations were washed into a scintillation fluid consisting of 0.3 gm/l of p-Bis (2-(5-phenyl-oxazolyl)/ benzene (POPOP) and 5 gm/l of 2,5-diphenyl-oxazole (PPO) in toluene and counted in a Packard liquid scintillation counting system.

Other Procedures

Hemolysis was estimated by absorbance measurements of supernatant fluids at 412 nM

Chemical fixation of irradiated parasitized cells was performed in a 2.5% solution of gluteraldehyde in 4% sucrose in 0.05 M sodium phosphate, pH 7.1 - 7.2 for 1o min at 4 C. The gluteraldehyde was removed by centrifugation and washing with 0.075M sodium phosphate, pH 7.3 in 0.075M NaCl.

Splenectomies were performed under sterile conditions on mice anesthetized with Relaxans. The spleens were exteriorized through a small incision and were removed by cautery. Sham-operated control mice received the same treatment with the exception that the spleen was not removed. Surgical skin clips were used to close the incisions. Splenectomies were done either one week before or 3 to 5 days after immunization.

Effect of Hemolysis of Immunogenicity of Irradiated Parasitized Erythrocytes.

The degree of immunity induced by irradiated parasitized erythrocytes was compared with that obtained with lysates of the same preparations. Two experiments were performed in which disruption of the parasite-host cell complex was accomplished either by passage through the Frenon pressure cell or by freezing and thawing. Mortality curves for animals in comparable groups were similar and the data from these two experiments were pooled. In spite of the survival of 80% of the animals which received intact irradiated parasitized cells (positive controls), all other animals died within 21 days.

The median survial times of negative controls (animals given no pretreatment) and experimental groups were similar (8 to 10 days) but three mice from three different experimental groups survived longer than any negative control animals. Both methods of disruption resulted in complete hemolysis as judged from the gross appearance of the preparations. Microscopic observations revealed many intact parasites after either freeze-thaw or treatment at 1000 pounds per square inch (psi) in the French pressure cell; higher pressures resulted in essentially complete disruption of parasites.

In order to determine whether or not a greater time interval between immunization and challenge enhances immunity, after treatment with antigens derived from disrupted cells, we performed a similar experiment in which the prechallenge period was extended. Irradiated parasitized erythrocytes were lysed at 1000 psi and used to immunize mice; positive and negative controls were included as before. Four weeks after immunization, one-half of the animals in each group were challenged; the remaining animals were challenged eight weeks after the last immunizing injection. It is evident that little immunity could be detected even when challenge was delayed. The survival of the single mouse in the experimental group challenged at four weeks is significant, however, since an untreated animal has never survived in any of our experiments. The survival of 100% of the positive control animals after challenge at 8 weeks is also noteworthy.

In order to further investigate the immunogenicity of disrupted parasites, an experiment was performed in which irradiated parasitized cells were disrupted at 1000 psi, the resulting preparation centrifuged at 27,000 x g, and the supernatant fluid and sediment each used as experimental immunogens. Control groups were also included, and the animals were challenged one week after the last injection. Median survival times were similar in negative control and experimental groups, but one mouse each in the supernatant fluid and sediment groups survived for 30 days.

Effects of Irradiation Dose on Immunogenicity and Protein Synthesis

Two experiments were performed to investigate the effect of increased levels of irradiation on the immunogenicity of parasitized erythrocytes. The first experiment assessed levels up to 200 Krad; in the second experiment, 400 and 800 Krad were also used. Because of the time required for the latter large irradiation doses, measures were taken to maintain all preparations at near 0 C during irradiation; heating effects can therefore be ruled out as a cause of the observed impairment

of immunogenicity. The median survival times of negative controls were similar to the two experiments (9 and 11 days respectively). In both experiments, a progressive dimunition in immunogenicity was noted as the irradiation doses increased.

In the third experiment, in vitro protein synthesis by irradiated parasitized cells was studied. Heavily parasitized and uninfected control heparinized mouse erythrocytes were washed in 0.15M NaCl and adjusted to 5 X 10^8/ml. Aliquots of the parasitized cells were irradiated at seven different dosages. After removal of samples for estimates of hemolysis, the suspensions were assayed for protein synthetic capacity. The results indicate little or no difference in the initial rate of isoleucine incorporated by nonirradiated cells or cells irradiated at 20 or 40 Krad, although the data suggest a dimunition in rate in the irradiated cells toward the end of the experiment. Higher levels of irradiation resulted in a progressive loss in protein synthetic capacity until at 200 Krad incorporation was indistinguishable from the uninfected erythrocyte control values. It was noticed during the course of this experiment that hemolysis was taking place in the reaction mixtures. Estimates were made of hemolysis in cell suspension samples taken after irradiation and held at room temperature in saline until the last sample was taken for estimates of isoleucine incorporation. It is apparent that both protein synthesis and hemolysis are functions of irradiation dosage but that the latter is more resistant to changes in dosage than the former.

Effect of Sulfadiazine on Immunogenicity of Irradiated Parasitized Erythrocytes

These experiments were designed to test the possibility that normally metabolizing parasites are required for immunogenicity. Since sulfadiazine is highly effective against P. berghei infections, it was reasoned that the drug would also inhibit metabolism in irradiated parasites. An experiment was therefore performed in which large doses of sulfadiazine were injected into mice prior to, during, and after immunization with irradiated parasitized erythrocytes. In addition to (a) positive and (b) negative controls as described above and (c) experimental mice immunized with irradiated parasites and given drug, two additional control groups were employed; these consisted of (d) animals which were treated with drug alone and (e) animals which were given nonirradiated parasitized erythrocytes and sulfadiazine. These last two groups were included to (1) serve as "drug only" controls to detect possible persistence of drug and to (2) test the efficacy of the

dose of drug used in preventing parasitemia due to nonirradiated parasites. One day prior to challenge, blood films were collected from the group (e) of animals which received nonirradiated parasites and sulfadiazine; all animals were negative.

The results demonstrate that immunization was achieved even though the animals were being treated with the drug. It can also be seen from these data that nonirradiated parasitized erythrocytes were immunogenic in drug treated animals. A total of three experiments of this type all indicated that sulfadiazine does not inhibit immunogenicity.

In an in vitro experiment, protein synthesis by non-irradiated parasites in the presence of sulfadiazine was investigated. No inhibition was observed during the 2 1/2 hour exposure to drug.

Effect of Gluteraldehyde Fixation on Immunogenicity of Irradiated Parasitized Erythrocytes

Since the integrity of the parasite-host cell complex may be crucial to immunogenicity, parasitized cells fixed in gluteraldehyde were tested for ability to induce a measurable immunity to challenge. In order to take advantage of any increase in sensitivity to be obtained from an extended time interval prior to challenge, groups of animals were held for either one week or four weeks before they were challenged. All positive control animals challenged at four weeks survived. Although median survival times were similar in the experimental and negative control groups, a single experimental mouse survived.

Effect of Splenectomy on the Resistance Produced by Irradiated Parasitized Erythrocytes

Four separate experiments were undertaken to determine the effects of splenectomy before and after immunization on the development and maintenance of immunity produced by irradiated parasitized cells. Immunized mice were given a total of approximately 5×10^8 irradiated parasitized cells. Negative control mice received injections of irradiated normal mouse erythrocytes or were untreated. Mice splenectomized before immunization were not resistant to the challenging infection and their median survival time (9 days) was similar to that in untreated mice (10 days) and mice receiving normal irradiated blood (9 days). On the other hand, the median survival time of sham-operated immunized mice was 18 days. Splenectomy after

immunization also severely affected the expression of immunity. Mice in this group died at a rate similar to that of negative control mice. The median survival time for mice splenectomized after immunization was 11 days, as compared to 19 days for sham-operated mice.

Sham-operation procedures conducted on mice either before or after immunization appeared to have an effect on resistance since there was a greater percentage survival and a greater median survival time in the positive control mice (untreated immunized) than in either of the two operated groups.

These studies demonstrate that optimal immunogenicity of irradiated parasitized erythrocytes is dependent on an intact parasite-host cell complex. The immunogenic effect persists in the presence of treatment with a chemotherapeutic agent. Disruption of parasitized erythrocytes either by passage through a pressure cell or by repeated freezing and thawing resulted in extensive, but not complete, loss of immunogenicity.

It is of interest to compare these findings with those of D'Antonio et al. who have detected immunogenic activity in fractions derived from parasitized mouse erythrocytes. Whereas these workers performed their studies with subfractions of the erythrocyte parasite complex, we worked either with the whole disruptates or with the sediment and supernatant fluids thereof. It is possible that in the present study the crucial antigenic substances were somehow destroyed or that their activity was masked by extraneous protein or by other material present in the suspension. However, activity was not significantly enhanced when the supernatant fluid containing the bulk of the hemoglobin was removed. A more likely explanation for the apparent discrepancy between these and the above mentioned findings is the fact that the other workers employed a host-parasite system in which virulence was less marked than in that reported here.

A possible explanation for the higher efficacy of intact parasitized erythrocytes as opposed to lysates is that antigen synthesis continues in vivo during the early hours (or days) after injection of irradiated parasites into the recipient. The fact that relatively low levels of irradiation only slightly inhibit protein synthesis has been demonstrated in P. knowlesi by Trigg et al. who suggest that this might account, in part, for immunogenicity. If this were the case, then "exoantigens" could be present in small quantities in the parasite extracts which show low level immunogenicity. The progressive reduction

of immunogenicity with increasing radiation dose might indicate a progressive loss of synthetic capacity, and this is indicated in this investigation. However, since lysis also increased with increasing levels of irradiation, a postulated direct inhibitory effect cannot be distinguished from inhibition associated with cell disruption. Interpretation of the interrelationship between these two phenomena is complicated since lysis proceeds during the protein synthesis experiments, and in fact, probably continues to occur for many hours. Studies of protein synthesis in irradiated parasitized erythrocytes under conditions of reduced lysis through the use of SH protective agents might be informative.

Pertinent to these considerations are the experiments which indicate no effect of sulfadiazine on immunogenicity. Since sulfadiazine would be expected to interfere with biosynthetic functions, it seems likely that the parasites of animals which received sulfadiazine on a daily basis would have an impaired ability for antigen synthesis. This speculation must be tempered by the fact that prompt inhibition of protein synthesis by P. berghei is not induced by sulfadiazine in vitro. It has also been reported that protein synthesis in malaria parasites is not inhibited by other drugs with similar sites of action. In any case, animals so treated were effectively immunized.

A recent contribution indicates that heat inactivated P. berghei parasitized erythrocytes are immunogenic. This observation may also help determine if there is a requirement for metabolizing parasites in the induction of immunity. Quantitative comparisons between the immunogenicity of heated and nonheated parasitized cells will be necessary to allow relevant interpretations.

Another hypothesis which can be offered to account for the requirement for intact cells concerns the fate of the damaged erythrocyte in vivo. Whereas minimally damaged erythrocytes are sequestered primarily in the spleen, more extensive erythrocyte alterations result in hepatic uptake. The spleen is clearly required for the induction of resistance by irradiated parasitized cells. Treatments which damage the parasitized erythrocyte membrane (i. e. increased radiation, fixation, pressure cell) may alter the patterns of sequestration and destruction of the irradiated parasitized cells in the recipient animal. This in turn may greatly effect the ability of the host to respond adequately to the challenging infection.

The loss of immunity observed when splenectomy was performed after immunization coincides with the work of others and emphasizes the importance of the spleen in the maintenance of immunity to malarial parasites.

Although gluteraldehyde fixation is routinely used to preserve ultrastructure, it did not preserve immunogenicity of the irradiated parasitized erythrocytes. This finding can be interpreted equally well in terms of inactivation of (a) biosynthetic capacity or (b) preformed antigen. It suggests that particle size alone is not the crucial factor responsible for the immunogenicity of intact parasitized erythrocytes. The significant protective effect observed in one mouse is interesting. Whether it is the results of specific immunization or nonspecific stimulation of indifferent defense mechanisms remains to be determined.

Enhancement of immunity by extension of the prechallenge interval is suggested in several of the experiments reported in this communication. This is in concert with reports of D'Antonio *et al.* who deferred challenge until 15 weeks after administration of antigen.

The finding that nonirradiated parasites in the presence of sulfadiazine are at least as effective as immunogens as irradiated parasites is interesting. Although this treatment resulted in 80% survival as compared with approximately 50% in the positive control group, no conclusion regarding comparative efficacy can be made.

The loss in immunogenicity of parasitized cells after lysis remains unexplained. If exoantigen production proves not to be involved, the problem can be thought of in terms of lability of antigen rather than in terms of the metabolic capability of the parasite. It is too early to speculate as to whether such lability, if applicable, might prove to be defined in terms of a relationship between organ or cell distribution and immunogenicity, protein denaturation, autolysis through hydrolytic enzymes, a combination or these considerations or some other as yet unsuspected factor(s).

3. The Effect of protective sera on *Plasmoidium berghei* in immunosuppressed rats.

The immunity to malaria has been looked at from a number of different views. All components of the immune response have been implicated in the immune clearance of malaria. The

protective effect of antibodies has been well documented, while the role of the retculoendothelial system has also been implicated. The role of cellular hypersensitivity has been implicated by studies on cell transfer and neonatal thymectomy. These studies have not examined the comparative effect of the various components of immunity in the response. The studies with antibody alone cannot rule out an effect of cellular immunity. The studies with cell transfer cannot rule out transfer antibody forming cells.

The present study is an attempt to explore in a comparative manner the humoral vs. cellular aspects of immunity to malaria when cell-mediated immunity was suppressed using a potent inhibitor, Anti-thymocyte Globulin (ATG).

ANIMALS

Rabbits: All anti-sera were produced in Albino New Zealand Rabbits bred at this institution. Rats: used in studies on the course of infection were Lewis rats ranging in weight from 130-170 gms unless otherwise noted. Sprague-Dawley rats (Walter Reed Strain) bred at this institution were used for in vivo assay of sera and maintenance of malaria parasites. All animals were maintained under conditions promulgated by the National Society for Medical Research. Malaria Parasites: Plasmodium berghei (NYU-2) has been maintained serially by weekly blood passage in Walter Reed Strain rats 12-15 days old. All smears were stained with Giemsa Stain. Parasitemias were counted per 10^4 erythrocytes unless more than 10% were parasitized.

Preparation of Anti-sera: Anti-thymocyte Serum (ATS) was prepared by two injections approximately 10^7 washed thymocytes from Walter Reed Strain rats immunized in Complete Freund's Adjuvant (CFA) a week apart. Starting ten days later, the rabbits were bled by cardiac puncture biweekly. At the end of two weeks, they were exsanguinated. The sera was separated and stored at 4 C, then pooled and stored at -20 C. The CFA is prepared from Incomplete Freund's Adjuvant (Difco) and 2 mg/ml M. tuberculosis H37Ra (Difco).

Anti-Macrophage Serum: Was prepared by collecting peritoneal exudates from Walter Reed Strain rats 4 days after intraperitoneal injection of light mineral oil, NF (Halsey Drug Co., Brooklyn, New York). The cells were washed three times in Hanks Basic Salt Solution (HBSS) and cultured in glass petri dishes in Medium 199 enriched with 10% fresh homologous rat serum and 1% Penicillin/Streptomycin (5,000 units each) for 3 days. The non-adherent cells were washed off the HBSS at 37 C.

The adherent macrophages then removed with a rubber policeman, washed once and emulsified in an equal volume of CFA and injected on the same schedule in preparation of the ATS. The sera were pooled and collected in the same manner also

Normal Rabbit Serum: A group of rabbits were immunized on the same schedule as the above sera but with 10 mg of Bovine Plasma Albumin emulsified in CFA.

Hyperimmune "Protective" Serum: (HPS). Two groups of 12 Lewis Rats were infected with P. berghei. After the peak parasitemia, the rats were reinfected weekly with large varying doses of P. berghei for 4 months. Sera were collected by retro-orbital venipuncture after that time. The rats were reinfected and bled at irregular intervals after that, animals were always bled at least 2 weeks after the most recent reinfection. A parallel group of rats were given an equivalent amount of normal Walter Reed Strain Rat erythrocytes on the same schedule to form a Control Rat Serum (CRS).

Preparation of the Globulin Fraction: The globulin fraction of the anticell sera and control sera were prepared by precipitation at 40% Saturation of Ammonium Sulfate ($Am\ SO_4$) at 4 C. Washed with 50% saturated $Am\ SO_4$ dialyzed vs. 0.01M phosphate buffer pH 7.2 and concentrated by lyphilization. The powder was redissolved in distilled water to give a final salt concentration of 0.1M phosphate buffer and the solutions were stored at -20 C. All preparations were sterilized by passage through Millipore filters prior to being given.

General Experimental Protocol: Lewis rats were injected with (ATG) intraperitoneally in a dose equivalent to approximately 1 ml of the original serum on days -2, -1 and day zero. Day zero was designated as the day of infection with malaria (or sensitization in the case of other antigens). Three hours after the last injection a complete blood count was performed. One hour after this, the animals were infected intraperitoneally with P. berghei infected rat erythrocytes. The AMS was used in a similar protocol except that the entire dose was given at one time on day zero, followed three hours later by infection with the malaria parasites. The exact dose of malaria parasites given in each experiment varied but was in the range of 10^7 parasitized erythrocytes except when mentioned.

Assay of the Anti Sera: In vitro Thymocyte agglutination was measured by microtitration as noted in Jasin et al. Macrophage cytotoxicity was measured by evaluation of cell viability after incubation for 30 min at 37 C with heat inactivated anti-sera in fresh guinea pig sera. The guinea pig sera was tested and

diluted to a non-toxic level. Peritoneal macrophages were obtained three days after stimulation of rats with sterile mineral oil. The macrophages were washed three times prior to use, initial concentration of cells was about 2.5 C 10^6/ml. Cell viability was measured by trypan blue dye exclusion, to an end point of 50% cell death.

In vivo: The anti-cell sera were given to Walter Reed Strain rats 120-140 gms on the schedule listed in the General Experimental Protocol noted above. The animals were sensitized with sheep erythrocytes (SRBC). At 7 and 14 days after sensitization the animals were tested for hemagglutinating antibody. The ATG blocked the formation of anti-SRBC antibody by an average of 5 doubling dilutions. When compared to animals given NRG, animals given the AMG were equal to or slightly higher in antibody titer to SRBC and the skin reaction to DNCB was not different from the control animals.

ATG and AMG were also tested in vitro for their ability to block clearance of carbon by the reticulo-endothelial system. The protocol used was outlined in the materials and methods section above. Three hours after the last injection of anti-cell globulin, the rats were injected with colloidal carbon intravenously. Clearance of carbon was measured by the method of Halpern. No difference could be detected in the clearance rate of control animals or any of the treatment groups.

The course of P. berghei malaria in Lewis rats of this age and size is a low parasitemia which rapidly becomes subpatent. Generally, there is a peak parasitemia about the first week of about 4-5 percent, which levels off and this drops to very low or subpatent levels during the second week. The effect of pretreatment with ATG on days -2, -1 and zero has the effect of causing a minor increase toward the end of the first week of infection. This is followed by a short period (3-4 days) of a relatively stable level of 8-12% of erythrocytes parasitized. During the second week of infection, however, there is a marked increase in the parasitemia reaching a mean peak of 30% of the cells infected at the time the uninfected group had become negative on peripheral smear. There was no increase in the mortality of the animals in this treatment group. However, there was a greater anemia in ATG treated animals, on day 18 mean hematocrit being 17.5% compared to 44.8% in controls. Further they appeared ill with jaundice, weight loss, ruffled fur and decreased activity. None of these features occurred in infected rats treated with NRG or in uninfected rats given ATG, but not infected with malaria.

ATG in vitro and AMG in vitro

The ATG and AMG were assayed for activity first using an in vitro assay. Thymocyte agglutination titer as shown in Table III was 1:1024 for ATG, but was 1:32 for AMG, a difference of five doubling dilutions. Conversely, when assayed for cytotoxicity for macrophages, the AMG had a titer of 1:256 while the ATG was 1:32. This shows the AMG had 3 doubling dilutions more activity in this system. Thus, while the in vitro assays show that the preparations are not completely specific, they are considerably more effective against the cell for which they were designed than is the other preparation.

It has been shown that ATG has an effect on macrophages. To show that increased and prolonged parasitemia was not due to the effect of the ATG on macrophages, an AMG was studied in a similar system. As shown in Table III, the ATG almost completely suppressed the antibody response to Sheep RBC compared to a mean titer of 1:8 compared to NRG. Conversely, the AMG as shown caused an increase in the titer of antibody to SRBC to a titer of nearly 1:55. There was an increase in mortality with 4/7. However, the AMG did not cause any increase in the level of parasitemia or in mortality. A third group was given a dose of AMG 1/3 as great on the basis of an OD_{280} reading. The peak of parasitemia was significantly lower in this group than in the controls. The mortality in this group was lower only 2/7 having died. Another group which was given an amount of AMG equivalent in protein concentration to the ATG had approximately the same course of parasitemia as the NRG treated group and there was no increased mortality. Thus, it appears that when an equivalent amount of activity against macrophage is given, as assayed by in vitro techniques, there was no increase in parasitemia nor a prolongation of course. It appears that the lower dose may have been somewhat protective, however, more work would have to be done to confirm this point. These data suggests that the activity of the ATG as used in these experiments (is not primarily exerting) its effect by acting on macrophages. Further evidence was obtained by showing that neither the ATG nor the AMG was able to alter the pattern of carbon clearance in vivo.

A hyper-immune anti-malarial serum, (Hyperimmune Protective Serum) or (HPS) was prepared as noted in the methods section. This material was pooled and tested in vivo for its ability to suppress an infection with P. berghei malaria as seen in Table IV. The HPS was given in doses of 0.25, 0.5, 1.0, either i.p. or i.v. 1 hour after, 1×10^6 parasites interperitoneally 1 hour previously. The results obtained are shown in Table IV where it will be seen that increasing doses of HPS had an increasing effect on blocking the parasitemia. For the first 4

TABLE IV

Effect of Hyperimmune Serum in Vivo

Group	Number	Antisera[a] HPS ml	NRS	Route[b]	Mean Percent Parasitemia Day 2	5	8
1	6	0	1.0	ip	0.10	.32	1.9
2	6	1.0	0	ip	0	.16	0.3
3	6	0.5	0.5	ip	.17	1.7	1.5
4	6	0.25	0.75	ip	.08	1.4	1.6
5	6	1.0	0	iv	0	0.80	0.75
6	6	0.5	0.5	iv	0	60	0.58

a HPS = Hyperimmune Rat Serum; NRS = Normal Rat Serum

b ip = intraperitoneal iv = intravenous

days after giving the HPS in all the groups there was suppression of the parasitemia to some degree. By the fifth and sixth days after treatment, the greatest difference between groups could be seen. By the eighth day all the rats had a significant parasitemia greater than 1% to RBC's parasitized in all groups, however, the two groups given 1 ml of the serum cleared more rapidly. The groups treated with 1 ml of the HPS either intravenously or intraperitoneally had a lower mean parasitemia than the other treatment groups, but were not very different from each other. It was decided, therefore, to use HPS in doses of about 1 ml per animal and to administer it intraperitoneally.

To evaluate the relative roles of humoral factors of HPS was used in conjunction with the ATG. The ATG or NRG was given as before on day -2, -1, and day zero. Three hours later the rats were infected intraperitoneally with 4×10^6 P. berghei. One hour later, half the rats in each group were given 1.24 ml of the HPS intraperitoneally. The rest of the rats in the groups were treated with CRS. The group given ATG plus CRS had a course similar to that seen in the ATG treated group in the previous experiment. There is a slight rise over the control parasitemia during the first week of the infection, leading to a pronounced rise in the second week of the infection. Those animals, however, that were given the HPS had a course which were treated with the NRG plus CRS. On the other hand, those animals which were given the NRG plus HPS also had a generally similar course. Unfortunately there was an increased mortality in both groups of ATG treated animals; 2/6 of the animals given ATG/HPS died and 3/6 those given ATG/CRS. However, most of the animals died in the first 24 hours and none had a high parasitemia. Cross pathology showed they died of massive respiration infection. The experiment was then repeated with the same protocol but the animals were given drinking water containing tetracycline to suppress the respiratory infection. To counteract the effect of the tetracycline on P. berghei, the dose of parasites given was increased to 2.1×10^8 given i.p. The results parallel those seen in the groups not given antibiotic, however, the course was much shortened and the maximum parasitemia was reduced markedly. There was, however, no mortality in this group.

The immune response to P. berghei malaria in rats was evaluated in this study by altering the immune response. The effect of immunosuppressive agents on the course of infection was measured by giving anti-thymocyte globulin (ATG) on the three days prior to infection. This caused an increase in the parasitemia, the animals became more ill by clinical evaluation and their hematocrit dropped markedly compared

TABLE III

Properties of ATG and AMG

	in vitro		in vivo
	thymocyte agglutination	macrophage cytotoxicity	Anti SRBC at day 11
ATG	1:1024	1:32	1:1.14
AMG	1:32	1:256	1:43
NRG	0	0	1:8

to the controls. Thus, it was seen that immunosuppression with a potent agent would cause a prolonged and more severe course of malaria in rats. These results are quite consistent with those of Barker and Power in mice and Spira et al. in rats.

The exact mechanism by which the ATG effects the response to malaria was not clear because the antibody is known to have several effects on the immune response. First and most prominent is its suppression of cellular immunity. Second, in some instances it can suppress the ability to produce antibody and third, it has been implicated as having antimacrophage activity in many cases. The last effect was examined first. A potent anti-macrophage globulin AMG was prepared against peritoneal macrophages. Tests in vitro showed that it had a considerably greater effect against macrophages than did the ATG while it had a minimal effect against lymphocytes. In vivo studies showed that where the ATG was markedly suppressive for antibody response to sheep erythrocytes the AMG did not lower the antibody level below the controls. Another parameter of macrophage function, phagocytosis, was measured in vivo carbon clearance. It was shown that there was no difference in the ability of an animal to clear particular colloidal carbon after treatment with control globulin or with either of the anti-cell sera.

Further, the AMG was evaluated for its effect on malaria infection. Animals treated with AMG had not only no increase in the level of parasitemia or the severity of clinical illness, but in fact a low dose of AMG seemed to cause a slightly lower parasitemia. This latter effect may have been related to a minimal stimulation of the reticuloendothelial system due to a submaximal dose. Thus, it would seem that the anti-macrophage activity present in the ARG cannot be implicated as the reason for the increased parasitemia and more severe course of malaria seen in immunosuppressed animals.

An effort was then made to determine the effect of antibody in these immunosuppressed animals. If in the face of severe immunosuppression, humorial factors would block the rise in parasitemias it would be strong evidence of a predominating role for humoral immunity over cellular factors. A hyperimmune protective serum was prepared by frequent reinfection of rats with P. berghei. This material was shown to be a potent but temporary inhibitor to patent parasitemia. This effect was seen as marked delay in becoming patent and slower initial rise of parasite over the course of five or six days after treatment with the protective material. Similar results were shown by Diggs and Osler. The latter authors also show that the effect was mediated by 7S IgG. The results of this experiment were that the protective serum completely blocked the increased

morbidity of a group of immunosuppressed animals. The parasitemia levels were no greater than controls. Further, the malaria did not persist although there is reason to suspect that the animals own defenses were still limited by the ATG. Unfortunately, the first time this was studied there was a high incidence of death from intercurrent infection, probably unrelated to the ATG induced immunosuppression. To overcome this last problem, this experiment was repeated in animals protected with tetracycline.

This was an experiment in minature since P. berghei is also affected by the drug. However, even when the animals were given tetracycline on a chronic basis, it was apparent that a very similar pattern of parasitemia was seen although absolute values are lower. The pattern of the response to ATG and protective serum during the course of malaria is quite consistent and shows that the effect is reproducible even in the face of an antibiotic treatment.

It has been apparent that complex mechanisms are involved in the immune response to malaria. Studies to test both cellular and humoral factors have both seemed to play a role in protection. Diggs and Osler showed, by use of hyperimmune serum, that there was a marked decrease in the parasitemia in subjects or animals pretreated with the sera. But other work has shown that the protection afforded by antibody is not complete and that there are relapses of parasitemia, which in several cases has been shown to be due to antigenic variation.

Other workers have shown the importance of cellular factors in this disease. Stechschulte and Brown found that neonatal thymectomy resulted in an increased and more prolonged parasitemia. Stechschulte and Phillips showed that transfer of spleen cells was associated with decreased parasitemia and a more complete immunity as evidenced by sterile immunity in rats treated with cells as compared with those protected with serum.

The data presented here have shown that ATG, a potent suppressor of cellular hypersensitivity, gives a course similar to that seen with neonatal thymectomy. Barker and Powers and Spira et al. found similar results on the course of parasitemia after immunosuppression with ATG. However, there is a disparity between these two reports in their assessment of the role of antibody. The former group found that there was no recovery until they could detect antibody by the fluorescenated antibody test. The latter group used a microprecipitation test and could detect no difference between the ATG treated and control rats, which led them to suggest that non-humoral aspects of the immune response were predominant. It has, however, been very

difficult to correlate protective antibody with any of the *in vitro* tests for antibody level in malaria. In the data presented in this paper, serum within proven ability to inhibit parasited *in vitro* showed that even in animals in which the cellular hypersensitivity is suppressed, the animals were able to survive and contain the infection as well as intact animals. This suggests that the primary means by which malaria is blocked is by antibody. The data presented do not rule out a rule of cell-mediated factors. Since ATG strongly affects these factors. In the situation of a large complex antigen, it is known that the thymus,thus presumably cellular factors, are important in production of antibody, the so-called helper cell. Brown has suggested that immunity to malaria might be dominated by such thymic helper cells process. The data in this report are compatible with such a hypothesis, but do not specifically give information about it. Possibly the antibody production to malaria may require some cellularly mediated action to start formulation while the malaria parasites would succumb to the antibody rather than some cytotoxic factor from the cells *per se*.

In summary, it would appear that all phases play some role in concert to give protection against malaria. Predominant in killing the parasite and keeping the infection within controllable limits are the humoral factors contained in protective serum. However, suppression of the cell mediated system as done with ATG allows the malaria parasite to persist at higher levels and for longer periods of time. Thus, there appears to be a combination of cellular factors and humoral factors with the latter suppressing the infection after sensitization through mechanisms requiring the former.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	63713A	3A663713D829	00	129
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c. CONTRIBUTING	CDOG 114 (F)			

11. TITLE (Precede with Security Classification Code)

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13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	2	70
FISCAL YEAR CURRENT 73	2	70

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Gamma globulin; (U) Biochemistry; (U) Antibody; (U) Fluorescent; (U) Isotope; (U) Metabolism

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 physiological pathology of malaria including the enhancement of non-specific resistance to infection and to determine how energy requirements are met within the parasite.

24(U) Study the effect of infection on the uptake and distribution of radiosotope-labelled amino acids, study the levels of enzyme activity in tissue extracts and alterations in protein and free amino acid contituents of blood and urine, study the development of relapses, and the pattern of parasitemias and fluorescent antibodies prior to, during, and following therapy. Investigate the use of immune gamma globulins as an adjuvant to chemotherapy in humans infected with drug resistant malaria.

25(U) 71 07 - 72 06 Human immunoglobulin G, isolated from plasma obtained in Nigeria, where Plasmodium falciparum malaria is highly endemic, was studied for its protective effect in Aotus trivirgatus infected with a strain of P. falciparum (Camp) isolated in Malaysia. Control monkeys were treated with comparable immunoglobulin prepared from plasma obtained in the U.S.A. A delay in onset of patency and an increase in survival time was observed in monkeys that received IgG from the malaria endemic area. Three Nigerian IgG treated monkeys survived challenge; one of these did not become patent. The data indicate that protection is mediated by IgG and not by contaminating materials. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*Available to contractors upon originator's approval.

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host Responses to Malaria

Investigators.

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1. The protective effect of African human Immunoglobulin G in Aotus trivirgatus infected with Asian Plasmodium falciparum.

Modification of the course of malaria infections by passively transferred antibody is a familiar phenomenon. Among the most noteworthy contributions are those of Cohen, et al. who clearly demonstrated that the IgG fraction of plasma collected in Gambia suppressed falciparum malaria contracted locally. Activity was not observed in control IgG from plasma collected in the United Kingdom. Sadun, et al. also demonstrated the therapeutic effect of human West African gamma-globulin against homologous falciparum malaria, in this case in chimpanzees. In addition, it was shown that the material can be used prophylactically. McGregor, et al. demonstrated that West African gamma-globulin was also effective against East African Plasmodium falciparum, suggesting antigenic similarity between the geographically distinct lines of the parasite. Sadun, et al. using the chimpanzee model studied the effect of African globulin on the Southeast Asian strain of P. falciparum. Although they observed a trend toward a lower parasitemia, the results were not considered conclusive. The present experiments were designed to pursue the study of the effect of West African immunoglobulin on Southeast Asian P. falciparum, using Aotus trivirgatus, the owl monkey, as the experimental host.

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 for several years in this laboratory. A history of this strain and the course of the disease it produces have been described. Plasma was obtained from blood collected at the Blood Bank of the University College Hospital of the University of Ibadan in Nigeria where malaria due to P. falciparum is highly endemic and from the Blood

Bank at the Walter Reed General Hospital, Washington, D. C. In a pilot experiment, control globulin consisted of Immune Serum Globulin, USP, obtained commercially. In all other experiments, control and experimental preparations were processed in parallel by the same methods. Plasma was clotted with calcium chloride and the serum collected by centrifugation. Isolation of IgG was accomplished on DEAE cellulose or DEAE Sephadex by batch methods. Protein concentrations were monitored by absorbance at 280 nm. The composition of serum protein preparations was examined by electrophoresis on cellulose acetate, immunoelectrophoresis and two-dimensional immunodiffusion against a potent anti-whole human serum using wells 2.5 nm in diameter and 1.5 nm apart. In addition, a commercially obtained anti-human transferrin was also used. Examinations of the preparations for immunoglobulins M and A were performed through the use of radial immunodiffusion plates obtained commercially. Standard methods were used for determining the toxicity of IgG preparations for the ten day old chick embryo.

Physical, hematological and blood parasitological examinations were performed on the Aotus monkeys and only apparently healthy animals were chosen for experimental use. The animals varied in weight from 500 to 1100 grams. Procedures for maintenance of the monkeys has been described. Proteins were administered intraperitoneally on a weight basis at the time of challenge by the intravenous injection of 1-3 X 10^6 parasitized erythrocytes obtained from a donor monkey. Thin blood films were obtained from the monkeys daily thereafter until dealth or until 30 days had elapsed. In spite of the efforts to exclude unhealthy animals from the experiments, microfilariae were seen in the blood of two animals after the experiments were begun. Since we have noted a decrease in susceptibility to P. falciparum in animals with filariasis (unpublished observations), these were excluded from the experiment. Postmortem examinations were performed routinely for evidence of inapparent disease. In three cases animals which died with low parasitemias were found to have intercurrent disease; one animal had massive peritonitis and two had severe bronchopneumonia with lung abscess. These animals were also excluded from further consideration.

In analyzing the data, two parameters were given major consideration; the prepatent period and the survival time. The prepatent period was defined as the time required for the first positive blood smear after injection of parasites. The day of death was taken as the day after the last blood film was obtained, since in a few cases the exact time of death (before or after midnight) was not recorded. Comparisons between groups of animals on the basis of these parameters were

made by the Mann-Whitney U Test and the 95% confidence level was chosen for significance testing.

The concentration of transfused human IgG in Aotus monkey serum was determined through the use of commercially obtained radial immunodiffusion plates. Although some cross reaction with a material in Aotus serum (presumably Aotus IgG) was evident, this did not interfere with quantitative estimates when measurements of human IgG were made in the presence and in the absence of monkey serum.

Passive hemagglutination tests were performed on globulin preparations by the method of Wellde, et al. as modified by McAlister.

Experiment 1

The pilot experiment consisted of observations in two monkeys which were inoculated with 1 X 10^6 parasitized erythrocytes and treated with globulin within the hour. The first animal, which weighed approximately 1000 grams, was given 200 mg of American globulin; the second monkey, which weighed approximately 800 grams, was given an equal amount of Nigerian globulin which had been lyophilized and stored for six years. The control animal became patent on the third day after injection and succumbed to the infection on day 9. In contrast, the animal which received Nigerian globulin did not become patent until day 12 and death was deferred until day 21. Based on this pilot trial, it was decided to examine the effect observed in detail.

Experiment 2

An experiment was performed in which serum prepared from plasma of Nigerian or American origin at a dose level of 30 ml per kilogram was given to monkeys immediately before challenge with 1 X 10^6 parasitized erythrocytes per kilogram body weight. Control animals became patent 4 to 10 days later whereas the experimental monkeys became patent on days 13 and 18 respectively. One of the animals (#533) which received immune serum exhibited a protracted course of parasitemia; on the day before death of the last control animal, the parasitemia in this monkey (#533) had risen only to 5%. It increased gradually thereafter to 17% on the 28th day of infection, then rapidly rose to 31% on the 29th day and to 73% on the 30th, the day before death. Monkey #531 exhibited an even more dramatic course; although it became patent on day 20, the parasitemia remained below 1% until day 28 at which time it rose to 2%; the peak parasitemia at 8% occurred on the 29th day, after which parasites could not be detected. The animal is still alive, more than one year after infection.

Experiment 3

The effect of isolated immunoglobulin on the course of the infection was next studied. The material was obtained by the DEAE cellulose method and was administered at a dose level of 200 mg per kilogram body weight. The challenge infection (2×10^6 parasitized erythrocytes per kilogram) was given approximately one hour after the globulin treatment. Whereas one of the animals which received Nigerian globulin became patent on day 3, the prepatent period for the other four experimental monkeys varied from 6 to 10 days; in contrast, all control animals became patent on the third day after infection. The first death in the Nigerian globulin treated group was on day 13 and the last on day 42. Postmortem examination revealed acute renal cortical necrosis as the cause of death in this latter case (Monkey #523). The etiology of the renal pathology was not clear, but it seems likely that it was unrelated to the malaria infection per se. The peak parasitemia in this animal was 7%. A uniformly progressive parasitemia resulted in the death of the control animals on days 11-14.

In spite of the fact that the prepatent period of only one experimental monkey was within the range of the controls, the group difference is not statistically significant at the 5% level. However, survival times were significantly different in the American and Nigerian IgG groups.

Since the immunoglobulin preparations used in experiment 3 were relatively impure (an 8 and 18% beta globulin contaminant in the Nigerian and American gamma-globulin preparations respectively by cellulose acetate electrophoresis), the possibility that some material other than IgG was responsible for the observed effects could not be lightly dismissed. We therefore performed an additional experiment using highly purified IgG in an attempt to minimize the possible effects of contaminating materials.

Experiment 4

The preparations were isolated from the plasma by the DEAE Sephadex method, and concentrated to 50 mg per ml. At this level, one of the preparations (American globulin) gave 2 lines on immunoelectrophoresis and in two-dimensional immunodiffusion against anti-whole human serum. On examination with anti-transferrin in two dimensional immunodiffusion experiments, a precipitin line could be detected. When the preparation was diluted with an equal volume of saline (i. e. at 25 mg of protein per ml) a single line was

seen with anti-whole serum and transferrin could no longer be detected. The experimental (Nigerian) IgG preparation revealed a single line in immunoelectrophoresis and two-dimensional immunodiffusion experiments at a concentration of 50 mg per ml, and the test for transferrin was negative. No contamination was observed in either preparation by cellulose acetate electrophoresis. Immunoelectrophoresis patterns of the proteins are illustrated in Fig. 1. No contaminant can be discerned in either IgG preparation in this photograph.

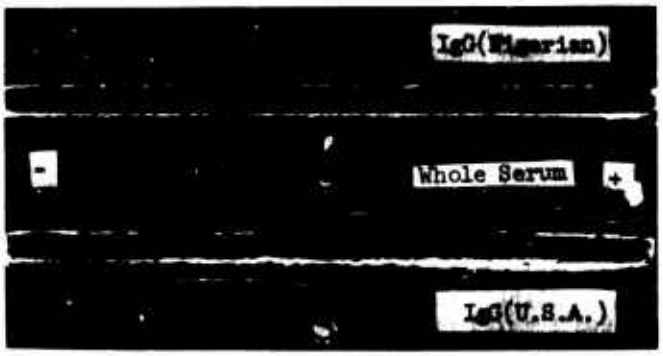
Hemagglutination tests for antimalarial antibody were performed on these preparations. Whereas the American IgG prep gave no hemagglutination at a dilution of 1:4, the Nigerian IgG preparation was reactive at a 1:512 dilution.

Both preparations were tested for possible endotoxin contamination in chick embryo toxicity tests; no evidence for toxicity was obtained with 1/10 ml volumes of the preparations at a concentration of 5 mg per ml.

Six monkeys were given the experimental Nigerian IgG and three the American preparation; two animals were given saline. IgG was administered at a dose level of 500 mg per kilogram body weight. The animals in the experiment ranged in weight from 510 to 760 grams. The globulin was administered intraperitoneally immediately prior to the intravenous injection of 2.5×10^6 parasitized erythrocytes per kilogram body weight. The control animals were patent on the second day after infection and they all were dead by the 13th day of the experiment. Whereas one of the experimental monkeys was also patent on day 2, patency in the other monkeys was delayed; three until day 5, one until day 10, and one indefinitely. This latter animal had not shown parasites on the 35th day after initiation of the experiment. There was a tendency for animals receiving American immunoglobulin G to succumb somewhat more slowly to the parasitemia than animals receiving saline only. In spite of the fact that there was a delay in onset of disease in four of the animals given Nigerian globulin, patency in two of the experimental animals was within the range of the controls. Nevertheless, the experimental group was significantly different from the controls with respect to both prepatent period and survival time.

Tests on serum taken from each monkey prior to injection of the human IgG or saline were all negative. It can readily be appreciated from these data that whereas high levels of human IgG were achieved in monkeys No. 605, 587, 609 and 614,

Figure 1



monkeys No. 579 and 608 had lower serum concentrations of the human immunoglobulin. Monkeys which were given American IgG (Nos. 581, 595 and 613) also exhibited high serum IgG levels. All sera from monkeys injected with saline were negative.

These studies demonstrate that human serum collected in West Africa, or the globulin fraction of such serum, extends the prepatent period and survival time of Aotus monkeys infected with a Malaysian strain of P. falciparum. In addition, some treated animals survived. In our experience, spontaneous cure is extremely rare. The results suggest that a degree of similarity exists between the protective antigens of these geographically remote strains of P. falciparum. This is the first convincing evidence, to our knowledge, of protection from a line of P. falciparum by antibody derived from a different continent. The results complement findings of others who have found partial immunity to heterologous challenge in humans and Aotus monkeys actively immune to P. falciparum.

The data indicate that the protection demonstrated is due to immunoglobulin G antibody. Although the presence of small amounts of other serum proteins cannot be ruled out, there is no reason to suspect that such contamination could be responsible for the effects observed. Small quantities of bacterial endotoxins have been shown to induce resistance to rodent malaria and could, therefore, conceivably be active in the present system. However, this is unlikely since no toxicity of the preparations for the chick embryo was detected. Most small molecular weight materials (viz. drugs) would be expected to have been removed by the extensive dialysis which was a part of the procedure for IgG preparation in Experiments 1, 3 and 4.

As observed in most studies of passive serum mediated immunity to malaria, the protection demonstrated was only transient in most of the animals. It can be speculated that parasites with an altered antigenic specificity might have emerged which were no longer reactive with the passively administered antibody. Antigenic variation of this type has been convincingly demonstrated for P. knowlesi and P. berghei infections. Alternatively, it might be considered that even with the relatively massive doses of protein used, the amount of protective antibody was too limited to destroy all parasites, and that the survivors replicated and ultimately killed the majority of the animals. Whether variant or species specific, it seems likely that the antigen(s) involved in the protective effects noted exist in at least two geographically separated

strains of P. falciparum; namely, those responsible for the development of the antibody obtained in Africa and in the challenge population. However, the possibility that the protective effect is due to antibody elicited in response to cross reactive nonplasmodial antigens cannot be ruled out.

To refer to the Camp strain in Aotus monkeys as an Asian strain of P. falciparum is perhaps presumptive, since by now a more accurate description might be "laboratory strain." The "Asian" designation is used only to indicate origin and not necessarily a similarity between the laboratory line and parasites to be found in the human population in Southeast Asia. The actual relationship between the antigenic structure of the parasites studied in these experiments and human pathogens can only be speculated upon. However, it is remarkable, in our view, that this laboratory parasite is sufficiently related to the pathogens in West Africa to allow the effects noted. Our results give more credence to the possibility that widespread geographic strain similarities may occur.

The examinations of the Aotus monkey sera for human IgG were informative. These determinations offer an explanation for the failure of the antibody treatment to protect certain individual monkeys; i. e. high IgG levels were either not achieved or were only transient in these animals. Whether these differences are due to technical factors or to peculiarities of the response of the individual animals to the foreign protein has not been determined.

The question of the quantitative relationship between the "cross protection" demonstrated in this investigation and "homologous protection" is an important aspect of humoral immunity in P. falciparum infections which has not yet been approached. It would be important to learn whether or not the protection observed is inferior to homologous protection. Attempts are being made to obtain the necessary materials to make such trials feasible.

2. The effect of Plasmodium berghei on the immune response in rats.

This study was designed to examine the effect of malaria on some of the parameters of the immune response. Although much effort has gone into examining the effect of the immune response on malaria infection, little work has been done on what the infection does to the host's ability to respond to other antigens. Malaria is a potent "immunogen" and prolonged exposure leads to elevated levels of immunoglobulins. Antibodies to malaria are found in high titers and "abnormal" antibodies are also seen.

In chronic P. malaria deposition of antigen-antibody complexes may lead to a nephrotic syndrome. Studies in mice on the effect of malaria infection on immunoglobulin production in intact germ free mice have shown an increased amount of immunoglobulin being formed. Studies by Barker showed that in unsensitized mice there was an enhancement of anti-sheep cell antibodies as measured by the Jerne plaque technique. It has been suggested that in certain more complex systems that malaria infection has a suppressive effect on the ability of the host to respond. Studies with several murine leukemias suggest that the animals succumb to the leukemia at a much earlier time when they have been infected with P. berghei yoelli. Conversely in a disease which is the result of immune complex deposition nephritis, namely NZB mice, Greenwood and Greenwood note that although the animals continued to have their basic underlying disease they no longer succumbed to chronic glomerulonephritis when infected with malaria. In another study, it was suggested that this effect might be mediated through suppression of the delayed hypersensitivity mechanism in that adjuvant arthritis was suppressed in rats treated with malaria. The following studies were designed to determine the mechanism of these effects on the immune response and whether they could be documented in rats.

Animals: Lewis strain female rats 100-130 gms. were used in all of these experiments. Rats were divided into groups according to a set of random numbers. Sprague-Dawley rats of the Walter Reed substrain maintained in this institution's own breeding colony were used for maintenance of malaria parasites. All animals were maintained under conditions meeting the standards promulgated by the National Society for Medical Research.

Malaria parasites: P. berghei (NYU-2) maintained by weekly passage in 12-15 day old Sprague-Dawley rats by intraperitoneal blood infection. All experimental animals were infected intraperitoneally with 2×10^7 malaria infected erythrocytes in Alsever's Solution. Parasitemias were counted in thin smears with Giemsa Stain; 1000 erythrocytes were counted unless there was more than 10% parasitemia. At various intervals, mice were subinoculated with 1 ml of blood from the study animals.

Sensitizations: 0.2 ml of sheep erythrocytes (1×10^7/ml) washed three times in normal saline, were injected intraperitoneally. Adjuvant arthritis was produced in the rats by injection of 0.1 ml Complete Freund's Adjuvant (CFA) made of Incomplete Freund's Adjuvant (Difco) with 0.6 mg of M. tuberculosis (H37Ra)/ml, subcutaneously at the base of the tail. Degree of arthritis was measured by estimating a joint score, based on 0-2 small

joints and 0-4 large joints according to the method of Currey and Ziff (1968). Scores were plotted for each animal and the area under the curve measured with a planimeter. The area was used in all calculations of means and statistical evaluations. Samples were collected from the retro-orbital plexus under ether anesthesia.

Anti-sheep cell antibodies were measured by a microagglutination technique in the presence or absence of 2-mercaptoethanol to estimate both 19S and 7S antibody against sheep cells as described previously (Jasin et al., 1968). Skin sensitivity to tuberculin was measured by intracutaneous injection of 0.2 ml of Purified Protein Derivating (PPD) (50 ugm/ml) in 0.2 ml Hanks Basic Salt Solution (HBSS). Induration was read in two diameters at 24, 48 and 72 hours. The product of the two diameters was recorded and used in the tabulations. Inflammatory stimulus was assessed in animals pretreated with 0.1 ml Dinitrochlorobenzene (BNCB) in acetone applied on the shaved abdomen. Two weeks later a test dose of 0.1 ml of 1%, 0.5% and 0.25% DNCB in acetone was applied to separate sites on the shaved backs of rats. Reactions were graded as 0.5=erythema, 1.0=induration, 2.0=marked induration and 3.0=necrosis. Results were tabulated by adding all positive scores for each animal. Evaluation was by rank ordering with Mann-Whitney U Test

The first experiment was to determine the effect of malaria infection on the antibody response to sheep erythrocytes (SRBC). One hour after giving SRBC, half the rats were infected intraperitoneally with P. berghei parasitized erythrocytes. On the fourth day after injection, the rats were further subdivided into four groups, one group with malaria and one control group, were treated with 320 mg/Kg Sulfalene subcutaneously in Peanut Oil. This dose is known to be highly effective in killing P. berghei in mice. Two similar groups were given Peanut Oil alone. As shown in Table I, there is no difference in the anti-SRBC between any of the sensitized groups. This was true for both 2-mercaptoethanol sensitive and resistant antibody. There was no appreciable change in the ratio of the two types of antibody as well.

The next set of experiments were designed to study the effect of malaria infection on an inflammatory response. Dinitrochlorpbenzene (DNCB) was used to cause the inflammation. In experiment 2, the malaria was given simultaneously with or prior to DNCB stimulation. In groups 1 and 2, the DNCB was initially given on day 0. Groups 3 and 4 were given the DNCB on day 14 after the infection and groups 5 and 6 were first given the DNCB on the 28th day after infection. Each was tested 14 days after their initial dose. The initial reaction to

TABLE I

Reciprocal geometric mean titer of anti-SRBC by microagglutination on day 7 and 14 before and after treatment with 2-mercaptoethanol (2ME)

	Treatment		Before 2ME		After 2ME	
Group	Day 0	Day 5	Day 7	Day 14	Day 7	Day 14
1	P. berghei	Sulfalene in oil	115.9	52.5	11.3	3.3
2	P. berghei	Oil only	64	37.3	5.6	3.4
3	Normal RBC	Sulfalene in oil	76.1	29.9	5.2	3.1
4	Normal RBC	Oil only	105	29.3	5.1	3.7

10% DNCB was severe in all animals and no attempt was made to quantitate them. The reaction to 1%, 0.5%, and 0.25% DNCB were quantitated as noted in the methods section. In Table IIA are the results when DNCB was given after infection with malaria. There was no significant difference between treatment groups.

In experiment 3, the effect on response to DNCB given before was studied. The results are shwon in Table IIB. All animals were exposed to DNCB on day zero. Group 1 is a control which was not infected. Groups 2 and 3 were infected 4 and 8 days after DNCB, respectively. Although in groups 2 and 3 there is a slightly lower mean value for inflammation, it is not significant statistically.

The effect of malaria infection on cellular hypersensitivity was studied next. The effect on adjuvant arthritis and tuberculin sensitivity were measured at various times in relationship to infection. Adjuvant arthritis is induced by injecting Complete Freund's Adjuvant (CFA) and approximately 11 days later the rats develop a severe polyarthritis, balanitis, conjunctivitis, and wasting. The same group of rats used in experiment 3 were given CFA 34 days after the initial exposure to DNCB and 10 days after the last testing with DNCB. This corresponds to day 26 and day 30 after infection with malaria in the respective groups. All the animals were negative on smear for parasites at that time, but most were still positive by mouse subinoculation. There was considerable variation in the arthritis within each group and neither of the two infected groups was statistically different from the control. The figures are relatively unchanged if the animals, which were not positive on subinoculation are deleted. Tuberculin reactions were also measured in these animals 6 and 15 days after CFA. The results are shown on Table III. On neither occasion there was a difference in the response to PPD in the three treatment groups.

The effect of giving the CFA simultaneously with or at closer intervals after the malaria infection were tested in Experiment 4. Here the animals were in paired groups. One of each pair was infected on day zero and/or the other given normal rat erythrocytes. Groups of rats were run in parallel pairs because of the variability of batches of CFA to produce arthritis. Groups 1, 3 and 5 were infected, while 2, 4 and 6 were not. Groups 1 and 2 were given CFA on day zero, groups 3 and 4 on day 11 and groups 5 and 6 on day 22.

As seen before, although there is considerable intragroup variation, this is not statistically significant nor was there consistent patterns of increased or decreased arthritis. The tuberculin reaction was measured on a number of occasions starting on day 14 and again showed no difference in the treatment groups.

TABLE II

Response to test doses of dinitrochlorobenzene (DNCB) as a measure of inflammation when infected with P. berghei before(Table A) or after(Table B)initial exposure to high base DNCB.

Group A	Injection on day zero with	Day Given High dose	Day Given Low dose	DNCB MTR[b]	Parasitemia
1a	P. berghei[a]	0	14	1.1 (0-2.5)	30.0[c]
1b	NRBC			1.0 (0.5-2.5)	0
2a	P. berghei	14	28	1.1 (0.5-2.0)	23.8
2b	NRBC			0.6 (0-1.5)	0
3a	P. berghei	28	43	0.8 (0.5-1.5)	21.8
3b	NRBC			1.0 (0.5-1.5)	0

Group B	High dose DNCB day	P. berghei given day	DNCB Tested Day	DNCB Tested MTR[a]	Parasitemia
1	0	None	15	1.9 (1.0-2.5)	0[e]
2	0	4	15(11)[d]	1.3 (0-2.5)	6.5
3	0	8	15(7)	1.3 (0.5-2.0)	3.57

In Experiment 5 the CFA was given first and the malaria subsequently. In this study rats were first divided into groups, then given CFA by groups, and finally they were infected with malaria 4, 7, 11, or 14 days later. One control was not infected but served as negative control for the repeated tuberculin testing.

When the animals are infected after the CFA was given, but before the onset of arthritis, there was a marked increase in the total joint disease. This increase was less dramatic but still evident when the malaria was given just about the time the arthritis developed. The difference from the control was statistically significant for the animals infected 7 days after giving CFA. The joint score was not statistically greater when the infection was at the time or after the onset of the arthritis. The tuberculin response had a similar pattern although not as striking and only statistically significant in the groups treated on day 4 after CFA.

The results of the last experiment were repeated in experiment 5 using larger groups of animals. In this study the animals were given CFA before being sorted into groups, to reduce any bias which might be introduced by injecting by groups. The rats were then divided into 3 groups of 15, however, one animal died of anesthesia in group 3 and was not included. All animals were given CFA on day zero as before. Group 1 was infected with malaria on the 14th day after CFA and group 2 infected on the 11th day. Group 3 was not infected. Parasitemias are approximately the same in the two infected groups. There again was a significant increase in the amount of arthritis when there has been infection with malaria This difference was statistically significant at $P < 0.01$ by Student's t. test. In this experiment, animals infected as late as 11 days after sensitizations with CFA had a significant increase in the amount of arthritis.

Infection with malaria parasites is a potent stimulator of the immune responses. High levels of immunoglobulins are found, and as well, there are high titers of antibody to the parasite. It could be expected that such a potent immunogen might have an effect on the immune response. Chronic infection in malaria has been associated in man with "abnormal" antibodies, such as biological false positive test for syphilis. Also seen may be immunologically mediated renal lesion leading to a nephrotic syndrome.

An effect on the immune response can be invoked as the mechanism for a number of phenomena seen in malaria infected animals. Salaman et al. noted a sharp decline in the production

of anti-sheep erythrocyte plagues in animals. A plot of the number of antibody forming cells was almost the mirror image of the parasitemia curve. Barker had similar results also using SRBC. Further studies by Salaman and Wedderburn showed that mice infected with any of several leukemias would die more rapidly if infected with P. berghei yoelli. Greenwood suggested that certain human diseases known to have a strong immunological component have a lower incidence in Nigeria, an area holoendemic for P. falciparum. He then studied the response to adjuvant disease, a disease mediated by cellular hypersensitivity, where he found a decrease in the level of arthritis, and also he studied NZB mice, thought to be a model for Systemic Lupus Erythematosus. The malaria infected mice had a much prolonged course and although they had a Coomb's antibody and hemolytic anemia did not get glomerulonephritis. This has been recently confirmed by Welton.

Other studies have shown that germ free mice have a more striking increase in IgG when infected with plasmodia. Barker showed that in unsensitized mice there is a marked increase in the baseline SRBC plague forming cells after malaria and that under the appropriate conditions saw an increase in spleen cells producing antibody to bacteriophage after sensitization during malaria infection. The difference between this study and those using SRBC as an antigen is the way it is processed.

In this paper, the effect of plasmodium infection on the immune response in rats was studied by several methods. Antibody formation to SRBC was measured after infection with P. berghei by microagglutination. Both the mercaptoenthanol sensitive and resistant antibody were measured, giving an estimation of 19S and 7S immunoglobulins. No difference was detectable between the infected and the control for both 7S and 19S antibody. Nor was there any apparent difference whether the malaria was treated or not. These results are parallel to those of Greenwood et al. in mice, although different from that of Salaman et al. and Barker. The reason for the different results is due to the differences in measuring the antibody. The earlier workers used the plague formation assay which measures the ability to make antibody at a given point in time. The microagglutination method used here tends to average these periods and gives an impression of ability to make antibody over a course of time. Thus, while the more specific test shows short-lived depressions of the ability to make an antibody the overall production is unchanged.

In the next series of experiments a more complex reaction to DNCB, was measured. This agent is a highly potent contact sensitizing reagent, however, in the doses used in this experiment, 0.25% to 1% the principle reaction is inflammatory and the predominant cell seen in the lesion is polymorphonuclear cell. Very few lymphocytes were seen and collections of lymphoctyes are rare. When the DNCB was given either before or after the malaria infection, there was no apparent difference between control and infected animals.

Then a study was made of systems which function largely through cellular hypersensitivity. This is a system in which arthritis is induced in rats by injecting Complete Freund's Adjuvant subcutaneously. The polyarthritis, which develops about 11 to 12 days later, is apparently mediated by a cellular hypersensitivity reaction to tuberculin. When the CFA was given a considerable time after the infection and when the malaria was subpatent although not cured, there was a non-consistent pattern in the arthritis response. One group was above and the other below the mean joint scores of the controls but neither was statistically different. In the groups given CFA simultaneously with, or only a few days later, P. Lerghei also showed no significant difference from the control animals.

However, when the CFA was given before infection with malaria, a rather unexpected result was obtained. If the malaria was given after CFA but before onset of the arthritis, there was a remarkable increase in the amount of the arthritis. Since adjuvant arthritis is variable, as noted before, this result was confirmed using a larger number of animals and making greater efforts to eliminate bias. Again, the same result was obtained, that is, increased arthritis in animals infected before the onset of arthritis.

By contrast, there was no difference between the infected animals and control with respect to tuberculin sensitivity. Reaction of the adjuvant arthritis but not the tuberculin reaction may be explained in a couple of different ways. Arthritis may be a more sensitive indicator of cellular hypersensitivity in the rat or it may reflect pathways that are not involved in delayed hypersensitivity. Neither of these possibilities can be excluded. However, there is evidence that the arthritis has an inflammatory component which can be separately suppressed. It is possible that certain stimuli which enhance the reticulo-endothelial system (RES) at critical times will increase adjuvant arthritis, since a similar enhancement can be seen with the Fab fragment of ATG, which is not immunosuppressive, but causes stimulation of lymphocytes in vitro. More work is planned on the study of this phenomenon.

These results are in partial conflict with the data of Greenwood *et al*. This can be explained on the basis that different strains of malaria were used, the critical timing of the malaria infection differences in rat stains used or the variability of adjuvant arthritis itself. It appears from the data presented that when given at a critical period, *P. berghei* increases the responsiveness of rats to certain stimuli.

This altered responsiveness in the face of malaria infection despite the increased arthritis, there was no increased response to DNCB. This difference is probably due to the short lived nature of the DNCB response (less than 72 hours) compared to the more chronic nature of the arthritis with its prolonged "incubation" period.

The mechanisms involved in this altered response remain unclear. Greenwood *et al*. suggest there might be a defect in dendritic macrophage processing of antigen by the RES in the mice infected. He does not, however, mention parasitemias in his animals. Lucia and Nussenzweig showed that carbon clearance was not increased unless the parasitemia was over 10%. It is thus hard to implicate altered phagocytosis as the mechanism for the enhanced response presented in this paper since the parasite level was considerably below this. Speculation that there was increased processing of antigen by stimulated macrophages would be a tempting explanation. However, there is little information on the role of the dendritic macrophage in cellular immunity. It is possible that subtle changes here may be important in the host's non-specific RES responses. Obviously, more work is needed in this area to elucidate the exact mechanism.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6525	72 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
71 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	63713A	3A663713D829	00	132
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Clinical Studies of Human Malaria (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 2600 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		
	72	1	35
	CURRENT		
	73	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

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

21. GENERAL USE

Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicine

ADDRESS:
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Canfield, LTC C. J.
TELEPHONE: 202-576-3268
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Hall, LTC A. P. DA
NAME:

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell

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

23. (U) Study pathophysiology of acute falciparum and vivax malaria, assess various modes of antimalarial therapy with respect to clinical responses and radical cure.

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.

25. (U) 71 07 - 72 06 Admissions to Walter Reed General Hospital for malaria infections have virtually ceased. Altogether, 21 patients with recrudescent falciparum malaria were treated with the investigational drugs, WR 33063 and WR 30090. All responded promptly and were cured. Coordination of the various volunteer centers and field investigations of the new drugs and drug combinations continue. Final analysis of the study from Vietnam with these 2 drugs showed both to be effective in 90 per cent of naturally acquired acute falciparum malaria. Blood level determinations of WR 33063 showed the single patient who failed to respond also have the lowest serum levels. A new Burmese strain of P. falciparum has been introduced into the volunteer test program and is presently being characterized. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132 Clinical studies of human malaria

Investigators.
Principal: LTC Craig J. Canfield, MC
Associate: LTC Anthony P. Hall, MC

Description.

The objective of this work unit is 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, and secure new strains of malaria for introduction into the volunteer test program. In addition, the various aspects of the pathophysiology of the disease have been studied.

Progress.

Admissions to Walter Reed General Hospital for acute or recrudescent malaria have virtually ceased. Recrudescent falciparum malaria has been treated with the investigational drugs, WR 33063 and WR 30090. Thus far, a total of 13 patients were treated with WR 33063 and 7 with WR 30090. All patients responded to therapy and were cured. The results of these studies and the field trial in Vietnam with these agents are being prepared for publication.

A prospective study of the treatment of recrudescent falciparum malaria in Vietnam with intravenous quinine and oral pyrimethamine and sulfisoxazole showed an 80% cure rate compared with a 33% cure rate for standard oral quinine, pyrimethamine, and sulisoxazole. The results of these studies have been accepted for publication.

A subsequent study of comparative blood levels achieved with oral or intravenous quinine showed significantly higher blood levels with intravenous quinine despite a 10% lower dose. These results were presented and have been published in abstract form.[1]

A study of the ethnic difference in susceptibility to falciparum malaria and subsequent recrudescence rates has been completed. Black Americans in Vietnam were less likely to develop acute falciparum malaria and if they did become infected, they were more likely to be cured with a single course of therapy. These studies were presented at the annual meeting of the American Society of Tropical Medicine and Hygiene and have been accepted for publication.

Project 3A663713D829 MALARIAL PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132 Clinical studies of human malaria

Literature Cited.

Publications:

1. Hall, A. P., Czerwinski, A. W., Madonia, E. C., and Evensen, K. L. Plasma quinine levels following tablets, capsules, or intravenous infusion. Clin. Pharmacol. Ter., 13:140, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6534	72 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. DOD'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	H.Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63731A	3A663713D829	00	135
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG114(F)			

11. TITLE (Precede with Security Classification Code)

(U) Experimental Pathology and Metabolism of Plasmodia (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 26 00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 09	72 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: Not Applicable
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 71	2	30
FISCAL YEAR CURRENT 72	2	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Pathology
ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Takeuchi, A., M.D.
TELEPHONE: 202-576-2677
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Jervis, H., Dr.Nat.Sc.
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U)Malaria; (U)Plasmodium; (U)Erythrocyte; (U)Phospholipid; (U)Biosynthesis

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 metabolic pathways in malarial parasites. To isolate, purify and identify by chemical, physical and morphologic means the subcellular components of the malarial parasites.

24. (U) Biophysical, biochemical and morphologic techniques are employed.

25. (U) 71 07-72 06 A paper, Ribosomes of the malarial parasites, Plasmodium knowlesi. I. Isolation, activity and sedimentation velocity was published in the Journal of Comparative Biochemistry and Physiology. Three papers dealing with lipid metabolism P. knowlesi were published in the Journal of Comparative Biochemistry and Physiology. In collaboration with the Division of Communicable Diseases and Immunology, a paper, Experimental infection with Plasmodium falciparum in Aotus monkeys. II. Observations on host pathology, was published.
Due to the departure of two investigators from this department, activity of the work unit 135 has been discontinued because of the lack of investigators capable of conducting this type of research. The study of lipid metabolism in plasmodia is being carried out by Dr. Rokus A. deZeeuw through an extramural research.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 June 72.

*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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 135 Experimenta pathology and metabolism of plasmodia
Malaria

Investigators.
Principal: Akio Takeuchi, M. D.
Associate: Helen R. Jervis, Dr. Nat. Sc.

Problem

To define the biosynthesis and metabolic pathway of lipid and phospholipid, one of the most important cell constituents of plasmodia.

Background

Earlier fine structural studies on various types of plasmodia demonstrated that cell walls and cytoplasmic membranes were rapidly synthesized and transformed (1,2,3,4). It was, therefore, postulated that the kinetics of phospholipid and lipid metabolism could be parallel to the membrane biosynthesis. In order to document this hypothesis, a series of investigations were initiated; the first step was isolation and fractionation of plasmodia which was solved with two years concentrated effort (4). With this new method, ribosomes of malarial parasites were successfully analyzed for the first time (5).

In subsequent investigations, the quantitative analysis of small samples of purified parasite lipid was successfully accomplished. This work was conducted with the assistance of Rokus A. deZeeuw, Analytical Chemist, State University, Groningen, Netherland, and his professor, Van Deenen, world expert on lipid research.

Approach to the Problem

To analyze the kinetics of phospholipids and lipids of isolated plasmodia by a combination of analytic chemical and biochemical methods.

Results and Discussion

Three papers dealing with lipid and phospholipid metabolism in malarial parasites have been published. Because two investigators departed this department, the research activities of metabolism of malarial parasites had to stop due to lack of investigators capable of conducting this type of research in the Division of Pathology.

1. The first paper entitled "Lipid composition of Plasmodium knowlesi membranes: Comparison of parasites and microsomal subfractions with host erythrocyte membranes" describes:

a. Plasmodium knowlesi has significantly more phospholipid and less cholesterol (phospholipid: cholesterol ratio = 5:2) than the host erythrocyte membranes of the Rhesus monkey (phospholipid: cholesterol ratio = 2:1).

b. Although both parasite and host red cell have the same major phospholipid components (phosphatidylcholine and phosphatidylethanolamine), other phospholipids differ significantly.

c. The red cell contains 12-14% sphingomyelin and 12% phosphatidylserine, whereas the parasite contains only 2-3% sphingomyelin, 8-10% phosphatidylinositol, and barely detectable levels of phosphatidylserine.

2. The second paper, "Incorporation of ^{33}P-orthophosphate into membrane phospholipids of Plasmodium knowlesi and host erythrocytes of Macaca mulatta," describes:

a. Incorporation of ^{33}P-orthophosphate into membrane phospholipids is eight to tenfold higher in Plasmodium knowlesi than in its host erythrocyte, using an in vitro system of short term incubation.

b. In the malarial parasite, ^{33}P activity is found primarily in the three major phospholipid classes, phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine, although the highest specific activities occur in two minor phospholipid classes, phosphatidic acid and phosphatidylglycerol. Host erythrocytes, in contrast, incorporate significant amounts of ^{33}P into one type of a phospholipid, phosphatidic acid, thus resembling erythrocytes of other mammalian species.

c. The dependency of parasite ^{33}P-phospholipid incorporation upon host erythrocyte glycolysis is suggested by the effects of various metabolic substrates and inhibitors.

3. In the third paper, "Incorporation of ^{14}C-labelled fatty acids into lipids of rhesus erythrocytes and Plasmodium knowlesi in vitro," the following result was obtained:

Project 3A663713D829 MALARIAL PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132 Clinical studies of human malaria

Literature Cited.

Publications:

1. Hall, A. P., Czerwinski, A. W., Madonia, E. C., and Evensen, K. L. Plasma quinine levels following tablets, capsules, or intravenous infusion. Clin. Pharmacol. Ter., 13:140, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6534	72 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	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	H.Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63731A	3A663713D829	00	135
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG114(F)			

11. TITLE (Precede with Security Classification Code)

(U) Experimental Pathology and Metabolism of Plasmodia (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 26 00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 09	72 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: Not Applicable d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	30
	CURRENT 72	2	30

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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ADDRESS:

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL, E.L.

TELEPHONE: 202-576-3551

19b. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Pathology

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Takeuchi, A., M.D.

TELEPHONE: 202-576-2677

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Jervis, H., Dr.Nat.Sc.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U)Malaria; (U)Plasmodium; (U)Erythrocyte; (U)Phospholipid; (U)Biosynthesis

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 metabolic pathways in malarial parasites. To isolate, purify and identify by chemical, physical and morphologic means the subcellular components of the malarial parasites.

24. (U) Biophysical, biochemical and morphologic techniques are employed.

25. (U) 71 07-72 06 A paper, Ribosomes of the malarial parasites, Plasmodium knowlesi. I. Isolation, activity and sedimentation velocity was published in the Journal of Comparative Biochemistry and Physiology. Three papers dealing with lipid metabolism P. knowlesi were published in the Journal of Comparative Biochemistry and Physiology. In collaboration with the Division of Communicable Diseases and Immunology, a paper, Experimental infection with Plasmodium falciparum in Aotus monkeys. II. Observations on host pathology, was published.
Due to the departure of two investigators from this department, activity of the work unit 135 has been discontinued because of the lack of investigators capable of conducting this type of research. The study of lipid metabolism in plasmodia is being carried out by Dr. Rokus A. deZeeuw through an extramural research.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 June 72.

(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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 135 Experimental pathology and metabolism of plasmodia
Malaria

Investigators.
Principal: Akio Takeuchi, M. D.
Associate: Helen R. Jervis, Dr. Nat. Sc.

Problem

To define the biosynthesis and metabolic pathway of lipid and phospholipid, one of the most important cell constituents of plasmodia.

Background

Earlier fine structural studies on various types of plasmodia demonstrated that cell walls and cytoplasmic membranes were rapidly synthesized and transformed (1,2,3,4). It was, therefore, postulated that the kinetics of phospholipid and lipid metabolism could be parallel to the membrane biosynthesis. In order to document this hypothesis, a series of investigations were initiated; the first step was isolation and fractionation of plasmodia which was solved with two years concentrated effort (4). With this new method, ribosomes of malarial parasites were successfully analyzed for the first time (5).

In subsequent investigations, the quantitative analysis of small samples of purified parasite lipid was successfully accomplished. This work was conducted with the assistance of Rokus A. deZeeuw, Analytical Chemist, State University, Groningen, Netherland, and his professor, Van Deenen, world expert on lipid research.

Approach to the Problem

To analyze the kinetics of phospholipids and lipids of isolated plasmodia by a combination of analytic chemical and biochemical methods.

Results and Discussion

Three papers dealing with lipid and phospholipid metabolism in malarial parasites have been published. Because two investigators departed this department, the research activities of metabolism of malarial parasites had to stop due to lack of investigators capable of conducting this type of research in the Division of Pathology.

1. The first paper entitled "Lipid composition of Plasmodium knowlesi membranes: Comparison of parasites and microsomal subfractions with host erythrocyte membranes" describes:

a. Plasmodium knowlesi has significantly more phospholipid and less cholesterol (phospholipid: cholesterol ratio = 5:2) than the host erythrocyte membranes of the Rhesus monkey (phospholipid: cholesterol ratio = 2:15).

b. Although both parasite and host red cell have the same major phospholipid components (phosphatidylcholine and phosphatidylethanolamine), other phospholipids differ significantly.

c. The red cell contains 12-14% sphingomyelin and 12% phosphatidylserine, whereas the parasite contains only 2-3% sphingomyelin, 8-10% phosphatidylinositol, and barely detectable levels of phosphatidylserine.

2. The second paper, "Incorporation of ^{33}P-orthophosphate into membrane phospholipids of Plasmodium knowlesi and host erythrocytes of Macaca mulatta," describes:

a. Incorporation of ^{33}P-orthophosphate into membrane phospholipids is eight to tenfold higher in Plasmodium knowlesi than in its host erythrocyte, using an in vitro system of short term incubation.

b. In the malarial parasite, ^{33}P activity is found primarily in the three major phospholipid classes, phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine, although the highest specific activities occur in two minor phospholipid classes, phosphatidic acid and phosphatidylglycerol. Host erythrocytes, in contrast, incorporate significant amounts of ^{33}P into one type of a phospholipid, phosphatidic acid, thus resembling erythrocytes of other mammalian species.

c. The dependency of parasite ^{33}P-phospholipid incorporation upon host erythrocyte glycolysis is suggested by the effects of various metabolic substrates and inhibitors.

3. In the third paper, "Incorporation of ^{14}C-labelled fatty acids into lipids of rhesus erythrocytes and Plasmodium knowlesi in vitro," the following result was obtained:

a. Plasmodium knowlesi rapidly incorporates large amounts of ^{14}C-labelled fatty acids from medium during a two-hour in vitro incubation, at levels ten to twelve times that of host erythrocyte membranes incubated under the same conditions.

b. In both the parasite and its host erythrocyte, 80-90% of the incorporated fatty acids appear in phospholipids.

c. The rapid incorporation of exogenous performed fatty acids into complex lipids of the parasite, together with prior evidence of minimal de novo biosynthesis of fatty acids by the parasite, is consistent with the interpretation that the malarial parasite depends largely upon its host as a source of fatty acids.

In addition, a study on experimental Plasmodium falciparum infections in the owl monkey was published in collaboration with DCD&I (Jervis et al 1972).

Conclusion and Recommendation

Due to the departure of two investigators from this department, activities of work unit 135 have discontinued because of the lack of investigators capable of conducting this type of research in the Division of Pathology. The study of lipid metabolism in plasmodia is currently being conducted by Dr. Rokus A. deZeeuw through extramural research.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 135 Experimental pathology and metabolism of plasmodia

Literature Cited.

References:

1. Aikawa, M., Cook, R.T., and Sprinz, H.: Fine structure of erythrocytic stages of Plasmodium knowlesi. Zeitschrft. Zellforsch v. mikrosk. Anatomie. 100: 271, 1969.

2. Terzakis, J.A., Sprinz, H., and Ward, R.A.: The transformation of the Plasmodium gallinaceum Oocyst in Aedes Aegypti mosquitoes. J. Cell Biol. 34: 311, 1967.

3. Ladda, R.L.: New insights into the fine structure of rodent malarial parasites. Milit. Med. 134 (Supplement): 825, 1969.

4. Cook, R.T., Aikawa, M., Rock, R.C., Little, W., and Sprinz, H.: The isolation and fractionation of Plasmodium knowlesi. Milit. Med. 134 (Supplement): 866, 1969.

5. Cook, R.T., Rock, R.C., Aikawa, M., and Fournier, M.J.: Ribosomes of the malarial parasite, Plasmodium knowlesi. I. Isolation, activity and sedimentation velocity. Comp. Biochem. Physiol. 39I: 897, 1971.

Publications:

1. Rock, R.C., Standefer, J., and Little, W.: Incorporation of ^{33}P-orthophosphate into membrane phospholipids of Plasmodium knowlesi and host erythrocytes of macaca mulatta. Comp. Biochem. Physiol. 40B: 543, 1971.

2. Rock, R.C.: Incorporation of ^{14}C-labelled fatty acids into lipids of rhesus erythrocytes and Plasmodium knowlesi in vitro. Comp. Biochem. Physiol. 40B: 893, 1971.

3. Rock, R.C., Standefer, J.C., Cook, R.T., Little, W., and Sprinz, H.: Lipid composition of Plasmodium knowlesi membranes: Comparison of parasites and microsomal subfractions with host rhesus erythrocyte membranes. Comp. Biochem. Physiol. 38B: 425, 1971.

4. Jervis, H.R., Sprinz, H., Johnson, A.J. and Wellde, B.T.: Experimental infection with Plasmodium falciparum in Aotus monkeys. II. Observations on host pathology. Amer. J. Trop. Med. Hyg. 21: 272, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6535	72 07 01	DD DR&E(AR)636

3 SUMMARY	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
71 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	63713A	3A663713D829	00	146
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11 TITLE (Precede with Security Classification Code)

(U) METABOLIC AND ENZYMATIC STUDIES OF NORMAL AND MALARIA INFECTED RED CELLS (09)

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 BIOLOGY

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
66 12	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 72	2	60
FISCAL YEAR CURRENT 73	2	60

19 RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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RESPONSIBLE INDIVIDUAL
NAME: Buescher COL, E. L.
TELEPHONE: 202-576-3551

20 PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
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ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Provide NAME, GRADE, TITLE, and Academic Institution)
NAME: McCormick, G. J., Ph.D.
TELEPHONE: 202-576-2447
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME
NAME DA

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 title of each with Security Classification Code.)

23. (U) Document metabolic alterations of human and red animal red blood cells when infected with malaria parasites and to assess the effect of antimalarial drugs on these alterations in order to develop new drugs effective against resistant falciparum malaria.

24. (U) Measure the effect of antimalarial drugs on morphologic growth, 14-C adenosine 14-C methionine or 14-C orotic acid incorporation during in vitro schizogony and observe utilization of metabolic precursors of nucleic acids; to measure folic acid reductase in parasite suspensions.

25. (U) 71 07 - 72 06 In vitro culture of P. knowlesi parasites has continued, both as a screening procedure for antimalarial activity and as an investigatory method for demonstration of drug potentiation. Analogs of purine bases, nucleosides and nucleotides are being screened and many of these compounds are effective in this system.

Using inhibition of 14-C orotic acid incorporation into DNA as a parameter for antifolic acid activity, drug synergism studies have shown that combinations of quinine with chloroquin and with pyrimethamine and trimethoprim with 5-fluoroorotic acid have additive effects in this system. Potentiation has been shown by combinations of sulfalene with trimethoprim and with an effective antimalarial quinazoline derivative. The results have corresponded to those expected on the basis of theory and experience in vivo. For technical rep.., see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Ju. 72.

* Applicable 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 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 136 Metabolic and enzymatic studies of normal and malaria infected red cells

Investigators.
Principal: COL Craig J. Canfield, MC
Associates: Gerald J. McCormick, Ph.D., Esther P. Jorolan, Ph.D., and Gloria P. Willet

Description.

The objective of this work unit is to 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 resistance falciparum malaria.

Progress.

A description of the application of the *in vitro* *P. knowlesi* malaria culture system to the study of antifolic acid activity of drugs has been published (1). This system is based upon the inhibition of incorporation of radioactivity from ^{14}C-orotic acid into DNA of the parasites. Dose response curves were reported for a sulfanilamide (sulfalene) and several inhibitors of dihydrofolic acid reductase. The counter-effect of p-aminobenzoic, folic and folinic acids against inhibitory concentrations of sulfalene and pyrimethamine were reported.

Study of synergism between antimalarial drugs has continued, using the ^{14}C-orotic acid system. Combinations of chloroquin with quinine, pyrimethamine with quinine, and trimethoprim with 5-fluoro-orotic acid were found to have additive effects in this system. Potentiation of effect was observed in combinations of sulfalene with trimethoprim and sulfalene with a derivative of quinazoline. The combination of sulfalene with the quinazoline derivative was also potentiating against *P. falciparum* in chimpanzee blood *in vitro*. These studies were presented at the Malaria Workshop at WRAIR and will be published as part of the proceedings.

Analogs of purine bases and nucleosides are being screened for antimalarial activity as measured by inhibition of growth and of incorporation of activity from ^{14}C-adenosine and ^{14}C-orotic acid into RNA and DNA and ^{14}C-methionine into protein of the parasites. Many of these compounds are effective in this system. One feature which apparently is associated with activity is the hydroxyl group at the 3'-(ribosyl)-position of adenosine. Analogs in which this group is missing (3'-deoxyadenosine, "Cordycepin"), replaced by an amino group, or sterically different (xylosyl-adenine) have shown inhibitory effects.

In a collaborative study with the Division of Experimental Pathology and Dr. de Zeeuw at State University, Groningen, The Netherlands, lipid fractions are being prepared from normal and malaria-infected plasma and red blood cells and from parasites. Results of lipid component analysis from several of these preparations were presented by Dr. de Zeeuw at the Malaria Workshop and will be published as part of the proceedings.

The in vitro cultivation system is being employed with the intraerythrocytic parasite babesia rodhaini in rodent (mouse and rat) blood. Using inhibition of incorporation of ^{14}C-methionine into protein as the parameter, various drugs are being evaluated. Pentamidine isethionate, an effective drug in vivo, was effective in this system in vitro. Studies of growth, utilization of nucleic acid precursors and of amino acids for protein synthesis are in progress.

In the study of dihydrofolic acid reductase, the assay using ^{14}C-folic acid as substrate has not successfully demonstrated the presence of the enzyme in P. knowlesi in rhesus monkey blood. Modifications of the technique are being attempted.

Project 3A063713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 136 Metabolic and enzymatic studies of normal and malaria infected red cells

Literature Cited.

Publications:

1. McCormick, G. J., Canfield, C. J., and Willet, G. P.: Plasmodium knowlesi: In vitro evaluation of antimalarial activity of folic acid inhibitors. Exp. Parasit. 30, 88-93, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6482	72 07 01	DD DR&E(AR)636

3 DATE PREV SUM'Y	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SE UNIT	7 REGRADING	8A ORG'N INSTR'N	8b SPECIFIC DATA-CONTRACTOR ACCESS	9 LEVEL OF SUM
71 07 01	D. CHANGE	U	U	NA	GP	[X] YES [ ] NO	A. WORK UNIT

10 NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A061371BD89	00	171
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 119 (U)			

11 TITLE (Precede with Security Classification Code)

(U) General Pharmacology of Antimalarial Drugs (09)

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
70 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)
PRECEDING FISCAL YEAR 72	8	350
CURRENT FISCAL YEAR 73	8	350

19 RESPONSIBLE DOD ORGANIZATION

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20 PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted] DA

PII Redacted

21 GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS
NAME: Einheber, A., Ph.D.
NAME: Rozman, R., Ph.D.

22 KEYWORDS (Precede EACH with Security Classification Code)

(U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicity
(U) Drug Metabolism; (U) Antimalarial Drugs; (U) Preclinical Pharmacology

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

23. (U) The technical objective is to develop and to exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purpose of these studies is to provide a basis for predicting the response of soldiers to antimalarials in a military environment and to fulfill requirements for submission of IND for clinical trials of new antimalarials.

24. (U) The approach will be to study the effects of antimalarial drugs in health, animals and to study the handling of antimalarial drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The effects of antimalarial drugs in diseased or injured animals will be studied in order to determine the effects of the drugs on disease and injury. The handling of antimalarial drugs by diseased and injured animals will be studied in order to determine the effect of disease or injury upon pharmacokinetics in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).

25. (U) 71 07 - 72 06 Pharmacodynamics studies in dog and/or monkeys were completed for WR 142,490, WR 158,122, WR 122,455 and WR 113,618. Pharmacokinetics studies using radioactivity labelled drugs in healthy mice were completed for WR 161,784, WR 125,676, WR 159,967, WR 122,458, WR 142,490 and WR 158,122. A test system for evaluating efficacy and toxicity of new antimalarial drugs in mice infected with P. berghei was standardized. A test system was standardized to evaluate the influence of induced hepatic microsomal enzymes on the efficacy and toxicity of new antimalarial drugs in normal and infected mice. For technical report see WRAIR Annual Progress Report, 1 July 1971-30 June 1972 and Claims for Investigational Exemptions for the appropriate New Drugs.

* Available to contractors upon originator's approval

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

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 171 General pharmacology of antimalarial drugs

Investigators
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC G. E. Demaree, CPT R. Caldwell, Dr. A. Einheber, Dr. R. Rozman, A. Berman

1. The general pharmacology of WR 158,122, WR 113,618 and WR 171,952 was studied in dogs. The results of these studies were reported in the appropriate claims for investigational exemptions for New Drugs.

2. The absorption and excretion of WR 142,490 and WR 158, 122 was reported in the appropriate claims for investigational exemptions for New Drugs.

3. Blood Levels of Phenanthrene Methanol Antimalarials.

Four phenanthrene methanols were examined separately for blood levels in mice receiving 80 mg/kg P.O. as an aqueous suspension.

a. WR 161,784 - ^{14}C:

The plasma level was maximal at 4 hours (the first sample) and decreased rapidly during the first 24 hours. There were still minimal detectable amounts evident in the plasma at the end of 10 days. WR 161,784 was not concentrated in mouse red blood cells.

b. WR 125,676 - ^{14}C:

Plasma level was maximal at 6 hours and decreased very rapidly thereafter. After 2 days there was essentially no detectable amount of radioactivity present in the plasma. WR 125,676 was not concentrated in mouse red blood cells.

c. Plasma level of WR 159,967 peaked at 8 hours. The red cell level remained relatively high for 2 days, with plasma level remaining at high levels for 4 days. However, there were still detectable amounts of radioactivity in both mouse red blood cells and plasma for 10 days. The maximum concentration in red cell was also reached at 8 hours.

d. WR 122,455 - ^{3}H:

Red cell and plasma levels both peaked at 12 hours. The red cell level of radioactivity was consistently higher than that of the plasma throughout the 14 day period. However, there was a relatively high level throughout the first 7 days, with gradual decrease at 10-14 days in both the plasma and red cells.

An additional experiment was done with WR 122,455 - ^{3}H to determine if blood levels of drug were proportional to drug dose. The mice were given either 5, 20, or 80 mg/kg orally, and blood levels were followed from 6 to 72 hours. A linear relationship exists between blood levels and drug doses. The 80 mg/kg dose showed blood levels of approximately 4 times that of the 20 mg/kg dose and 16 times that of the 5 mg/kg dose. The 20 mg/kg dose gave blood levels approximately 4 times that of the 5 mg/kg dose.

4. Influence of pretreatment of mice with phenobarbital on the toxicity and efficacy of antimalarial drugs.

a. Background:

The technical objective is to develop and to exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purpose of these studies is to provide a basis for predicting the response of soldiers to antimalarials in a military environment and to fulfill requirements for submission of IND for clinical trials of new antimalarials. Drug metabolizing enzymes are induced by pretreatment with phenobarbital. In these studies the roles of metabolites in toxicity and efficacy are assessed indirectly. The results may be used to point to antimalarials that may be derived as metabolites of new chemicals. Adjunctive therapy may also be employed to improve the therapeutic index of new or established antimalarials through metabolic modifications.

These experiments are designed to screen for potential metabolic roles in the toxicity and efficacy of candidate antimalarials.

b. Methods:

Female CD-1 mice 7-9 weeks old were intraperitoneally inoculated with freshly prepared inocula of drug-sensitive KBG 173 strain of P. berghei to produce infection. Antimalarial drugs were given in a solution or suspension in

0.9% saline containing 0.4% polysorbate 80 and 0.2% methylcellulose (MCT) as a gastric gavage. Control animals received the vehicle only. All doses are given as mg/kg of salt.

Liver microsomal enzymes were induced by three daily I.P. injections of phenobarbital sodium (100 mg/kg) dissolved in water; controls received three daily I.P. injections of water. This phenobarbital pretreatment reduced hexobarbital sleeping time from 30 minutes to about 5 minutes. The mice infected on the first day of phenobarbital treatment (day 0) and the antimalarial drugs were given on day 3, the day following the third phenobarbital injection. Toxicity studies were performed on uninfected animals.

Parasitemia was estimated on days 3, 7, 10 and 14 from at least 250 RBC in thin blood films expressed as percentage of cells parasitized.

c. Results:

The pretreatment of uninfected mice with phenobarbital reduced acute toxicity of primaquine, proguanil and cycloguanil but not WR 40,070 (Table 1).

Primaquine given as a single large oral dose produced a swelling of the snout and tongue and grossly observable necrosis of the diaphragm (confirmed by microscopy). There were also some crusty secretions and dryness of the eyes observed after large doses of primaquine. These signs were also ameliorated by pretreatment with phenobarbital.

Phenobarbital alone had no effect on the course of parasitemia; it did not significantly affect the efficacy of WR 158,122 or primaquine. Phenobarbital pretreatment reduced the efficacy of WR 142,490 and increased the antimalarial potency of proguanil or cycloguanil (Tables 2, 3, and 4).

d. Discussion:

These data clearly demonstrate the feasibility of the use of an in vivo, integrated test system to screen and evaluate the potential metabolites for more potent or less toxic antimalarials. These results can also be interpreted to indicate a potential additional adjunctive role for phenobarbital in the therapy of malaria. The fact that the toxicity of primaquine, cycloguanil and proguanil is reduced by phenobarbital pretreatment at the same time that

the antimalarial potencies of proguanil and cycloguanil are increased while that of primaquine is minimally depressed suggests that the metabolites of these agents are more potent than or as potent as the parent compound as well as being less toxic. The nature of these metabolites should be investigated in order to isolate, synthesize and test these potentially superior antimalarial drugs.

The fact that phenobarbital fails to potentiate the potency of primaquine and virtually abolishes the efficacy of WR 142,490 argues against but does not rule out a potential adjunctive role for phenobarbital. This potential clinical application of phenobarbital should be considered as these experiments progress.

TABLE 1

Effect of Phenobarbital Pretreatment on the Toxicity of Some Antimalarials

Drug	Dose (mg/kg)	Pre-treatment	Percent mortality	Number of animals
WR 40,070	720	W	63	30
		P	67	30
Proguanil	100	W	62	50
		P	18*	50
	160	W	93	30
		P	63*	30
Cyclo-guanil	775	W	75	12
		P	17*	12
	1280	W	100	12
		P	42*	12
Primaquine	160	W	8	12
		P	0	12
	320	W	61	23
		P	0*	23
	640	W	90	10
		P	10*	10
	95	W	75	12
		P	17*	12
	110	W	75	12
		P	33*	12

*Significantly less than water pretreatment group ($p < .05$) by t test.

TABLE 2

Effect of Phenobarbital Pretreatment on Antimalarial Activity of WR 158,122 or WR 142,490 in Mice Infected with P. berghei. (5×10^5 Parasitized RBC)[b]

Drug	Dose (mg/kg)	Pre-treatment[a]	Responses to Therapy[d] day 3	day 7	day 10	day 14
MCT[c]	-	W	2.4(12)	86.4(8)	- (1)[f]	- (1)
MCT	-	P	2.4(12)	75.2(11)	- (1)	- (1)
WR 158,122	20	W	2.2(12)	3.4(12)	28.4(12)	86.4(8)
WR 158,122	20	P	2.2(12)	2.2(12)	17.8(12)	43.2(11)
MCT	-	W	2.0(12)	72.2(12)	- (2)	- (0)
MCT	-	P	2.0(12)	73.2(11)	52.4(3)	- (1)
WR 158,122	20	W	2.2(12)	2.6(12)	12.4(12)	43.2(7)
WR 158,122	20	P	2.4(12)	3.8(12)	25.2(11)	49.2(9)
WR 142,490	10	W	2.2(12)	0.8(12)	1.4(12)	20.4(12)
WR 142,490	10	P	2.2(12)	7.2.(12)[e]	75.6[e](3)	- (1)
MCT	-	W	1.6(12)	76.0(10)	- (1)	- (0)
MCT	-	P	2.0(12)	71.4(10)	- (1)	- (0)
WR 142,490	20	W	2.2(12)	0.8(12)	0.8(12)	2.6(12)
WR 142,490	20	P	2.0(12)	34.6[e](12)	44.8(6)[e]	62.8(4)[e]

a. Animals received water (W), 1% body weight or Phenobarbital Sodium 100 mg/kg (P) for 3 days prior to therapy (days 0, 1, 2).
b. Number of parasitized RBC injected I.P.
c. Vehicle only.
d. Median parasitemia as percent parasitized RBC. Numbers in parentheses represent number of surviving mice.
e. Significantly greater than water pretreatment group (Sign rank test).
f. Parasitemia values not reported for less than 3 survivors.

TABLE 3

Effect of Phenobarbital Pretreatment on Antimalarial Activity of Primaquine in Mice Infected with P. berghei

Primaquine Dose (mg/kg)	Pre-treatment[a]	Inoculum[b]	Responses to Therapy[d] day 3	day 7	day 10	day 14
0[c]	W	10^7	12.0(12)	82.2(10)[e]	- (0)	- (0)[g]
0	P	10^7	12.0(12)	80.4(10)[e]	- (0)	- (0)
160	W	10^7	14.0(12)	1.0(12)[e]	1.4(12)	20.8(11)
160	P	10^7	12.6(12)	0.8(12)[e]	1.6(12)	42.4(10)
0	W	10^5	0.8(12)	58.4(11)	41.6(3)	- (1)
0	P	10^5	0.8(12)	54.0(11)	57.2(4)	- (2)
160	W	10^5	0.8(12)	0.6(12)	1.2(11)	10.8(10)
160	P	10^5	0.8(12)	0.8(12)	1.2(12)	20.4(9)
0	W	5×10^5	2.0(12)	72.2(12)	- (2)	- (0)
0	P	5×10^5	2.0(12)	73.2(11)	52.4(3)	- (1)
40	W	5×10^5	2.2(12)	0.8(12)	8.2(12)	37.6(7)
40	P	5×10^5	2.4(12)	1.6(12)[f]	12.4(12)[f]	51.6(7)[f]
0	W	5×10^5	1.6(12)	76.0(10)	- (1)	- (0)
0	P	5×10^5	2.0(12)	71.4(10)	- (1)	- (0)
20	W	5×10^5	1.8(12)	3.0(12)	21.2(11)	48.8(7)
20	P	5×10^5	2.0(12)	18.6(12)[f]	69.0(12)[f]	70.4(5)[f]

a. Mice received water (W), 1% of body weight or phenobarbital sodium (P), 100 mg/kg for 3 days prior to therapy (days 0, 1, 2).
b. Number of parasitized RBC injected I.P.
c. Vehicle only.
d. Median parasitemia as percent parasitized RBC. Numbers in parentheses represent number of surviving mice.
e. Measurements made on day 6 because of severity of disease.
f. Significantly greater than water pretreatment group (Sign rank test).
g. Parasitemia values not reported for less than 3 survivors.

TABLE 4

Effect of Phenobarbital Pretreatment on the Antimalarial Activity of Proguanil or Cycloguanil in Mice Infected with P. berghei

Drug	Dose (mg/kg)	Inoculum	Pre-treatment	Responses to Therapy[d] day 3	day 7	day 10	day 14
MCT[a]	-	10^5[b]	W[c]	0.8(12)	42.2(12)	61.8(4)	- (1)[e]
	-	10^5	P	0.4(12)	40.0(12)	72.0(5)	- (2)
Cyclo-guanil	50	10^5	W	0.8(12)	3.2(12)	8.0(12)	52.0(11)
	50	10^5	P	0.8(12)	0.8(12)[f]	4.6(12)[f]	40.8(11)
Pro-guanil	50	10^5	W	0.4(12)	0.8(12)	6.0(12)	39.6(11)
	50	10^5	P	0.4(12)	1.2(12)	2.6(12)[f]	13.6(11)[f]
MCT	-	10^5	W	2.2(12)[f]	57.0(12)	45.8(4)	- (2)
	-	10^5	P	1.4(12)[f]	62.2(12)	59.6(5)	- (2)
Cyclo-guanil	50	10^5	W	1.6(12)	5.6(12)	19.8(12)	39.6(7)
	50	10^5	P	1.2(12)	1.2(12)[f]	6.8(11)[f]	26.4(9)[f]
Pro-guanil	50	10^5	W	1.2(12)	2.8(12)	17.0(12)	52.0(7)
	50	10^5	P	1.0(12)	0.8(12)[f]	3.6(12)[f]	18.0(9)[f]
MCT	-	5×10^5	W	2.0(12)	71.2(9)	68.0(3)	- (0)
	-	5×10^5	P	2.6(12)	71.8(10)	- (1)	- (1)
Cyclo-guanil	50	5×10^5	W	2.0(12)	4.2(12)	28.0(9)	68.0(7)
	50	5×10^5	P	2.0(12)	5.2(12)	24.4(9)	62.8(7)
Pro-guanil	50	5×10^5	W	2.2(12)	2.0(11)[f]	23.8(10)	48.8(4)
	50	5×10^5	P	2.0(12)	0.8(12)[f]	2.6(12)[f]	13.2(4)[f]

a. Vehicle only.
b. Number of parasitized RBC injected I.P.
c. Mice received water (W), 1% body weight or phenobarbital sodium (P), 100 mg/kg I.P. for 3 days prior to therapy (days 0, 1, 2).
d. Median parasitemia as percent parasitized RBC. Numbers in parentheses represent numbers of surviving mice.
e. Parasitemia values not reported for less than 3 survivors.
f. Significantly less than water pretreatment group (Sign rank test).

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 171, General pharmacology of antimalarial drugs

Literature Cited.

Publication:

Rozman, R., Berman, A., Gibson, R., and Hutchinson, A. Distribution and excretion of 2,4-diamino-5-piperonyl pyrimidine, a new antimalarial, in the mouse. The Pharmacologist 13:269, 1971.

PROJECT 3A062110A830
BIOSENSOR SYSTEMS

Task 00
Biosensor Systems

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6441	72 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
71 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	3A062110A830	00	055
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE (Procede with Security Classification Code)

(U) Development and Evaluation of Improved Biological Sensor Systems (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

001700 Animal Husbandry 011800 Operations

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 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 72	3.5	240
	CURRENT 73	4	260

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Detector System; (U) Dogs; (U) Genetics; (U) Selective Breeding

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 more intelligent and sensually acute dog which is physically and temperamentally better suited for military purposes than is now generally available.

24. (U) This study is being made in response to the approved (11 Dec 68) US Army QMDO, Detector System Military Dog (USACDC Action Control Number 12527). Critically evaluated AKC registered dogs were purchased as foundation stock. The progeny of these are closely evaluated by recognized tests designed to reveal the superior individual. These in turn are selectively bred and their offspring similarily tested, evaluated and accomplish the objective.

25. (U) 71 07 - 72 06 Forty-five litters produced 291 weaned puppies. Present kennel population consists of 245 German Shepherd Dogs. Following a decision to concentrate only on the German Shepherd, the breeding of German Short Haired Pointer-German Shepherd Dog crosses was terminated and the German Short Haired Pointers and Draathaars were sold at public auction. During the year a total of 337 dogs were transferred to other activities including the Mine/Tunnel Dog Training Center at Ft. Benning, DOD Dog Center at Lackland AFB, WRAIR, Ft. Monmouth, and the State Department. Puppy socialization and imprinting was intensified by beginning at 4 weeks of age rather than at 8 weeks. Improvements to the physical plant included the installation of an automatic watering system, construction of semi-protective roofing for key portions of the kennel areas and the building of a second confidence course. Consultant visits to this organization were made by national authorities. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 June 72.

*Available to contractors, upon originator's approval

Project 3A062110A830 BIO SENSOR SYSTEMS

Task 00, Bio Sensor Systems

Work Unit 055 Development and evaluation of improved biological sensor systems

Investigators

Principal: COL Merida W. Castleberry, VC
Associates: CPT Jeffrey M. Linn, VC; CPT Jerry F. Hardisty, VC; 1LT Stephen E. Scalera, MSC

OBJECTIVE: To develop a more intelligent and sensually acute dog which is physically and temperamentally better suited for military purposes than is now generally available.

BACKGROUND: This study is being made in response to the approved US Army QMDO, "Dector System, Military Dog", (USACDC Action Control Number 12527). Seven breeds of dogs, including crosses, were studied by the University of Maryland for behavioral evaluation and selection 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 breeding stock purchased especially for this purpose was selectively bred to produce superior progeny. These are in turn closely evaluated by recognized tests designed to reveal the superior individual. Line breeding combined with progeny testing of each generation is being used to accomplish the objective.

PROGRESS:

A. Breeding Program

1. Forty-five litters produced 291 weaned puppies.

2. Present kennel population consists of 245 German Shepherd dogs.

3. Three hundred and fifty-nine dogs were transferred to other activities or sold at public auction:

Walter Reed Army Institute of Research	286
Fort Benning, GA	19
Lackland Air Force Base, TX	16
Fort Monmouth, NJ	13
State Department	3
Public auction	22

4. Puppy evaluation: Evaluation procedures remained unchanged during the period of this report.

B. Special Projects

1. Evaluation of the German Short Haired Pointer, the German Wire Hair Pointer and crosses of these breeds with the German Shepherd Dog was discontinued. Those not shipped to Fort Benning, GA were sold at public auction. The advantages offered by the natural hunting instincts of these dogs was more than off-set by the difficulty of training them to sit-alert on mines and trip wires.

2. The game of ball retrieval or "fetch" has continued to be stressed as one of our principle means of socialization. A dog was partially trained to detect mines and trip wires by use of "forced retrieve", a training technique recommended by our consultant, Mr. W.R. Koehler. This dog was subsequently sent to Fort Benning but did not do as well as was expected. This was probably due to the new handler and the different training technique employed there.

3. The relationship of animal heart rates and of adrenal-pituitary function to natural aggressiveness and self-confidence has been explored (1-2-3). Essentially the findings indicate that the more dominant animals have a higher resting EKG and a higher magnitude of adrenal-pituitary response to stress than in their subordinate litter mates (4). The validity of these observations as applied to a large canine program has not been established. If such physiological testing could assist as a prognostic evaluator of future military dogs, the additional effort would indeed be worthwhile. This question formed the theoretical framework for a definative study outlined by Dr. M.W. Fox, our canine behaviorist consultant. The required telemetering and read-out equipment for heart rate determination was obtained. Part of this was on-hand at the WRAIR. The necessary laboratory equipment and expertise for plasma cortisol determination was found to be locally available. Actual work began recently.

4. Our puppy socialization program, which begins formally at four weeks of age was changed recently from only petting and play to include meaningful commands and accustomation to collar and leash. Rag play, and praise are used as rewards. It is all very low key but the results of this "imprinting" have been most gratifying. We have had puppies that will aggress an "intruder" at seven weeks of age and track at 10 weeks.

C. Consultant Visits: During the past year this Division was visited by its canine behavioral consultant, Dr. M.W. Fox, Ph.D., BVM, MRCUS; by Dr. Wayne H. Riser, DVM, who monitors our hip and elbow dysplasia control

program and by Mr. L. Wilson Davis, our evaluation and training consultant. Mr. Ernest H. Hart has recently accepted an invitation to act as our breeding consultant.

D. Facilities: Over half of our outside kennel facilities were roofed by corregated fiberglass supported by a welded angle iron framework.

E. Equipment:

1. An automatic watering system was installed in all cages except those in the whelping area.

2. The following telemetry equipment was received:

Transmitter
Receiver
ECG duplifier-recorder

Discussion: Man has always selectively bred animals. It is by this process that more meat, wool, eggs, milk, and black orchids have been developed. To expect to develop a more tempermentally stable, more intelligent, and physically better dog than is now generally available for military use, then, is an expectation that brooks no failure. The principal problem is that of truly selecting the best animals as breeders. It is for this reason that this Division is engaged in examining the possible correlation of cardiac and adrenal function values to the visible manifestations of superiority. Should these physiological values prove meaningful, not only would our breeding program be strengthened, but our monetary costs and manhour expendatures could be significantly reduced by early more positive recognization of the non-productive puppy. Additional time could be devoted to imprinting and socializing the genetically better puppies. This environmental enrichment through learning and increased human contact would maximize their genetic advantages and be of great assistance in developing a superior working dog. The Australian Army, following a visit to this facility, has recently initiated their own canine breeding program. They have named their project the "Psychogenetic Breeding Programme". The name is descriptive.

CONCLUSION: Reasonable genetic objectives are attainable through advantageous matings, patience, and work. There is every reason to expect the successful development of a remarkably improved military dog within the next decade.

RECOMMENDATIONS: None.

Project 3A062110A830 BIO SENSOR SYSTEMS

Task 00, Bio Sensor Systems

Work Unit 055, Development and evaluation of improved biological sensor systems

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