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AD-A225 259

GRANT NO: DAMD17-85-G-5011  
DAMD17-88-Z-8014

TITLE: TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS  
DISEASES OF MILITARY IMPORTANCE IN EQUATORIAL ASIA

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REPORT DATE: June 22, 1989

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
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## REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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| 1a. REPORT SECURITY CLASSIFICATION<br>Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |       |                                             | 1b. RESTRICTIVE MARKINGS                                                                                                             |                                                       |                                  |
| 2a. SECURITY CLASSIFICATION AUTHORITY                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |       |                                             | DISTRIBUTION / AVAILABILITY OF REPORT                                                                                                |                                                       |                                  |
| 2b. DECLASSIFICATION / DOWNGRADING SCHEDULE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |       |                                             | Approved for public release;<br>distribution unlimited                                                                               |                                                       |                                  |
| 4. PERFORMING ORGANIZATION REPORT NUMBER(S)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |       |                                             | 5. MONITORING ORGANIZATION REPORT NUMBER(S)                                                                                          |                                                       |                                  |
| 6a. NAME OF PERFORMING ORGANIZATION<br>Institute for Medical Research                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |       | 6b. OFFICE SYMBOL<br>(if applicable)        | 7a. NAME OF MONITORING ORGANIZATION                                                                                                  |                                                       |                                  |
| 6c. ADDRESS (City, State, and ZIP Code)<br>Jalan Pahang, 50588<br>Kuala Lumpur, Malaysia                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |       |                                             | 7b. ADDRESS (City, State, and ZIP Code)                                                                                              |                                                       |                                  |
| 8a. NAME OF FUNDING / SPONSORING ORGANIZATION<br>U.S. Army Medical Research & Development Command                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |       | 8b. OFFICE SYMBOL<br>(if applicable)        | 9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER<br>Grant No. DAMD17-85-G-5011 & DAMD17-88-Z-801                                      |                                                       |                                  |
| 8c. ADDRESS (City, State, and ZIP Code)<br>Fort Detrick<br>Frederick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |       |                                             | 10. SOURCE OF FUNDING NUMBERS                                                                                                        |                                                       |                                  |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |       |                                             | PROGRAM<br>ELEMENT NO.<br>62787A                                                                                                     | PROJECT<br>NO. 3M1<br>62787A870                       | TASK<br>NO.<br>AN                |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |       |                                             | WORK UNIT<br>ACCESSION NO.<br>001                                                                                                    |                                                       |                                  |
| 11. TITLE (Include Security Classification)<br>TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF MILITARY IMPORTANCE IN<br>EQUATORIAL ASIA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |       |                                             |                                                                                                                                      |                                                       |                                  |
| 12. PERSONAL AUTHOR(S)<br>M. Jegathesan                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |       |                                             |                                                                                                                                      |                                                       |                                  |
| 13a. TYPE OF REPORT<br>Final Report                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |       | 13b. TIME COVERED<br>FROM 2/1/85 TO 4/30/89 |                                                                                                                                      | 14. DATE OF REPORT (Year, Month, Day)<br>1989 June 22 |                                  |
| 15. PAGE COUNT<br>54                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |       |                                             |                                                                                                                                      |                                                       |                                  |
| 16. SUPPLEMENTARY NOTATION<br>The period February 1, 1985 - February 1, 1988 is for DAMD17-85-G-5011                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |       |                                             |                                                                                                                                      |                                                       |                                  |
| 17. COSATI CODES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |       |                                             | 18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)                                                    |                                                       |                                  |
| FIELD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | GROUP | SUB-GROUP                                   |                                                                                                                                      |                                                       |                                  |
| 06                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 13    |                                             | RA I, Malaysia, malaria, drug resistance, circumsporozoite, sporozoite, scrub typhus, <u>Plasmodium falciparum</u> , <u>P. vivax</u> |                                                       |                                  |
| 06                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 03    |                                             | P. malariae, P. ovale, Rickettsia tsutsugamushi, Ehrlichia                                                                           |                                                       |                                  |
| 19. ABSTRACT (Continue on reverse if necessary and identify by block number)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |       |                                             |                                                                                                                                      |                                                       |                                  |
| <p>The malaria immune status of various Malaysian populations (military, civilian) was characterized and numerous assays were developed for this purpose. The presence of antibody specific for the circumsporozoite proteins of <u>Plasmodium vivax</u>, <u>P. malariae</u> or <u>P. falciparum</u> did not convey immunity against infection with one or more of these three malaria species. Chloroquine and fansidar remain suitable prophylactic antimalarials in many areas of Peninsular Malaysia.</p> <p>The indirect immunoperoxidase test kit for the diagnosis of rickettsial diseases was developed, documented to be superior to conventional serological methods, and distributed throughout Southeast Asia.</p> |       |                                             |                                                                                                                                      |                                                       |                                  |
| 20. DISTRIBUTION / AVAILABILITY OF ABSTRACT<br><input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |       |                                             | 21. ABSTRACT SECURITY CLASSIFICATION<br>Unclassified                                                                                 |                                                       |                                  |
| 22a. NAME OF RESPONSIBLE INDIVIDUAL<br>Mrs. Virginia M. Miller                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |       |                                             | 22b. TELEPHONE (Include Area Code)<br>301/663-7325                                                                                   |                                                       | 22c. OFFICE SYMBOL<br>SGRD-RMI-S |

## FOREWORD

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| DTIC TAB           | <input type="checkbox"/>                   |
| Unannounced        | <input type="checkbox"/>                   |
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| By                 |                                            |
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| A-1                |                                            |

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## INTRODUCTION

BACKGROUND: With the beginning of a new U.S. Army Medical Research and Development Command (USAMRDC) Grant in February 1985, the Institute for Medical Research (IMR) in collaboration with the USAMRU-M undertook a drastic redirection in research priorities. For the 10 years prior to 1985 the primary effort of the Unit was directed toward research on the rickettsial disease, Scrub Typhus. Success in this endeavor, particularly the confirmation through human trials that doxycycline was extremely effective both prophylactically and clinically against Scrub Typhus, lead to the decision by the USAMRU-M Commander and the IMR Director, with the concurrence of the Director of WRAIR and the Commanding General, USAMRDC to redirect the efforts of the IMR/USAMRU-M collaborative program away from scrub typhus and toward characterization of the immune status of various groups of Malaysians at risk to a disease of considerably greater military relevance, malaria. Seventy-five percent of the new grant funds were allocated to support malaria research, while 25 percent were utilized to complete ongoing Rickettsia research efforts, transfer rickettsial disease technologies and secure and assure the diagnostic capabilities of the IMR, the only rickettsial disease reference laboratory in the Asia-Pacific region. This shift also brought the research efforts of the IMR/USAMRU-M collaborative research program into synchrony with at least one of the major research programs of the Walter Reed Army Institute of Research, i.e., malaria immunology and the development of an inexpensive and efficacious vaccine against the

malaria parasites Plasmodium falciparum and P. vivax. This change in research program was undertaken with a second goal in mind, that of assisting the IMR to rejuvenate its own malaria research efforts. In short, a switch to malaria research was of mutual interest and benefit to both the Malaysian and United States governments.

The Walter Reed Army Institute of Research malaria vaccine efforts are directed toward developing a vaccine against P. falciparum and P. vivax sporozoites. Accordingly, several WRAIR overseas research laboratories were conducting longitudinal and seroepidemiological malaria studies to provide immunological data for the vaccines and to provide well studied sites for eventual vaccine trials. In this respect, Malaysia was viewed as a very promising site for future vaccine trials because of the endemicity of both P. falciparum and P. vivax in certain areas of Peninsular Malaysia and in Sabah in eastern Malaysia. Furthermore, there are major differences in the vectors, parasite drug resistance and the ecology of malaria in Malaysia compared to field sites being studied by DOD personnel in neighboring countries and throughout the world.

Unlike some of the other overseas laboratories with large pools of manpower and diverse programs directed toward several important tropical diseases, the IMR/USAMRU-M had limited manpower which was concentrated on a single tropical disease of declining importance as a military threat, Scrub Typhus. The shift in the primary research program was a major and dramatic undertaking, requiring (a) extensive departmental reorganizations, (b) the acquisition of officers trained and

skilled in malaria field and laboratory research and (c) the retraining a vast majority of the Malaysian employees.

A strict plan to implement the transition to malaria research was formulated and put into effect immediately upon approval of the grant. This plan involved 3 different phases during the grant period. The first phase consisted of the initial 3-6 months of the grant when equipment, facilities and departments were reorganized and training of the existing staff was undertaken. The second phase involved months 3-12 of the grant period and concentrated on the development of laboratory expertise, evaluation and characterization of the malaria immune status of Malaysian army recruits, Plasmodium in-vitro cultivation, the establishment of an Entomology Laboratory and entomological field survey techniques, training of the professional and technical staff, identification of potential field sites and determination of the efficacy of Fansidar and chloroquine for the prophylaxis of malaria in select Malaysian military groups. The third phase involved a more indepth study of the relationship between measured malaria specific immune responses and resistance/susceptibility to infection with P. falciparum, P. vivax and P. malariae, and the completion of efficacy trials for Fansidar and chloroquine in Police Field Force jungle training areas. Additional malaria research projects of opportunity also evolved along with the growing efficiency and expertise of the collaborative IMR/USAMRU-M malaria research program.

FACILITIES: One aspect of the laboratory redirection required immediate attention, adequate space and facilities were not available for the proposed malaria research. This problem was resolved by the transfer of laboratory space from the Division of Medical Ecology, IMR. This space was ideally located adjacent to the existing rickettsial disease research facilities and nearly doubled the available malaria work space. Upon acquisition of this space and the reorganization of several departments, the facilities were renovated and remodeled to provide adequate space for parasite culture, serology/immunology laboratory space, entomology laboratory space and insectaries.

Later, a temporary field laboratory was designed and built about 300 km northeast of Kuala Lumpur in Gua Musang, Kelantan State. This laboratory was built in a malaria endemic area and specifically used to monitor and culture P. falciparum isolates for drug susceptibility studies. This area of Peninsular Malaysia is being watched very carefully by malaria control workers as major tracts of forest are being cleared and numerous immigrants, including Thai, Burmese and Indonesian workers, and their families are moving into the area and bringing with them chloroquine as well as Fansidar resistant strains of P. falciparum.



DEPARTMENT REORGANIZATIONS: Very early in the grant period major organizational adjustments were made within the USAMRU-M division of the IMR which included establishment of new departments of Parasitology and Entomology, along with the dissolution of the departments of Microbiology and Acarology. In addition, the department of Clinical Epidemiology was revitalized with additional personnel and training. Eventually, the Immunology and Serology sections were combined into a separate, all Malaysian section, the Serology Research Service Section. This section was formed to served as the backbone of all malaria and rickettsial immunology and seroepidemiological studies. All of these departmental and section adjustments were done in the least disruptive manner by transferring personnel already in the Unit to positions most appropriate for their skills. With these organizational adjustments the IMR/USAMRU-M malaria effort was quickly converted and attuned to both laboratory and field based malaria research.

PERSONNEL: The USAMRU-M has been recognized for many years as a premier rickettsial diseases research facility with exceptionally well trained laboratory and field personnel. The shift to malaria research promoted extensive in-house training conducted by the professionals in the Unit; sending laboratory personnel to training courses and calling in consultants to advise and train personnel in the laboratory. Some of these actions included: 2, one week malaria identification training courses conducted by the renowned malaria WHO expert malaria consultant, Mr. Yap Loy Fong for 4 professionals and 2 senior laboratory technologists; a one week training course at the Armed Forces Research Institute of Medical Sciences (AFRIMS), in Bangkok, on malaria cell-mediated immunity for 2 professionals and one Medical Technologist; a 2 week Immunological Technology Training course in Singapore attended by a Medical Technologist; a one week malaria workshop at AFRIMS attended by a Medical Technologist; a 6 week training period for one Laboratory Assistant learning proficiency in the WHO Drug Susceptibility Test Kit in Vanuatu, Solomon Islands; and a one and half week training period for the Medical Officer and new Chief, Department of Entomology in the field in Sabah, studying malaria epidemiology and entomological techniques in association with Dr. J. Hii. Consultants called in to assist the Unit were: Dr. A.I. Cole, College of Veterinary Medicine, University of Illinois (Ehrlichia sennetsu expertise); Dr. H. Tanaka, University of Tokyo, (E. sennetsu expertise); MAJ Robert A. Wirtz, Dept. of Entomology, WRAIR (Sporozoite ELISA

expertise); COL Carter L. Diggs, Headquarters, WRAIR (malaria expertise); MAJ W. Ripley Ballou and MAJ Daniel M. Gordon, Dept. of Immunology, WRAIR (malaria epidemiology and immunology expertise); Dr. V. Navaratnam, National Drug Research Center, University Science, Malaysia (expertise in drug susceptibility and drug levels in serum); and Dr. Richard Carter, University of Edinburg (malaria transmission-blocking expertise).

## BODY OF REPORT

### 1. Application of a WRAIR Developed ELISA to measure Vector Transmission Rates of Malaria in Malaysia

The dissection of Anopheles salivary glands and the subsequent microscopic detection of malarial sporozoites therein has been the standard technique for determining the malaria infective status of anophelines for years. This technique, however, has at least 2 major limitations: (1) dissections can only be done on fresh specimens, and (2) there is no morphological means to differentiate the species of Plasmodium, based on the sporozoite. In the mid-1980s, researchers at the WRAIR developed Enzyme-Linked Immunosorbent Assays (ELISA) for both P. falciparum and P. vivax sporozoites. These assays utilize the monoclonal antibodies (MABs) 2A10 and NMRI-3, for P. falciparum and P. vivax sporozoites, respectively.

In 1985, the 2 above ELISAs were incorporated into the malaria research efforts of the IMR/USAMRU-M to assist the WRAIR in an evaluation of their sensitivity and specificity for sporozoites of the 2 malaria species as they occur in Malaysia. Assays conducted at the WRAIR on sporozoites of P. falciparum and P. vivax strains from numerous world wide locations indicated the MABs in use were highly specific and possibly universal, however additional tests were needed from Malaysia. In addition, once the 2 assays were validated in Malaysia, they would serve as an important tool for identifying and characterizing potential malaria vaccine test sites in Malaysia.

After a period of training personnel and procuring the equipment and facilities for the assays, initial microscopic and ELISA surveys of An. maculatus salivary glands from Perak and Kelantan States revealed that the assays were specific for P. falciparum and P. vivax sporozoites. In addition, the circumsporozoite protein for each parasite species produced in mature oocysts was also identified by these assays. In 1986, this capability was offered as a service by the IMR/USAMRU-M to malaria control groups and researchers throughout Malaysia, and specimens from both Peninsular Malaysia and Sabah were tested routinely. In addition, over 1,700 An. farauti from Vanuatu were tested in support of a collaborative field study conducted in conjunction with the IMR based, World Health Organization Antimalaria Team. In this last effort 4 farauti specimens were found positive for P. falciparum sporozoites. Unfortunately, however, many other specimens sent to the Unit or collected by the Unit were not being identified by the assays, even though sporozoites were seen in the glands under the microscope. Hypothetical explanations for these discrepancies were: (A) the presence of Plasmodium species such as malariae, cynomolgi, traguli, etc., for which there were no monoclonal antibodies; (B) the presence of P. falciparum and/or P. vivax sporozoites that were coated by a CS protein antigen sequences not recognized by the monoclones in use, (C) improper storage technique for specimens between the time of collection and the ELISA Assay; (D) contamination of the storage medium NP40; (E) poor technique in the collection and handling of the salivary glands in the

field and laboratory; (F) contamination of dissection tools; and (G) overdilution of the gland aliquot, i.e., lack of sensitivity.

By September 1987, only 19 (38%) of 50 specimens that were sporozoite positive by dissection had been identified to either P. falciparum or P. vivax. This identification problem was causing serious doubts as to the validity of the ELISA as a practical tool in malaria work in Malaysia. At this time major efforts were taken to resolve this problem: (1) discontinuing the ELISA for oocysts and concentrating only on infective (sporozoite positive by dissection) mosquitoes; (2) more rigid quality control in performance of the assays; (3) developing additional sampling methods for preserving sporozoites for the assay; and (4) obtaining monoclonal antibodies for P. malariae and P. cynomolgi sporozoites from CDC and NYU for evaluation of previously non-reacting sporozoites. These efforts were highly successful and by September 1988, 46 (95.8%) of 48 gland positive (by dissection) An. maculatus were assayed and shown to have P. falciparum, P. vivax, P. malariae, or double or triple sporozoite infections of these 3 species. No P. cynomolgi sporozoites were identified.

These results demonstrate the value of the sporozoite ELISA assays. The ELISAs add a very valuable piece of information to the malaria epidemiological picture, i.e., the role the vector plays in transmitting (no. of infective bites/unit of man-exposure time) each Plasmodium species in a study area. This information is very important for formulating vector control programs in areas where more than one vector is present and the vectors may not be equally susceptible to or capable of

transmitting the different Plasmodium species in the area. This information is also extremely important for developing predictive models for malaria transmission in future malaria vaccine field trial sites.

## 2. In vitro Cultivation of P. falciparum

The establishment of a malaria research laboratory with the capability of in vitro cultivation of the human malaria parasite P. falciparum is necessary for the characterization of parasite drug resistance patterns, the production of antigen for serological procedures, and as an adjunct to determination of the malaria immune status of various populations in Malaysia.

In 1985, the newly formed Department of Parasitology established a malaria laboratory and initiated an in vitro cultivation technique for P. falciparum. The methodology used was that of the Malaria Laboratory, Division of Experimental Therapeutics, WRAIR. The first Malaysian isolates were propagated in the latter part of 1985.

Early in the initiation of this technique a procedure was developed for the introduction into culture of field collected malaria-infected blood samples, without regard to blood type, e.g., AB positive serum and O positive red cells. In this procedure infected blood samples were placed in culture in the presence of A positive plasma. Ten to 15 days later the parasitized red cells were subcultured in A positive red cells and A positive plasma. All isolates cultivated in the laboratory were originally adapted into culture using this procedure.

The establishment of this cultivation technique was completed within a one year period. Since 1985 numerous P. falciparum isolates have been cultured. The importance of this technique and the P. falciparum cultures to the IMR/USAMRU-M malaria research program cannot be over-emphasized. The technique, isolates and antigen from the cultures were essential basic components for many of the projects described below.

### 3. Evaluation of the Malaria Immune Status of Malaysian Army Soldiers

In Malaysia, the infectious disease of most concern to the military forces is malaria. Therefore, it is of great potential utility to determine the malaria immune status of military recruits. Of equal interest and importance is an understanding of the progressive changes in their immunity to P. falciparum, P. vivax and P. malariae, as they move from areas of low incidence to areas of high incidence, for example, from urban base to jungle. Furthermore, the evaluation and comparison of the population's immune status with the actual clinical incidence of malaria by species and characteristics of the infecting parasite should provide for the Malaysian Armed Forces operationally useful predictive data.

A comprehensive study requiring 5 bleeds over a 2 year period was begun in May 1985 with the collection of blood samples from 461 newly inducted Malaysian Army recruits at Port Dickson. All samples were negative for malaria and filaria. At the end of the six month recruit training program, the 385 subjects that



were still available were bled again - the remainder had failed to successfully complete the course, been medically discharged for reasons unrelated to the study or had withdrawn from the service. A third bleed was performed in April/May 1986, six months after the subjects had been posted to their units throughout the country. Despite the wide dispersal of the study group to 63 units in 27 locations throughout Malaysia, 348 (90.4%) were located, interviewed and bled.

Unfortunately, after 2 more bleeds (and 1 more year) only 187 of the original 461 study subjects (40.6%) had completed 5 bleeds. This underlines the difficulties encountered in follow-up studies of rotating military personnel. Analyses of the sera of the 187 subjects, based on the (IFA) and the Avidin-Biotin-Glucose Oxidase immunoassay (ABC-GO), indicate that most of the subjects initially had a low to insignificant antibody titer against P. falciparum bloodstage antigens. Those that did possess an antibody titer, initially, showed a steady decline in that titer with time.

Only 2 positive cases of malaria were found in the subjects after deployment, one a mixed infection (P.f./P.v.) and the other a single infection (P.f.). A blood sample from the last case was cultured in vitro and tested against chloroquine and mefloquine using the radioisotope microdilution method. The isolate was susceptible to both antimalarials.

#### 4. Fansidar and Chloroquine Chemoprophylaxis of Malaria among the Police Field Force in Central Malaysia

A high incidence of malaria occurred among commandos training in Central Peninsular Malaysia during FY85. In Malaysia P. falciparum resistance to chloroquine has been reported, but reports of resistance to Fansidar (sulfadoxine-pyrimethamine) are limited to a number of isolated case reports and lacked documentation. However, the widespread and southward movement of Fansidar resistance in Thailand demanded that the drug resistance patterns of the malaria parasite in Malaysia be closely monitored.

Members of the Malaysian Police Field Force (PFF), a group which represents a potential target population for the efficacy testing of malaria vaccines, are routinely prescribed Fansidar and chloroquine for malaria prophylaxis. In most PFF units this regimen is quite effective. However, members of two units, the North Brigade, which operates in the area of the Thai/Malaysian border, and the Commando Training School, located in Central Peninsular Malaysia, experienced a high incidence of malaria. Of 65 trainees undergoing a 4 month training course at the Commando Training School, 16 developed malaria; one of these progressed to cerebral malaria. Because of the suspected failure of chemoprophylaxis, rather than the presence of fansidar resistant P. falciparum, we initiated an investigation in a subsequent group attending the Commando Training School.

Eighty members of the Malaysian PFF were studied for eight weeks (one week before, three weeks during, and four weeks after

a jungle training exercise) while operating in a jungle training site. The area of jungle operations was highly malaria endemic as confirmed by entomological surveys and by mass blood surveys of Orang Asli (aborigine) populations residing in or near the area. Strict compliance with antimalarial medications (Fansidar and chloroquine) was assured. Despite this, one member of the PFF developed falciparum malaria during the study period.

Cultivation of P. falciparum isolates from the Orang Asli in the operational area and from the infected PFF member allowed the assessment of drug sensitivity by the radioisotope microdilution technique. All collected isolates were sensitive to both chloroquine and Fansidar. Serum drug levels of chloroquine and Fansidar were performed in Australia at the Army Malaria Research Unit and adequate concentrations of the drugs were demonstrated in all of the PFF subjects. The difference in malaria incidence between the study group and previous groups appears to be due to carefully supervised compliance in the study group rather than to the presence of chloroquine or Fansidar resistant strains of malaria in the area.

Reports of Fansidar and chloroquine resistance in Peninsular Malaysia, particularly near the Thai border, are likely to continue. Such resistance poses a serious threat to the efficacy of established treatment and prophylactic regimes in these areas of Malaysia. Before regimes are changed, however, confirmation of resistance claims should be studied with the possibility of non-compliance of prophylaxis as an alternate explanation.

5. Development and Evaluation of an Animal Model for the Study of the Peripheral Vascular Pathology of Cerebral Malaria

There is presently no suitable model for the study of the pathogenesis of cerebral malaria in vivo. The clinical syndrome, seen in man infected with P. falciparum, is thought to result from altered permeability of the blood-brain barrier or mechanical blockage of the nutritive vascular beds of the brain. Plasmodium berghei infection in the hamster results in cerebral lesions very similar microscopically to those seen in the brains of humans following death due to P. falciparum infection. The hamster is unique in that its cheek pouch may be everted for microscopic examination in the anesthetized animal. The pouch contains a complete vascular bed made up of arterioles, precapillary sphincters, capillaries and venules.

During FY85 we combined the hamster cerebral malaria model of Rest and Wright and the hamster cheek pouch model of Fulton and Jackson in an attempt to observe, in vivo, the pathogenesis of vascular lesions and then to correlate their development with the changes seen in the contralateral pouch and the brain on postmortem. Distinct and progressive vascular pathology was demonstrated in the pouch circulation of infected hamsters. The lesions, observable microscopically in the anesthetized animal, include a reduction in the number of apparently functional capillaries, sequestration of mononuclear cells, adherence of spherical erythrocytes to venous endothelium and aggregation of mononuclear cells and erythrocytes on vessel walls.

Histopathologic examination of the brain and pouch of P. berghei infected hamsters demonstrated changes very similar to those seen in vivo in the cheek pouch, changes not unlike those described in the brains of humans after death due to cerebral malaria. The changes included early (ca. day 3-4) sequestration of mononuclear cells in the venous vessels and an apparent reduction in the lumen size of 4-7um capillaries concurrent with a reduced frequency of observation of erythrocytes within them. Also, beginning on day four, multifocal hemorrhages were noted throughout the parenchyma and meninges of the brain and occasionally in the pouch. Later, (days 5-12) aggregation of erythrocytes and sequestration of monocytes was observed; adherence of apparently spherical erythrocytes to vessel walls, common in vivo, was typically not noted on postmortem. Subsequent studies in anemic control animals show similar spherical erythrocytes adhering to endothelial walls in vivo, and suggest that the cells seen in large numbers in the infected animals are reticulocytes. Preliminary electron micrographic studies of the tissues suggest that the observed capillary failure is the result of degeneration of individual endothelial cells followed by their subsequent protrusion into the lumen.

In 1986, an evaluation of the effects of dexamethazone (0.7 mg/kg daily) given on days 1-12, 4-12 or 7-12 post-challenge demonstrated that the capillary lesions and aggregation of cells noted previously can be prevented but not reversed by the drug; treatment must begin long before clinical signs of cerebral malaria develop.

6. Evaluation and utilization of the hamster cheek pouch model of cerebral malaria

Evaluation and utilization of the hamster cheek pouch as a model for the study of cerebral malaria was continued throughout 1987. Two hematropic parasites, P. berghei and Babesia microti, were used to further evaluate the model: each gave significantly different results. Plasmodium berghei duplicates the condition of cerebral malaria in the hamster model, while B. microti does not. Since dexamethasone (0.7 mg/kg), given subcutaneously, was shown in 1986 to prevent the failure of nutritive capillary (4-7 um) function, but would not reverse this pathological change once it occurred, the investigation of promising antimalarial drugs was initiated. Preliminary studies with Qinghaosu (QHS) indicate that 50 mg/k QHS administered daily, subcutaneously in corn oil, for three days starting on day 10 of infection, will clear the parasitemia and allow survival of the hamsters. Evidence of parasite destruction is present within six hours of QHS administration.

7. Evaluation of malaria transmission and immunity among the Orang Asli residing near Sungei Siput, Perak State, Malaysia

Little information is available regarding the seasonal dynamics of malaria transmission and immunity among populations residing in malaria endemic regions of Malaysia. Longitudinal studies to assess these parameters are needed to establish baseline data on the natural immunity of such populations to various malaria antigens as well as seasonal changes in the

population's immunological profile. In addition, epidemiological and entomological studies on the seasonal changes in malaria transmission are needed to provide valuable information by which to assess these areas as possible malaria vaccine field evaluation sites. With these goals in mind, a longitudinal study was initiated in August 1985, in an Orang Asli population residing in the Pos Legap Valley, near Sungei Siput Perak State, peninsular Malaysia.

Malariometric surveys were conducted on a bimonthly basis among Orang Asli living in the Pos Legap Valley, Perak between August 1986 and January 1988. Over 1,200 individuals live under constant exposure to malaria infections in 16 villages in the study area. A spleen rate of 71% in children less than 10 years old qualified the area as hyperendemic for malaria, by WHO standards. Overall malaria prevalence rates of 37-40% occurred among all individuals sampled and the prevalence approached 60% in children less than 10 years old. Nine 8-day surveys provided the following results:

(A) 1900 of 4726 slides were positive for malaria parasites (40.2%). (B) Prevalence rates by Plasmodium species were: P.f. = 39.6%, P.v. = 28%, P.m. = 10.8%, P.f./P.v. = 12.6%, P.f./P.m. = 3.2%, P.v./P.m. = 3.7%, and P.f./P.v./P.m. = 2.1%. (C) Anopheles maculatus is the sole malaria vector in the area, with gland positive rates averaging 2.75% for the study period, and infective bites/man/year averaging 32.3 for the 6 kampungs surveyed, (D) Clinical illness associated with parasitemia was the exception rather than the rule. (E) The analysis of serum samples for antibody to blood stage malaria antigens revealed

consistently high geometric mean titers (GMT), increasing with age, while malaria prevalence and incidence decrease with age. These results suggest the development at an early age of an effective malaria immune response.

#### 8. Antimalarial drug susceptibility testing of Malaysian isolates of P. falciparum

Until recently, the only method available within Malaysia to evaluate the drug response of P. falciparum was the World Health Organization's Micro In Vitro Drug Sensitivity Test Kit. The purpose of our project was to determine, through the use of a radioisotope microdilution method, the antimalarial activity of several drugs against isolates of P. falciparum collected from naturally acquired malaria infections in Malaysia.

During this project, seventy isolates were evaluated. All isolates were collected from the Orang Asli. The mean (geometric) inhibitory concentration (IC-50) values (ng/ml) were as follows: chloroquine, 10.00; amodiaquine, 4.66; mefloquine, 2.80; quinine, 40.55; halofantrine, 1.53; enpiroline, 3.02; and pyrimethamine, 21.37. The IC-50 (50% inhibitory concentration) is defined as the inhibitory drug concentration corresponding to 50% inhibition of the uptake of radiolabeled hypoxanthine by the parasites as compared to the nondrug-treated controls. Only four of 70 isolates tested exhibited resistance to chloroquine (IC-50 >60 ng/ml), and three isolates exhibited borderline resistance to mefloquine (IC-50 = 10-11 ng/ml). All other antimalarial values fell well below resistance values. The lack of drug



pressure in areas where the Orang Asli reside may account for the high prevalence of chloroquine sensitive P. falciparum. The value of this finding is that chloroquine is, and can still be considered as an effective, useful, and inexpensive drug for the treatment of falciparum malaria in peninsular Malaysia.

9. Development of an avidin-biotin immunoassay to detect antimalarial antibodies against blood-stage antigens of P. falciparum

In recent years, a highly specific and sensitive avidin-biotin complex (ABC) technique, which is based on the strong affinity between the glycoprotein avidin and the vitamin biotin ( $K_D = 10^{15} \text{ M}^{-1}$ ), has been used in many fields for the detection of antigens. The purpose of this study was to adapt the ABC technique for immunohistochemical detection of antibody specific for P. falciparum. To avoid the interference caused by endogenous activity of HRP and AP in red blood cells (RBC) we used glucose oxidase (GO) as the enzyme marker.

We combine the avidin-biotin-glucose oxidase complex procedure (ABC-GO) with light microscopy to detect P. falciparum specific antibody. Thin blood films were prepared from culture material of P. falciparum and fixed with acetone. Antibody was detected by successive incubations with test serum, biotinylated goat antihuman antibody, avidin-biotin-glucose oxidase complex, and glucose oxidase substrate. In the presence of reactive serum, a blue precipitate formed on the parasites and could be visually observed with a 40 x objective. Sera from patients with single infections of P. vivax or P. ovale were unreactive. No

cross-reactivity was observed with sera from patients with rheumatoid arthritis, filariasis, amebiasis, schistosomiasis, dengue, scrub typhus, leptospirosis, or toxoplasmosis. The sensitivity of ABC-GO is comparable to that of the IFA.

10. Determination of the malaria infective status of the vector, Anopheles maculatus, in malaria endemic areas of Malaysia

Anopheles maculatus has been recognized as a primary vector of malaria in peninsular Malaysia since the 1920's. However, during the last 8 years data have rapidly accrued in studies conducted in Thailand, that confirm that An. maculatus known from India to the Philippines, is a complex of distinct species. These chromosomal, crossing, electrophoretic, behavioral and morphological data help clarify the old enigma of why maculatus in Malaysia is a good vector, yet not a vector in some other Southeast Asian countries.

USAMRU-M studies of An. maculatus were re-established in 1985, with primary efforts conducted in the Pos Legap Valley, Perak State. Sporozoite positive maculatus were found during this study in the Malaysian states of Selangor, Pahang, Kelantan and Perak. All of the specimens of maculatus collected to date in peninsular Malaysia appear to represent one single species. In fact, of the 9 sibling species now recognized in the Maculatus Complex, only maculatus (form E) has been found in peninsular Malaysia. Form E is apparently the major vector in the Maculatus Complex, as nearly all of the other 8 species have not been incriminated elsewhere as malaria vectors.

In August 1988, a 2 year entomological study of intense hyperendemic malaria in 6 Orang Asli settlements in the Pos Legap area of Perak, Malaysia, was completed. This study involved 11,165 man-hours of human-bare-leg-collections and resulted in 3,857 captured and dissected anophelines of 7 species. Anophles maculatus (Form E) constituted 92.8% of all the anophelines collected and was the only species found positive by dissection, for Plasmodium sporozoites. Of 3,508 maculatus dissected, 92 contained sporozoites in the salivary glands for a cumulative average sporozoite rate of 2.62%, the highest ever recorded for maculatus. Sporozoite rates for 43 separate surveys in the 6 villages ranged from 0 to 20.5%. Sporozoites in the glands of maculatus were identified by ELISA as single or mixed (double and triple) infections of P. falciparum, P. vivax and P. malariae. P. cynomolgi was not detected in the study.

Calculations of daily sporozoite inoculation rates, monthly risk of contact with sporozoite inocula and the number of sporozoite exposures per man per month were high, the latter ranging between 1.83 to 4.57 for the 5 study villages. However, these calculations varied considerably from village to village and even for outside and inside collections within a given village. These variable inoculation rates are viewed as very important for estimating human exposure rates to the various species of Plasmodium in the study area, and for understanding the malaria circumsporozoite antibody kinetics in the human population. Overall, the accrued data reemphasize the extremely efficient vector capabilities of An. maculatus in maintaining

high levels of multi-species malaria in Orang Asli populations.

#### 11. Characterization of the Sexual Stage Antigens of Malaysian Isolates of P. falciparum

The effects of anti-malaria gametocyte antibodies in humans on the extrinsic development of the malaria parasite in the mosquito are very poorly understood, yet human derived gametocyte specific immunoglobulins hold prospect as a mechanism (transmission blocking immunity) of interrupting the P. falciparum transmission cycle in Malaysia. In order to study the effects of such a mechanism the sexual stage antigens of Malaysian isolates of P. falciparum have been characterized as to their gametocyte/gamete antigens using monoclonal antibodies developed to the 48/45K and the 230K antigen on the gametocytes, and 3 monoclonals to the 25K antigen on the gametes. These results indicate that the proteins on the surfaces of these sexual stages are highly conserved among Malaysian isolates.

#### 12. Development of an improved field incubator

A sturdy, reliable, temperature consistent portable incubator was required to support in the field evaluation of the effects of antimalarial drugs on fresh isolates of P. falciparum. The incubator supplied by WHO for use with the WHO microtechnique of evaluating P. falciparum is bulky, flimsy in its construction, and at times it is highly unreliable for maintaining the precise temperature needed.

A modification of the WHO supplied field incubator was developed for use in performing the WHO in vitro microtechnique for assessing the drug response of P. falciparum to antimalarial

drugs. The unit is compact, sturdy, and reliably maintains the incubation temperature at  $36.6^{\circ}\text{C}$  ( $\pm 0.1^{\circ}\text{C}$ ) for 32-34 hr. A maintenance-free, rechargeable battery provides power, and a battery charger is used to recharge the battery. The incubator functioned optimally under sustained and extreme field conditions.

13. Development of a sensitive indirect immunoperoxidase assay for the detection of antimalarial antibodies to P. falciparum blood-stage antigens.

The indirect fluorescent antibody assay (IFA) is an epidemiological tool frequently used by malariologists. However, the requirement for a fluorescent microscope to read the IFA result has restricted its widespread use.

We developed a new rapid malaria immunoperoxidase assay using HRP in place of FITC to allow the serological measurement of antimalarial antibody by light microscopy. Acetone-fixed thin blood films prepared from cultured Plasmodium falciparum were used as the source of antigen. This malaria immunoperoxidase assay is as sensitive as, and occasionally more sensitive than, the IFA. It is easy to perform and the antigen used does not show cross-reactivity with sera from nonmalarial diseases.

14. Development of an visually read ELISA system for the measurement of antimalarial serum reactivity.

Since the introduction of the enzyme-linked immunosorbent assay (ELISA), this procedure has frequently been applied to the diagnosis of malaria. The test generally consists of a crude

soluble antigen, which is first used to coat a microtiter plate, and a solution of an anti-human immunoglobulin conjugated to an enzyme marker. However, many enzyme markers present limitations regarding visual endpoint determinations and substrate requirements.

We developed a visual, enzyme-linked immunosorbent assay using urease (ELISA-U) as the enzyme marker was adapted for rapid detection of antibody against P. falciparum. Flat-bottom, 96-well microtiter plates were coated with P. falciparum soluble antigen obtained by saponin and NP-40 treatment of parasite cultures. Antibody was detected by successive incubations with test sera, urease-conjugated rabbit antibody, and urease substrate. Reactive sera developed a definite and easily visualized purple color. Sera from patients with single infections of P. vivax or P. ovale were unreactive. No cross-reactivity was noted with sera from patients with rheumatoid arthritis, filariasis, amebiasis, schistosomiasis, dengue, scrub typhus, leptospirosis or toxoplasmosis. The procedure can be performed at room temperature and completed within 1 hr. The sensitivity of the assay is comparable to that of the indirect fluorescent antibody test at all but the lowest dilutions tested.

#### 15. Therapeutic and histological efficacy of Qinghaosu (QHS) and its Analog, Arteether (AE), in the hamster model of severe P. berghei malaria

Lethal dose (LD50) and effective dose (ED99) studies involving Qinghaosu (QHS, = Artemisinin) and Arteether (AE) have been completed. The LD50 study for QHS in hamsters demonstrated

no toxicity at 200 mg/kg in corn oil. The ED99 (the effective dose level to cure 99% of infected animals treated) value for QHS was 20-40 mg/kg given daily for 3 days. Single doses of QHS were not effective for clearance, but animals were able to survive the crucial episode and maintain a moderate parasitemia. LD50 studies for AE indicated a single dose of 87 mg/kg and 3 daily doses of 27 mg/kg were lethal for uninfected hamsters. The ED99 of 2.8 mg/kg daily for 3 days in sesame seed oil produced parasite clearance and not toxicity. Lesions in the cerebral tissue were seen in positive controls. Lethal doses of AE in uninfected hamsters demonstrated renal tubular necrosis and pigment deposition. Qinghaosu and Arteether are effective antimalarials at therapeutic ranges, but clearance cannot be achieved in a single dose. Subtherapeutic levels provided enough parasite clearance to allow animals to overcome the "cerebral" phase of disease progression and survive to study termination, regardless of initial parasitemia.

16. Investigation of the significance of circumsporozoite protein specific antibody in the natural transmission of P. falciparum and P. vivax malaria in an aboriginal population of central Peninsular Malaysia.

During the period March - July 1988, an intense 16 week malaria study was conducted of 275 subjects from 9 kampungs in the Pos Legap valley. Week 0 sera from 275 study subjects revealed P. vivax circumsporozoite (CS) antibodies (anti-NS1 V20 malaria vaccine construct) in 72% of the subjects, while P. falciparum CS antibodies (anti-R32tet malaria vaccine

construct) were detected in 85%. After age stratification, the prevalence of these CS antibodies was shown to increase with age, reaching about 90% in 10-19 yr olds (P. vivax) and ranging from 60% in 0 to 4 yr olds to 90% in 10 yr old subjects (P. falciparum). Parasite rates for both species were highest in the 0 to 4 yr old category, then declining with age thereafter. There is an inverse relationship with age between increased prevalence of CS antibodies and parasite rates. Entomological surveys were conducted biweekly throughout the first 14 weeks of the study. These studies revealed that an average of 2% of the An. maculatus were infective during the study period. Calculations based on the sporozoite rate and other entomological parameters predicted a monthly malaria risk for the study subjects of exposure to infective maculatus approaching 0.90 throughout the 16 week period. Sixty of the subjects were parasitemic at the time of radical treatment during week 0. After radical treatment there was a gradual increase, from zero post treatment infections, in the cumulative incidence of malaria infections from week 7 through week 15. By the end of week 15, 96 of the 275 (35%) subjects had developed parasitemia. Week zero median antibody titers were not significantly different between the 96 subjects who developed malaria after radical cure and the 179 who did not. Assays for antibodies against asexual blood stage antigens of P. falciparum and P. vivax (using P. cynomolgi as a capture antigen) were carried out using the ABC-GO assay. The majority of subjects tested had anti-P. falciparum and anti-P. vivax blood stage antibodies irrespective of the



occurrence of circumsporozoite antibodies. Data from this study revealed that natural boosting of CS specific serum reactivity (titer), in response to mosquito delivery of sporozoites, occurs in both the presence and the absence of blood stage parasitemia. The half-life of booster phase CS specific antibody for both CS constructs is approximately 28 days. Also, there is a poor correlation between the presence and intensity of CS specific serum reactivity and the absence of blood stage parasitemia. Analysis of data, still continues in the laboratory in preparation for publishing several papers.

17. Comparison of a bioassay using P. falciparum and HPLC for the detection and measurement of cycloguanil, a metabolite of proguanil, in human plasma.

The increasing spread of chloroquine resistant P. falciparum and occasional adverse reactions to fansidar have prompted a reevaluation of previously used antimalarials such as proguanil. A bioassay for detection and measurement of plasma proguanil that does not rely upon the use of expensive HPLC equipment, but is as sensitive as the HPLC, is most desirable.

A bioassay utilizing H-hypoxanthine as the index of parasite viability and measuring 50% inhibitory concentrations of cycloguanil, a metabolite of proguanil, alone and in human plasma was compared to the traditional HPLC method. The HPLC proved to be the more sensitive method for detecting plasma cycloguanil. The minimum detectable concentration by bioassay was 20 mg/ul, where as it was 5 mg/ul by HPLC.

Although HPLC was found to be the more sensitive of the two

methods employed in our study, our results support previous contentions that the bioassay can be used to either provide drug data in laboratories that do not possess an HPLC system and it can be used as well as a supplement to the HPLC for assessing biological activity of antimalarial drugs.

## 18. Seroepidemiological Surveys of Rickettsial Diseases in Sabah, East Malaysia.

Very little is known about the prevalence of rickettsial disease in East Malaysia. The vector mite of R. tsutsugamushi, the etiologic agent of scrub typhus, and known tick vectors of the spotted fever group of rickettsiae, are common in Sabah. Therefore, rickettsial diseases such as scrub typhus and tick typhus could be unrecognized causes of a portion of many of the undiagnosed febrile illnesses occurring throughout East Malaysia.

A seroepidemiological survey of selected populations in various localities was conducted in collaboration with the Sabah Medical Department. Of 837 blood samples collected in the antibody prevalence surveys, 72 (8.6%) had SFGR specific antibody at a titer of  $\geq 1:50$ ; in 64, the titer was 1:50 and in 8 samples it was 1:100. Seven samples (0.8%) had R. tsutsugamushi specific antibody titers of  $\geq 1:50$ ; 5 at 1:50, 1 at 1:100 and 1 at 1:200. In Luasong Camp, 52 of 315 (16.5%) people surveyed had SFGR specific antibody at a titer of  $\geq 1:50$ , a rate significantly higher than that of the remaining population ( $p < 0.0001$ ). There was no statistical difference in the occurrence of positive SFGR antibody titers between the sexes or between age groups in either the total study population or the Luasong Camp population ( $p > 0.10$ ). The seven individuals that demonstrated elevated antibody titers to R. tsutsugamushi came from 5 different villages. Five were adult males, one a woman, and one a young

girl. In none of the blood samples tested was antibody to typhus group rickettsiae detected.

In none of the 383 febrile patients from which paired blood samples were collected was there serological evidence of active infection with any of the rickettsial organisms studied. Thirty of the patients (7.8%) had a SFGR specific antibody level of 1:50, and 2 patients (0.5%) had R. tsutsugamushi specific antibody at a titer of 1:50. There was no evidence of antibody to typhus group rickettsiae in any of the samples tested. Sixteen of the 97 (16.5%) patients studied at the Apas Bulung Health Centre had SFGR specific antibody levels of 1:50. This was significantly more frequent than in the remaining population ( $p < 0.0001$ ).

The two forest dwelling populations surveyed (Apas Bulung and Luasong Camp) had nearly four times the rate of occurrence of SFGR antibody (16.5%) of the other groups (4.5%).

#### 19. Chemoprophylaxis of Scrub Typhus in Malaysian soldiers

To date, an effective scrub typhus vaccine has not been developed. Previously, prevention has been by vector control or chemoprophylaxis with chloramphenicol, an antibiotic shown to cause unacceptable risk as a prophylactic agent. Subsequently tetracycline replaced chloramphenicol. Our study was undertaken to determine and compare the efficacy of each of two different doxycycline, a long acting tetracycline, regimes for the prevention of scrub typhus in a military situation, to determine the prevalence of scrub typhus in mammalian populations, and to

examine the dynamics of the vector mite populations in operational areas occupied by Malaysian Army field troops.

Two battalions of Malaysian soldiers were randomly and evenly divided into 3 groups and given either doxycycline 200 mg once weekly, plus a placebo once weekly, doxycycline 200 mg twice weekly or a placebo twice weekly. Blood samples were collected before, on three occasions during, and after a two month jungle operation. The field trial was completed in July, 1984. A total of 667 soldiers entered the study, with 548 of these being issued the study medications and each donating blood on four or more occasions. Serological and hematological testings were completed in early 1987.

The incidence of clinical scrub typhus was extremely low during the study period. Only one overt case of the disease was detected in the study population. There was serological evidence that an additional 14 volunteers were subclinically infected with R. tsutsugamushi. Unfortunately the incidence of disease was low and insufficient to allow definitive conclusions to be made as to the efficacy of doxycycline for the prophylaxis of scrub typhus in Malaysian soldiers participating in a prolonged field exercise in Peninsular Malaysia.

There was a high drop out rate of volunteers. Of the 667 volunteers who entered the trial 127 (19%) were lost to follow up at various stages of the trial because of reassignments, courses, and annual leave. An additional 80 (12%) reported non-compliance with project medications. Information gained by assaying selected serum samples for doxycycline levels suggests that non-compliance of medications was considerably more extensive than

reported. Over 30% of the volunteers complained of medication side-effects (an equal proportion in each of the three treatment groups). The high rate of non-compliance is attributed in part to the association of side-effects with the consumption of project medications.

In addition, 21.6% of the study population had R. tsutsugamushi specific antibody at the time the trial began, thus indicating their prior experience with the etiologic agent of scrub typhus. Thus, many of the volunteers possessed an undefined degree of immunity to the disease under study. Other factors, such as volunteers not reporting for various reasons and the occurrence of non rickettsial febrile illness, are thought to have adversely affected this study.

The high rate of non-compliance with project medications indicates that for future drug chemoprophylactic trials the ingestion of each and every dose of project medication must be supervised.

## 20. Identification and Antigenic Analysis of Rickettsia tsutsugamushi Strains Endemic to the Asia-Pacific Region

Information on the prevalence and distribution of R. tsutsugamushi strains is essential if an effective scrub typhus vaccine is to be developed. Eight prototype strains are predominant in isolates from Peninsular Malaysia, Thailand, Taiwan, the Philippines, Hong Kong, Australia and the islands of the Northern New Hebrides and Santa Cruz groups. Although isolates have been collected from a large segment of the endemic

region, isolates from some of the countries on the region's periphery have yet to be obtained and characterized.

Six isolates of R. tsutsugamushi were obtained from Dr. N. Tachibana of the Miyazaki Medical School, Miyazaki, Japan. One of the 6 was characterized as Gilliam-like by the direct fluorescent antibody test (DFA). The remaining 5 isolates rendered adult ICR mice immune to lethal (Karp) challenge but could not be characterized by the DFA, nor could these isolates be propagated in cyclophosphamide treated (immunosuppressed) adult ICR mice. Ten R. tsutsugamushi isolates were received from Dr. H. Tanaka and Dr. M. Murata of the University of Tokyo in a reciprocal exchange for Malaysian R. tsutsugamushi isolates. Four isolates proved virulent for adult ICR mice and were shown to be Gilliam-like by both the DFA and IFA. Of the remaining 6 isolates, 4 demonstrated peak titers to Karp strain and 2 to TA686 by the IFA test. All isolates rendered adult ICR mice immune to lethal intraperitoneal Karp strain challenge. These results suggest that, unlike in Malaysia, the Gilliam strain of R. tsutsugamushi represents the major endemic serotype in Japan.

#### 21. Investigation of the Seroepidemiology of Ehrlichia Sennetsu in Malaysia

Sennetsu rickettsiosis, a human febrile illness resulting from infection with Ehrlichia sennetsu, has not been well documented in Malaysia. Infectious mononucleosis (IMN), which is commonly reported in Malaysia, is believed to be caused by the Epstein-Barr virus and is characterized by fever, lymphadenopathy, headache, pharyngitis, and malaise. The disease

classically affects young adults resulting in considerable and prolonged morbidity, but only very rarely does it prove fatal. Because sennetsu rickettsiosis produces an almost identical clinical picture to this in western Japan, and in fact, is often clinically diagnosed as IMN, this seroepidemiological investigation was undertaken to determine if E. sennetsu could be a cause of IMN-like illness in Malaysia. This is of practical importance as sennetsu rickettsiosis responds well to tetracycline therapy and IMN does not.

In FY 85, 1,452 serum samples from 790 patients were examined for E. sennetsu specific antibody. Testing was performed using P388D1 derived E. sennetsu antigen propagated within the Unit. Twenty eight (3.5%) of the patients tested had titers  $\geq 10$ . When the criterion titer for a positive serology was  $\geq 20$ , 3 patients (0.4%) were positive. All cases studied were derived from separate groups which included febrile Malaysian fever of unknown origin (FUO) cases, a healthy European population, and febrile Malaysian soldiers participating in a field trial designed to evaluate doxycycline as prophylaxis for scrub typhus.

These findings suggest that there may be within Malaysia a sennetsu or sennetsu-like agent which may be responsible for a small number of FUO's.



## 22. The Effect of Tetracycline Therapy on the Host Defense of Mice Infected with Ehrlichia sennetsu

Ehrlichia sennetsu is the etiological agent of a well defined human disease syndrome known as sennetsu fever. This disease has been described as a cause of febrile illness in western Japan, and evidence suggesting its low level existence in Peninsular Malaysia was presented in the previous study (above).

Although the bacteriostatic tetracyclines have proven efficacious in the treatment of rickettsial diseases, including human E. sennetsu rickettsiosis, the chemotherapeutic effect of oxytetracycline on the control of, and the development of immunity to, a primary E. sennetsu infection has not been thoroughly examined.

Researchers have found the mouse to be an optimal in-vivo model for the isolation and investigation of E. sennetsu, and previous studies have indicated that mice infected with E. sennetsu respond to oxytetracycline chemotherapy, but uniformly succumb to infection following cessation of therapy. Since there have been few other examinations of the splenic infectious burden following infection, the ehrlichial burden in the spleens of normal infected mice and the diminution in those receiving various oxytetracycline chemotherapeutic regimes was examined to determine the most efficacious therapy.

Two studies of mice treated with oxytetracycline were conducted. In the first, mice were challenged with 1,000 50% mouse lethal doses (MLD) of E. sennetsu. The regime of chemotherapy was initiated at designated times proximate to infection of Balb/c mice with E. sennetsu. Therapy initiated 10

days post-infection resulted in a significant decrease of the infectious burden in the spleen followed, upon termination of therapy, by the establishment of a chronic infection. Specific antibody was detectable by the IFA on day 12, peaked on day 25 and persisted throughout the study period. All animals survived a potentially lethal E. sennetsu backchallenge given one month following cessation of therapy. Mice in which therapy was initiated simultaneously, with the E. sennetsu inoculation, in contrast, exhibited no detectable ehrlichia organism in the spleen either during or following the cessation of the antibiotic treatment. These mice developed minimal levels (geometric mean titer  $\leq 12$  of antibody, were not chronically infected and did not survive backchallenge).

Because the results of the first study were at odds with the results of previously published data, a second study was conducted to see if a lethal E. sennetsu recrudescence is a function of the infection dose. This study increased the dose of E. sennetsu from 1,000 to 5,000,00 MLD. All 22 mice receiving a 28 day course of oxytetracycline, initiated on the day of infection, survived for 3 months following the cessation of treatment. When sacrificed, 21 of 22 mice showed no evidence of E. sennetsu infection either by IFA or by subinoculation of donor tissues. All 10 mice in which a similar treatment was initiated 10 days post-inoculation also survived, however, through subinoculation all were shown to harbor E. sennetsu rickettsiae.

The findings of the second study supported the results of the first study, i.e., demonstrating that E. sennetsu infected mice do not die following cessation of oxytetracycline treatment. Furthermore, survival does not appear to be dependent upon the infective dose. These results suggested that chemoprophylaxis with rickettsiostatic levels of oxytetracycline usually prevents the establishment of an E. sennetsu infection in mice, and does not allow the development of a long lasting immunity. However, a delay of treatment until the time when morbidity becomes evident results in the establishment of an enduring immune response.

### 23. Evaluation of an Immunoperoxidase Test for the Serodiagnosis of Scrub Typhus in Malaysia

Scrub typhus is a common though frequently undiagnosed caused of febrile illness throughout rural Malaysia. Traditional serodiagnostic methods employed to measure human R. tatusugamushi specific antibody include the Weil-Felix test and the indirect fluorescent antibody (IFA) test. Though quite simple to use, the Weil-Felix test is unreliable for the serodiagnosis of rickettsial diseases. The IFA test, though highly sensitive and specific, requires a fluorescence microscope, and the test must be read before fluorescence fades. A recently developed modification of the IFA test, the indirect immunoperoxidase (IIP) test, is specific, sensitive, can be easily read with an ordinary light microscope and unlike the IFA provides a permanent slide record of the result.

In 1985, a parallel study was conducted on 95 febrile patients from several health centers in Peninsular Malaysia to compare the new IIP with the Weil-Felix test and the IFA. The sensitivity and specificity of the USAMRU-M modification of the IIP test was compared with the sensitivity and specificity of the Weil-Felix and IFA tests by measuring rickettsial antigen specific activity of IgG and IgM. Acute and convalescent sera were collected from 50 R. tsutsugamushi isolate-positive human patients and from 45 febrile patients diagnosed as having a disease other than scrub typhus. The IIP was significantly more sensitive than the Weil-Felix ( $P < 0.05$ ) in detecting IgM in acute and convalescent sera. The IIP test was significantly more sensitive than the IFA test in detecting IgG in acute sera ( $P < 0.05$ ) though sensitivity was comparable using convalescent sera. There were no significant differences in specificity among the three tests.

After documenting the advantages of the IIP test over the Weil-Felix and IFA tests for the serodiagnosis of scrub typhus, we initiated a planned period of over 2 years for training personnel and evaluating the use of the kit in medical facilities throughout Malaysia. Training courses were designed for medical technologists from the southeast asian region and the test was adapted to a small easily carried kit for rapid and easy distribution. Between 1986-1988, 4 training courses were conducted and 22 hospitals in Malaysia, 3 laboratories in Thailand (AFRIMS-U.S. Component, AFRIMS-Thai Component and Songkhla Hospital), NAMRU-2 Manila and NAMRU-2 Jakarta Detachment received IIP Test Kits. In addition, a member of the new USAMRU-

Korea staff was trained in the use of the kit, and that laboratory will receive a kit before USAMRU-M closes on the 4th of July 1989. Participating hospitals and laboratories submitted selected positive and negative serum samples as well as the corresponding immunoperoxidase stained slides to the IMR/USAMRU-M for corroboration of test results. Two additional training courses conducted in early 1989 were the last provided by the USAMRU-M.

To date, evaluation of the test kit and its usage has revealed a typical user response. Routine usage of the kit ranged from non-active to very active in 10 Malaysian hospitals, while all 3 laboratories in Thailand and one of the 2 Navy laboratories can be classified as very active. Quality control, though often difficult to coordinate, revealed a variety of usage errors, ranging from complete failure to utilize control sera to excellence in performance of the test. Invariably, when the test was conducted correctly it performed excellently. The highest quality of performance (accuracy of performing and interpreting the test) was demonstrated by 10 of 22 laboratories in Malaysia (the General Hospital, Kuala Lumpur, consistently being the best), 2 laboratories in Thailand and the NAMRU-2 Jakarta Detachment. All of the laboratories currently performing the test rely heavily upon the USAMRU-M/IMR for both the supply of standardized IIP test kit antigens and quality control assurance of proper kit utilization.

The provision of quality assurance and assistance will continue to be offered to each participating laboratory in

exchange for sera, developed test slides and test data. IMR technicians have been thoroughly trained in the test procedures.

24. Evaluation of intragastric inoculation as an efficacious means of infecting monkeys with R. tsutsugamushi.

Rickettsia tsutsugamushi, the etiological agent of scrub typhus is normally transmitted to man by the bite of an infected chigger of the genus Leptotrombidium. Macaca fascicularis monkeys are susceptible to experimental inoculation with various strains of R. tsutsugamushi via the intradermal, subcutaneous and intravenous routes. Furthermore, oral vaccination has been successfully used to protect human populations against infectious diseases such as Q-fever, typhoid, cholera and poliomyelitis. Experiments were initiated to determine if oral inoculation might be an efficacious means of infecting monkeys with R. tsutsugamushi. If so, it might be feasible to produce an oral vaccine for scrub typhus, using an attenuated strain of R. tsutsugamushi. Five Macaca fascicularis monkeys were inoculated, via the intragastric route, with 10<sup>7</sup> 50% mouse lethal doses of the Karp strain of R. tsutsugamushi. The monkeys were examined for clinical signs, weighed and their rectal temperatures recorded on days 0 through 58, 90, and 120 postinoculation. Blood samples were collected for rickettsial isolation studies and serum antibody determination. None of the monkeys developed clinical evidence of disease. The blood profile of all test animals remained within normal limits. Mice inoculated with the blood of these monkeys did not become ill and were not immune to

lethal Karp challenge, and serum samples collected from the test monkeys did not react with Karp antigen by indirect immunofluorescent antibody assay. These data suggest that M. fascicularis cannot be infected with R. tsutsugamushi via the intragastric route.

#### 25. Utilization and support of the indirect immunoperoxidase test kit for rickettsial disease diagnosis

During the last several years, IMR based USAMRU-M scientists designed, developed, tested and distributed the Indirect Immunoperoxidase (IIP) Test Kit for Rickettsial Disease Diagnosis. This kit provided a very rapid and accurate means of diagnosing scrub, murine (endemic) and tick typhus. Distribution of the kit, originally confined to Southeast Asia, has now reached the U.S., Japan and Korea. The USAMRU-M provided quality control checks for hospital and research laboratories using the Kit. In 1987 the test kit was distributed to 22 hospitals in Malaysia, 3 laboratories in Thailand and 1 each in Indonesia and the Philippines. Quality control efforts revealed a variety of usage errors, ranging from outstanding excellence in performing the test to complete failure to utilize control sera. A High quality of test performance was demonstrated by 10 laboratories in Malaysia, 2 laboratories in Thailand and the Indonesian laboratory. During 1988, 85 quality control assays were conducted. In general, local hospital facilities in Malaysia demonstrated excellence in performing the test. A total of 3,742 antigen slides were supplied to hospitals and laboratories in Malaysia and Thailand during the year.

## CONCLUSIONS

The two USAMRDC grants that provided the funds to support the research herein described formed the fiscal foundation upon which the IMR malaria research effort was revitalized and became, once again, internationally recognized for excellence.

The malaria immune status of various military and civilian populations was characterized and in the process several new or modified immunological assays were developed. Disappointingly, there is no evidence that the presence of a high degree of serum reactivity for the circumsporozoite proteins of P. vivax, P. falciparum or P. malariae convey protection to infection with any of these species of malaria.

Our development of the indirect immunoperoxidase (IIP) test kit for the diagnosis of rickettsial diseases along with our extensive training program in use of the kit, our quality control program and the distribution of the kit throughout Southeast Asia have not only provided for the first time a decentralized, sensitive and dependable means of accurately measuring serum reactivity to scrub, murine and tick typhus, but as well, the availability of the IIP kit has significantly heightened the regional awareness of rickettsial diseases.



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A reference format was not utilized in the development of this final grant report.

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## APPENDIX

No document are appended to this report.