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SYMPOSIUM ON
MALARIA IMMUNOLOGY
AND
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Proceedings, together with conclusions and recommendations of AID malaria strategy consultants

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MALARIA IMMUNOLOGY AND VACCINATION RESEARCH WORKSHOP

I. Introduction

In response to an invitation extended by the American Public Health Association on behalf of the Agency for International Development, the principal investigators directing the 11 AID-financed projects in the area of malaria immunology and vaccine research, together with their research associates, met with a group of AID consultants, members of the AID Research Advisory Committee, and senior officials of AID and APHA from January 14 through January 16 1981, at facilities provided by the Uniformed Services University of the Health Sciences in Bethesda, Maryland. Consonant with established AID practice, senior officials of the World Health Organization, the Pan American Health Organization, and a considerable number of U.S. Government Departments and Agencies concerned with malaria immunology, and scientists conducting related but non-AID funded research attended and participated in the interchange of information which the workshop was designed to make possible.

The workshop was keyed to the achievement of three principal goals:

A. A "state of the art" review of progress achieved (and, pari passu, areas where progress had not been achieved) against the several project objectives of the eleven AID-financed projects in the fields of malaria immunology and vaccine research. This review was designed to make available to all investigators in the AID network, and to others participating in the workshop, not only the results achieved but the experimental approach employed in 1.
each project, including both positive and negative conclusions.

In addition to the presentations by members of the "AID network", workshop participants were provided with presentations by other scientists whose research lies within the areas of malaria immunology and vaccination, but whose efforts are not AID-supported.

B. An initial effort to apply the Program Evaluation and Review Technique (PERT) to identify, in logical sequence, the total number of activities required to reach the ultimate goal of producing viable anti-malaria vaccines. In its completed form, the PERT chart will not only identify the activities required and their interaction and interdependence, but also the timetable for each activity, the list of responsible individuals, the decision points with respect to continuation of lines of exploration, funding, and initiation of new activities in the hypothetical sequence. It will also provide structural guidelines for the kinds of research which will be most proximately related to achievement of AID's goal, and will therefore direct if not dictate the nature of future AID requests for research proposals. Such guidelines must of course retain an element of flexibility, but will nevertheless be useful not only to AID officials charged with project monitoring responsibilities but also to investigators as they formulate subsequent research proposals.

2.
C. A review and updating of the 1978 AID malaria vaccine research strategy. Concomitant with this strategy review, the priorities which have guided AID in approving and financing proposals for contract investigations in malaria immunology and vaccine research were submitted to critical reevaluation to determine the relative weight to be given to individual avenues of investigation. The progress reports and information exchange (which required over two thirds of the time of the workshop) made it apparent that a major fraction of the outstanding projects had indeed been keyed to the top priority item -- improvement of the continuous-culture system -- and that project success in this key field rendered further efforts lower on the priority list.

Active participation in the workshop by five members of AID's Research Advisory Committee provides the RAC with first-hand project knowledge which will contribute to future consideration of proposed AID-supported research.
II. Background

The Agency for International Development has supported research on malaria immunity and vaccination since 1966, beginning with a single contract with the University of Illinois. This project was designed to determine the feasibility of developing a vaccine against human malaria and involved both the testing of sporozoite and erythrocytic antigens and the *in vitro* cultivation of these parasite stages. Although initial progress was gradual rather than dramatic, the biological feasibility of successful vaccination against malaria was convincingly demonstrated by this project as well as by other laboratories.

Through the National Academy of Sciences, AID sponsored a malaria vaccine workshop in 1974 to examine the state of the art of malaria immunology and vaccine research. The workshop produced a number of concrete results:

1. To identify the areas of critical research need, through a review of the then-current status of research on malaria immunity and vaccination,
2. To provide a basis for a more specific AID focus on research approaches and priorities, and
3. To stimulate increased interest and cooperation among scientists working in this field.

Following the 1974 workshop, AID convened a group of well-qualified consultants to assist in developing a strategy for malaria vaccine research by advising the agency regarding the elements of such research which warranted priority attention, the availability of...
institutional facilities, known expertise in the field, and interest. Equally significant, the consultants discussed the need for cooperation and collaboration among investigators, including those outside the AID-funded program. The Agency's first priority-based research strategy was adopted in 1975.

Consistent with the identified priorities, AID issued requests for proposals which resulted in the selection and funding of additional research projects on \textit{in vitro} cultivation of malaria parasites; following a significant breakthrough in the continuous culture of parasites, AID advertised for a second time for contractors to work in special study areas of \textit{in vitro} cultivation of the erythrocytic stages of malaria parasites. At the present time, the AID collaborating network of malaria immunity and vaccination investigators (and their staffs) encompasses some 11 projects.

In June 1978, a second AID workshop was held to review the forward progress of the AID collaborative network and to update the 1975 strategy. Problems and priorities identified in the previous strategy statement were still considered valid but because of the breakthrough in developing a continuous-culture system and because of the degree of progress on several related problems, the expert consultant team considered the highest priorities for future activities to be as follows:

1. Continuation of improvement of the continuous-culture system with particular reference to eliminating components not suitable for producing an antigen to be used in man. This would include replacement of components in short supply such as \textit{Aotus} serum and red cell supporting components.

2. Characterization and purification of antigenic material.
3. Other secondary objectives as follows:

a. Development of in vitro correlates based on cellular or humoral responses which would provide a test for immunity.

b. Development of better synchrony in continuous cultures.

c. Development of an alternative model system to permit use of the large numbers of animals needed for greater statistical validity in certain studies.

d. Study of geographically distinct strains of malaria with respect to their biological and biochemical characteristics both in vivo and in vitro.

e. Encouragement of the elimination of unnecessary duplication but support of planned replication of significant findings.

f. Insistence on the publication of confirmatory studies whether results are positive or negative.

g. Promotion of a program of exchange through observation visits and/or work visits between project personnel of appropriate laboratories, including some outside the AID network.

h. Continuation of the present level of support of sporozoite immunity research.

i. Deferral of any expansion of effort in the development of breeding facilities.

Like its predecessor workshops, the 1981 Malaria Immunology and Vaccination Research Workshop, convened at facilities provided by the Uniformed Services University of the Health Sciences in response to an invitation extended by the American Public Health Association on behalf of AID, had the goals of a "state of the art" review of progress achieved both within and outside of the "AID collaborative network" of investigators and a review and updating of AID's malaria vaccine research strategy. In addition, for the first time, it became possible to explore and (tentatively) to identify the total number of activities required to reach the ultimate goal of producing viable anti-malaria vaccines.
III. Workshop Proceedings

A. Introductory

1. Dr. Kessler's welcome to the workshop participants stressed the developing concern and skepticism as to the feasibility of the goal of malaria eradication, the need for a new approach to the problems of resurgent malaria, the need for the development of appropriate technology (of which an effective vaccine is an example) and the importance of developing appropriate systems for applying such technologies. New vaccines must indeed be developed, but equally innovative methods for making them widely available are also needed.

2. Dr. Joseph recapitulated AID's history of support for malaria immunology and vaccine research (now running at the level of $3 million annually) and took note of a number of circumstances which bear on and have influenced AID's desire for the present workshop:

   a. The need to be aware of the on-going work of WHO's Division of Tropical Disease Research (which has funded previous investigations of at least one of the currently AID-supported malaria vaccine research investigators) and to optimize relations between TDR-supported research investigators and those in the "AID Collaborative Network".

   b. The strong probability of budget stringencies over the next few years, and the need to concentrate research funding in those areas most proximately related to the development of viable malaria vaccines.

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1 Dr. Susi Kessler
   Director, International Health Programs
   American Public Health Association

2 Dr. Stephen Joseph
   Deputy Assistant Administrator
   Bureau for Development Support
   Agency for International Development

3 See Section II, Background
c. The anticipated need for applied research in relation to field trials of malaria vaccines which, if not presently available, are no longer beyond the horizon.

Dr. Joseph extended his wishes for successful completion of the important workshop efforts, and assured AID's close consideration of the conclusions reached.

3. Dr. Richter described the origins of the Uniformed Services University of the Health Sciences (USUHS), the relation of its curriculum to problems to be encountered by the uniformed services (including malaria) and the need to understand and possibly prevent environment-related disease. Such a need mandates and has in fact resulted in a vigorous research program -- related, indeed, to problems faced by the military, but not for that reason unique to the military. The research work of USUHS is in fact widely applicable. The USUHS wishes to be a part of many research communities, is an element in the AID Collaborative Network, and therefore welcomes the Malaria Immunology and Vaccination Research Workshop.

4. Dr. Brackett stressed a point made earlier by Dr. Kessler, the use of a viable malaria vaccine will constitute an example of appropriate technology, but felt that no single solution to the problem of malaria is to be expected. Vaccine will take its place in the arsenal of weapons to be employed against malaria -- along with chemotherapy, vector control, and other available tools. But it may in the

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1 Capt. Tor Richter, M.D.
Associate Dean
School of Medicine, USUHS

2 Dr. Robert Brackett
Warner - Lambert/Parke Davis
end add the critical input required to make malaria control feasible in areas where effective control has yet to be achieved. Interest in immunization, dormant for 20 years, has been stimulated by recognition of the limitations of the chemotherapy and pesticide approaches.

Dr. Brackett cautioned that, at least prior to the present workshop, evidence of successful immunization had been limited to blood-stage parasites and their antigens -- sporozoite protection only -- with no possibility of a massive immunization program. He identified the lack of a sufficient antigen supply as the main drawback -- enough for research, but no large-scale vaccine production.

Dr. Brackett took note of one agenda topic -- the introduction of the critical path method of analysis (CPM). His own experience with CPM had been good, he reported. The use of CPM can help answer the critical questions: what are the prospects for a viable vaccine, and when?

Dr. Brackett concluded that two new technologies, hybridoma technology and genetic engineering -- based on the work of principal investigators present in the workshop -- provided helpful, powerful new research tools, each of which will play an important role in the development of any vaccine which will be significant in combatting malaria in the "developing" countries.

5. Dr. Trigg described the several WHO research programs, efforts to strengthen research efforts in various countries and the work of the

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1Dr. Peter Trigg
Scientific Working Group of Malaria
World Health Organization
Scientific Working Groups. He concurred with Dr. Brackett: Malaria vaccine, when and if available, will be important, but will constitute only a part of the total antimalaria effort. He felt, further, that a single vaccine is not the end target: sporozoite, merozoite and other vaccines should all be in the target area.

Like Dr. Joseph, Dr. Trigg took note of present and potential budget stringencies. Research is expensive, and WHO must restrict its inputs to the best proposals. The WHO Malaria Immunology program (IMMAL) which had an availability last year of about $900,000.00 equivalent, will probably have no more in the year ahead.

Dr. Trigg also echoed Dr. Joseph's stress on the need for close collaboration between AID-supported and WHO-supported researchers. That such collaboration exists is demonstrated by the overlap of the two groups of investigators. But it can and should be improved.
B. Principal Investigators Progress Reports

1. Large Scale Cultures of Human Malaria (P. falciparum) and Their Use for Immunization and for Antigenic Analysis of the Parasites

Principal Investigator: Dr. William Trager
Institution: Rockefeller University

a. Purification of merozoites from culture and their use for immunization of Aotus monkeys

A line of FCR-3/Gambia has been maintained in the knobby state in continuous culture in flow vessels. Once a month, it has been reselected for knobbiness and resynchronized. Just after resynchronization the material has been used to start large cultures (each with 5 ml packed red cells). These were grown and expanded during a 5-day period and then resynchronized by combined gelatin-sorbitol treatment. Approximately 80 hours later (about 1 3/4 cycles) the material was harvested.

Schizonts were concentrated with gelatin and incubated in red cell extract; the merozoites were collected and purified by centrifugation through Percoll.

The freshly prepared merozoites were mixed with an intralipid emulsion containing Pfizer's adjuvant CP-20961 and injected intramuscularly with 3 Aotus monkeys of karyotype 3. Three controls received the same amounts of emulsion and adjuvant with a small amount of normal human red cells in saline, corresponding to the estimated number of contaminating erythrocytes present in the merozoite preparation. The monkeys have now received 3 injections at intervals of 1 week (and were scheduled for challenge on January 23, 1981 -- the week following the workshop).

b. Separation of parasite antigens with special reference to those concerned in providing protective immunity

Antigenic constituents of the parasites have been separated by gel electrophoresis and the binding of antibody from immune human and monkey serum to these antigens measured. Sera that inhibited growth of the parasites in vitro strongly bound relatively large amounts to proteins with apparent molecular weight of 45,000 to 50,000.

c. Further observations on knobby and knobless lines

The line of FCR-3/Gambia selected once a month with gelatin is about 95% knobby.

A continuous culture line of FCR-3 kept in flow vessels has remained
about 50% knobby, 50% knobless.

The knobs have been visualized at the light microscope level through the use of Nomarski interference optics combined with videotape image enhancement (equipment of Dr. R. Allen and of Dr. S. Inouye at M.B.L. Woods Hole).

d. Clone prepared by microscopic selection

This was prepared from the continuous culture line of FCR-3 (50% knobby). It grows well in flow vessels and produces gametocytes under appropriate conditions. It appears to be of intermediate knobiness. Its drug sensitivity has not yet been determined.

e. Reduction in serum content of medium

Good initial growth has been obtained in medium with only 1% serum supplemented with serum albumin and proteose peptone.

f. Studies on chemotherapy in vitro

Methylation inhibitors, a series of metal chelators, and certain quassinoids from plants have been found to give high antimalarial activity in vitro. Most active were sinefungin giving complete inhibition at 0.3 \( \mu \text{M} \), 2-mercaptopyridine-N-oxide at 0.08 \( \mu \text{M} \), and simalikalactone D at 0.004 \( \mu \text{M} \). Chloroquine is effective against sensitive strains at about 0.1\( \mu \text{M} \).
2. **In Vitro Cell Culture of the Exo-Erythrocytic Stage of P. berghei**

Principal Investigator: Dr. Michael Hollingdale

Institution: Biomedical Research Institute

This report described the first full cycle of development in tissue cell culture of the exoerythrocytic (FE) stage of *P. berghei*.

Sporozoites of *P. berghei* were isolated in normal mouse serum from salivary glands of *Anopheles stephensi* mosquitoes which had fed 21 days previously on *P. berghei* (ANKA strain) infected NMRI mice. Sporozoites were added to cultured cells, either directly by disrupting salivary glands or after purification through discontinuous gradient centrifugation and elution from ion exchange columns.

Embryonic rat cells were poorly infected by *P. berghei* sporozoites, but a line of human embryonic lung cells (line W138, from American-type tissue collection) proved uniquely susceptible. W138 cells were grown on 1 cm² coverslips in NCTC-135 medium containing 10% fetal bovine serum, 50 U/ml penicillin and 50 micrograms/ml streptomycin, and after the addition of sporozoites, cultured at 37°C in 5% CO₂ in air. Trophozoites were seen after 18 hours of incubation and multinucleate schizonts measuring about 30 microns mean diameter at 48 hours. Segmentation occurred by 60 hours and merozoites were released by 68 hours. Indirect immunofluorescence antibody (IFA) tests with mouse anti-RBC and mouse anti-sporozoite serum showed that the in vitro development of *P. berghei* schizonts was similar to such development in vivo. Moreover, W138 cells containing schizonts removed by trypsinization from coverslips and injected intraperitoneally into mice produced a patent red blood cell infection after 7 days.

The susceptibility of W138 cells to *P. berghei* sporozoites also allowed the first observation of the entry of sporozoites into a cell and the initiations of exoerythrocytic development. IFA and phase microscopy suggest that the entry mechanism initiates from bulb formation at the center of the sporozoite, transfer of specific sporozoite antigen(s) to the cell surface, causing invaginations, and the entry of a "rounded-up" sporozoite into the parasitophorous vacuole. The parasitophorous membrane continued to show specific sporozoite antigen(s) throughout the full cycle of exoerythrocytic development as detected by mouse immune serum and by a hybridoma monoclonal antibody which reacted with a sporozoite surface antigen, induced the circumsporozoite (CSP) reaction and conferred protection to mice by passive transfer.

In contrast, a hybridoma monoclonal antibody also reactive with a sporozoite surface antigen, but which did not produce CSP or confer protection, only reacted with the parasitophorous membrane at 24 hours but not when schizogony was complete at 48 hours.
Thus, it is suggested that a sporozoite antigen may play a role in the entry of sporozoites into a susceptible cell and that this antigen may be related to the induction of protective immune response.

Of interest was the observation that irradiated sporozoites (60 Cobalt, 8,000 - 15,000 rads) also entered by the same mechanism and transformed into a small, trophozoite-like structure. However, subsequent development was rare and did not produce mature schizonts or merozoites. Thus, irradiated sporozoites, which induce a complete protective response in mice, are capable of entering susceptible cells but do not develop the exoerythrocytic cycle.
3. **In Vitro Cultivation of Malaria Parasites**

Principal Investigator: Dr. Monte Bawden

Institution: Gorgas Memorial Institute of Tropical and Preventive Medicine

This report described progress to date toward objectives of the AID contract with the Gorgas Memorial Institute of Tropical and Preventive Medicine, the first AID-financed parasite culture contract outside the United States.

a. **Strain Isolation**

Professional contacts have been established with scientists in six Latin American countries: Colombia, Guatemala, Honduras, Panama, Peru, and El Salvador. A field trip has been made to Colombia to isolate and collect strains of *P. falciparum*, and, additionally, *P. vivax* and *malariae* as desired. Three strains of *P. vivax* have been isolated in Panama, but did not survive culture efforts.

b. **Continuous Cultivation**

Methods for continuous *in vitro* cultivation of malaria parasites have been established in the Gorgas laboratory in Panama. Initial problems with a growth inhibitor have been overcome, and *P. falciparum* is now being successfully cultured by the candle jar method, not only in RPMI medium but also in a new medium, "Richter's IMEMZO and HEPES (10mM) with Zinc and Insulin" (Richter's is an Earle's medium base).

The advantages of Richter's are: (1) it is a ready-to-use sterile liquid which is stable for 6 months at 4°C; (2) a working stock may be prepared once a week for current use; such a stock is stable if stored at 4°C; (3) there is less opportunity for contamination, because there is only one additive step in the preparation of the working stock; (4) the medium is available from a commercial source: Associated Biomedic Systems, Inc., Buffalo, New York.

c. **Area Training Efforts**

On a continuing basis, assistance in malaria parasite culture is being provided to other laboratories in Latin America. Assistance takes the form of technical advice, materials and culture-adapted *P. falciparum* strains.

d. **Gametocyte Production in Culture**

Studies of gametocyte production in culture have been initiated, using proven techniques. These studies have not, at this point, progressed sufficiently to enable them to produce results.
4. Sporozoite-Induced Immunity in Limian Malaria

Principal Investigator: Dr. Ruth Nussenzweig

Institution: New York University

Original specific research goals of this project were: determination of optimal conditions for immunization of rhesus monkeys with *P. knowlesi* sporozoites; immunization with sporozoites of *P. knowlesi* of its natural host, i.e., *Macaca fascicularis*; characterization of surface antigen(s) of sporozoites; and study of sporozoite-host cell interaction. In addition, because of rapid progress in the area of antigen characterization, through the use of hybridoma methodology -- not foreseen in the original application -- a supplement has been granted recently.

a. Immunization of rhesus monkeys with *P. knowlesi* sporozoites

This line of research by Dr. A. Cochrane in collaboration with Dr. R. Gwadz of NIH is a continuation of a project initiated in the preceding contract period, related to earlier rhesus immunization studies using *P. cynomolgi* sporozoites.

Findings can be summarized as follows: Immunization of rhesus monkeys was performed with gamma-irradiated sporozoites of *Plasmodium knowlesi*. Levels of antisporeozone antibodies were monitored by immunofluorescence, sporozoite neutralization, and the circumsporozoite precipitate reaction, and appeared to correlate well with protection. Only the intravenous route was effective in inducing both protection and antisporeozone antibodies. Immunization with sporozoites mixed with Freund's complete adjuvant failed completely to induce protection and resulted in a minimal antibody response.

Mechanisms of resistance to sporozoites probably involve the interaction of the host's immune system with the parasite's surface antigen(s). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of surface-labeled, partially purified sporozoites followed by autoradiography revealed the presence of a small number of labeled proteins in the extract. The molecular weight of this main surface antigen of sporozoites of *P. berghei* was different from that of sporozoites of *P. knowlesi*.

Since publication in the Bulletin of the WHO, Vol. 57, (Suppl. 1): 163-173, data on additional immunized animals confirm the correlation between good antibody response and development of protection; a poor antibody response indicates lack of development of protective immunity. Additionally, it has been demonstrated that antisporeozone antibodies are transferred via the placenta to newborn rhesus, and that the same phenomenon occurs in newborn Gambian babies, whose mothers live in an area of high malaria endemicity (Nardin et al., submitted for publication).

Initial attempts have been made to demonstrate the transfer of protection, through passive serum transfer. The results varied on different occasions. They will be much more meaningful when repeated by using monoclonal antibodies.
b. **Immunization of M. fascicularis with sporozoites of P. knowlesi**

This approach is being initiated now at the NIH by Dr. R. Gwadz, by determining the degree of susceptibility to sporozoite-induced infection of M. fascicularis of different geographic origins. [The NYU laboratory's high demand of sporozoites of P. knowlesi for antigen characterization and also, until recently, for the immunization of rhesus, as well as space contraints in the NYU animal facility, have delayed the onset of this research line.]

c. **Characterization of surface antigens of sporozoites**

This research by Drs. N. Yoshida, P. Potocnjak, V. Nussenzweig and R. Nussenzweig was initiated in early 1979, and has since the spring of that year been pursued using hybridoma methodology. As usual, the P. berghei-mouse model has been used first as an experimental system; this methodology has since June of 1980 been applied to the P. knowlesi system.

Findings have been the subject of two recent publications (Science, 1980, 207, 71-73, and J. Exp. Med. 1980, 151, 1504-1513). An additional manuscript is in publication (J. Immunol., in press), and two others are being prepared.

Basically, it has been shown that the protective antigen of P. berghei sporozoites is uniformly distributed over the surface of salivary gland sporozoites, and that it is a differentiation antigen. A monoclonal antibody, which reacts with a surface protein of molecular weight 44,000 has been shown to be protective since it neutralizes sporozoites in vitro and confers protection to mice upon passive transfer of as little as 10 μg of purified antibody. Continuing investigation of the biosynthesis and turnover of this antigen appears to indicate intracellular precursor(s).

Other ongoing studies involve the detection of cross-reaction among surface antigens of sporozoites of different rodent malarial species, which can only be detected through hybridoma methodology.

Using monoclonal antibodies in his analysis of surface antigens of P. knowlesi, Dr. A. Cochrane has obtained nine monoclonal antibodies which react with a sporozoite surface antigen, seven of which give an identical pattern of immunoprecipitation with 35S-methionine labeled sporozoites, i.e. are directed against the same molecule. The two other antibodies are directed against a different surface compound, which so far remains undetected despite use of a number of different labeling procedures.

Functionally these antibodies are distinct. Various antibodies of the type which recognizes one same molecule do neutralize sporozoites, i.e. abolish their infectivity for rhesus monkeys. The two other types, which also fail to produce a CSP reaction, fail to neutralize sporozoites, under the same experimental conditions. Dr. E. Nardin has developed a hybridoma against a surface component of P. vivax, as well as P. falciparum sporozoites. Both give CSP reactions with the respective parasites. These purified monoclonal antibodies and/or their Fab fragments will be used for functional assays, i.e., to determine whether they abolish sporozoite infectivity in vitro.
d. Studies on sporozoite-host cell interaction

This work, primarily performed by Dr. H. Danforth, in collaboration with Drs. M. Aikawa, A. Cochrane and Nussenzweig, and published in the J. Protozool., 1980, 193-202, can be summarized as follows:

Sporozoites of P. berghei adhere to, and are internalized by peritoneal mouse macrophages. Part of this process is active, since cytocholasin B treated macrophages, which fail to ingest inert particles, are still penetrated by P. berghei sporozoites.

Serum is essential for both sporozoite adhesion and internalization. A rather striking species specificity in this interaction was observed in the sense that serum and macrophages had to be derived from a susceptible host species, in order for sporozoite-host cell interaction to occur in vitro. Dr. F. Zavala, supported by the World Health Organization, is planning to pursue this and other related approaches in the NYU laboratory.
5. In Vitro Cultivation of Human Malaria

Principal Investigator: Dr. Jerome Vanderberg

Institution: New York University

This report described progress toward the principal goal of the contract: to develop methods for in vitro cultivation of gametocytes of human malaria, and subsequent infection of mosquitoes with these gametocytes.

a. Increased gametocytemia

Studies were undertaken on induction of P. falciparum gametocytes in culture. It was demonstrated that a reduction of the hematocrit from the normal 12% to 6% during the process of induction permitted an approximately doubled gametocytemia. The procedure has now been adopted as standard at Dr. Vanderberg's laboratory.

b. Gametocyte Maturation

It was demonstrated that the addition of hypoxanthine (50μg/ml) to cultures of gametocytes permitted the full maturation of the gametocytes. Gametocytes from cultures 12 - 14 days old produced oocyst infections when fed to mosquitoes. All groups of mosquitoes fed on these gametocytes developed oocyst infections that continued to normal salivary gland infections. Only rare mosquito infections were found when hypoxanthine was omitted from the culture medium.

c. Infectivity of In Vitro Cultivated Gametocytes

Studies with P. berghei ookinetes formed in vitro from gametocytes (taken from hamster blood) showed that these ookinetes were infective when fed to mosquitoes. Thus, in vitro-formed ookinetes should be capable of further in vitro development.

d. Synchrony in Continuous Cultures

Experience under this project of synchronization of parasite stages by sorbitol treatment has been previously reported. It has now been shown that a second sorbitol treatment 34 - 36 hours after the initial treatment produces a highly synchronous population of ring stages that go on to form almost 100% of schizonts at a given time.

e. Human Serum Replacement

It was demonstrated that it is possible to "wean" away P. falciparum parasites from a culture requirement for human serum by a gradual step-
wise reduction of the serum concentration and a concomitant addition of peptones over a period of time. Calf serum or other animal sera were required in the culture at the same time. Parasite growth under these conditions was as satisfactory as with human serum.
6. The Preparation and Evaluation of Merozoite Antigens in the Immunoprophylaxis of Malaria

Principal Investigator: Dr. Karl H. Rieckmann
Institution: University of New Mexico

a. Preparation of Contaminant-Free Merozoites

The preparation of merozoite specimens of *P. falciparum* which contain little or no host erythrocyte material was achieved by using two different procedures. In the first procedure, spontaneously-released merozoites were separated from host cell contaminants by using a combination of low-speed centrifugation and a succession of membrane sieves. In the second procedure, merozoites were obtained by the gentle rupture of schizont-infected cells and isolating them from erythrocyte stroma by free-flow electrophoresis. Although merozoites are less mature when obtained by the second procedure, larger quantities of purified parasites can be harvested for immunization studies. Immunization studies with clean merozoites are in progress to determine the relative efficacy of both these preparations in protecting monkeys against *falciparum* malaria.

b. Antigens in Supernatant Culture Media

During the process of parasite maturation and the collection of merozoites, it was observed that parasite-derived proteins were released into the supernatant culture media. Inhibition of parasite growth in vitro by sera obtained from mice which had been administered such supernatants suggests that "supernatant" parasite antigens might be capable of inducing a protective immune response in monkeys.

c. Breeding of Aotus Monkeys

Aotus monkeys in the small colony at the University of New Mexico (17 adult pairs) continued to breed successfully, each pair producing an average of about one viable offspring per year. Although most of the 63 viable offspring resulted from matings of wild-caught *Aotus trivirgatus griseimembra* from Colombia, 3 were hybrid offspring of 2 phenotypically-different pairs of Aotus monkeys from Bolivia and Colombia, and one was an offspring of a pair in which both parents were born in our colony. The breeding performance among pairs of the same karyotype was similar to that observed among pairs in which the 2 members had different karyotypes. Gentle handling of these primates seems to be the most important factor contributing to a successful breeding program.

d. Aotus Monkey Alternatives

The relative scarcity of Aotus monkeys emphasizes the need to assess the value of potential immunogens using animals such as mice and rabbits.
Mice which were hyperimmunized with parasites or parasite material from supernatants produces antibodies which inhibited the growth of *P. falciparum* *in vitro*. Examination of serum specimens from immunized rabbits by means of the fluorescent activated cell sorter (FACS) showed that there was antibody activity directed against the knobs on the surface of infected erythrocytes. A larger number of human or *Aotus* sera can be screened for the presence of knob-specific antibodies using the FACS than is possible by electron microscopy.

**e. Monoclonal Antibodies**

The production of monoclonal antibodies which are specific against single antigenic determinants of *P. falciparum* was carried out in order to assist in the identification and purification of functional antigens. The species, strain and stage specificity of 50 antibodies has been determined and studies are now in progress to evaluate their inhibition of *in vitro* parasite growth.
7. **Continuous In Vitro Cultivation of Various Strains of P. falciparum at High Parasitemia, Production and Purification of Antigens, and Vaccination Studies**

Principal Investigator: Dr. Wasim A. Siddiqui

Institution: University of Hawaii

a. **Strains and isolates of P. falciparum in continuous culture**

Two strains (FUP and FVO) of *P. falciparum* have been in continuous culture for the last four years. Recently four isolates from the Philippines, a strain from New Guinea and a strain from Cameroon have also been isolated in continuous culture.

b. **Continuous in-vitro cultivation at high parasitemia**

Using the static culture flask system, the FUP and FVO strains of *P. falciparum* have been adapted to grow at high parasitemia. Large quantities of antigen are produced by bi-weekly harvest of parasites at a parasitemia of 20-30%. A new design of culture vessel has been developed under the project to scale-up the production of antigen.

c. **Culture medium devoid of human serum**

The project has successfully demonstrated the complete replacement of human serum by commercially available calf serum and proteose peptone. This has greatly facilitated the large-scale production of *P. falciparum* antigen.

d. **Enrichment of late schizont parasites to harvest merozoites**

In the early phase of this project, the Bovine Serum Albumin (BSA) gradient system was used to concentrate a merozoite-enriched segmenter stage of *P. falciparum* obtained from continuous in-vitro culture. For the past year, experiments with Percoll density gradient centrifugation have produced results as good as those obtained with the BSA gradient system. The Percoll gradient system has many advantages over BSA: Percoll gradient is stable and cheaper; larger samples can be handled with ease; it can easily be removed from the medium because of its impermeability to biological membranes; and it is nontoxic so that it does not affect the viability of biological materials.

e. **Isolation and purification of P. falciparum parasites**

It has been shown that commercial saponins of high-hemolytic quality, when used in the right quantity, will render soluble a part of the erythrocytic membrane and break up over 90% of the rest into tiny debris which fuse only very slowly. After saponin lysis, parasites can be separated from red cell debris by differential centrifugation. The remaining erythrocytic membrane contaminant was not detectable by SDS-PAGE and Laurell's rocket immunoelectrophoresis.
f. Adjuvant substitution

A few years ago a report was published from the University of Hawaii laboratory showing the first successful immunization of Aotus trivirgatus griseimembra monkeys against P. falciparum infection. In this study, the use of Freund's complete adjuvant was found to be essential for effective immunization. For the last three years, a continuing effort has been in progress to find a substitute for FCA for an effective immunization against P. falciparum. It has now been found that P. falciparum-merozoite immunization, using 6-O-Stearoyl-MDP with liposomes as an adjuvant, protects against homologous infection with intraerythrocytic stages of the normally lethal dose of P. falciparum parasites. The most important observation was the absence of any side reactions at the site of injection. Recently it has also been found that P. falciparum-merozoite immunization, using another synthetic adjuvant - CP-20,961 - was effective in protecting against a lethal homologous challenge.

These experiments demonstrate the necessity of a suitable adjuvant in combination with parasite antigen to achieve effective immunization in Aotus monkeys against P. falciparum.
8. The Development of a Model for Antigenic Analysis of Cultured Plasmodium falciparum

Principal Investigator: Dr. James Jensen

Institution: Michigan State University

This report described progress toward, and problems encountered in the development of an animal model other than the Aotus monkey.

a. The Rabbit Model

Work in the MSU laboratory has centered on the development of alternate animal models, other than the Aotus monkey, for the basic analysis of soluble parasite antigens. Efforts have concentrated on the rabbit, the serum of which can be used in P. falciparum cultures.

Rabbits are bled for several weeks to accumulate a pool of serum that will be used to supplement the culture medium used to grow the parasites. Supernatant fluid from the cultures is used as an antigen source after high-speed ultracentrifugation to remove all particulate materials and to obtain a post-ribosomal fraction that is then concentrated by passing through a 5,000 MW ultra-filtration membrane. These antigens are then used to immunize the same rabbit whose serum was used to grow the parasites. The resulting antiserum contains immunoglobulins against the human erythrocyte and the parasite antigens. The antiserum is then adsorbed with human erythrocytes to remove the anti-erythrocyte immunoglobulins and the adsorbed antiserum used to detect antigens in the culture supernatant fluid. Results have revealed several parasite exoantigens; some are parasite species specific and others are shared among several different strains of P. falciparum. Indirect fluorescent antibody (IFA) techniques were used to test for parasite antigens.

The serum from non-immunized rabbits, however, had high (up to 1:320) titers of antiplasmodial antibody as detected by IFA. These rabbits were all infected with various species of Eimeria. Accordingly, coccidia-free rabbits were obtained; in sera derived from these rabbits, no antiplasmodial/coccidial antibodies were detected by IFA. This finding places in question the plasmodial antigens detected in the first experiments and strongly suggests that rabbits used for research on malarial antigens be coccidia-free.

b. Soluble Antigens

Other observations on soluble antigens obtained from culture supernatants demonstrated that they produced marked lymphocyte blast transformations in both previously infected and previously uninfected individuals, suggesting that soluble parasite exoantigens contain mitogenic components that produce non-specific activation of lymphocytes in
individuals not previously exposed to malaria. These unfractionated exoantigens also produce marked hyperpyrexia in rabbits — and thus may be responsible for malarial fevers.
9. Ultrastructural and Cytochemical Analysis of Malarial Antigens

Principal Investigator: Dr. Susan G. Langreth

Institution: Uniformed Services University of the Health Sciences

One of the more recent additions to the network of AID-supported projects, the primary focus of this research program is ultrastructural and cytochemical analysis of malarial antigens. On a collaborative basis, the expertise of the principal investigator as a cell biologist and electron microscopist is available to other members of the AID Network.

a. Localization of Antigens

A key objective is the localization and characterization of those components of blood-stage P. falciparum infections, both in the parasite and in its infected host cell, which are immunologically significant. The two antigenic surfaces which are currently of most interest are the parasite surface at the merozoite stage and the infected erythrocyte surface, particularly the knobs on mature-stage infected cells. An immunocytochemical sandwich assay, which uses territin as the marker on the secondary antibody, continues to be useful in probing the antigenic nature of these surfaces. This assay has been used to demonstrate the unique antigenicity of the knobs and the antigenicity of the merozoite surface coat. Additional procedures, using gold, peroxidase, and PHP as markers, are being developed in order to locate and characterize internal antigenic components.

b. Cytochemical Characterization

One of the advantages of EM cytochemistry is that it is possible to identify and characterize components in situ in heterogeneous, "dirty" preparations and to determine the nature of the microcontaminants. Emphasis is placed on glycoprotein characterization and the radioautographic tracing of components synthesized by the parasite and processed through its Golgi apparatus. The structures of particular interest are the knobs, Maurer's clefts, parasitophorous vacuole, merozoite cell coat, rhoptries, and micronemes.

c. Knob Structure and Function

Structural analysis of the infected erythrocyte membrane, with particular reference to the knobs, is being undertaken, utilizing freeze-fracture and scanning electron microscopy. Determination of the function of knobs is being investigated in vivo by comparative studies of Knob-bearing (K+) and knobless (K-) lines of P. falciparum. Current results indicate that the reactivity of K+ and K- merozoites to immunosera is the same, as far as can be determined by present EM immunocytochemical procedures. Monospecific antibody probes may show differences, however.
d. Purity and Integrity

Ultrastructural analysis of blood-stage *P. falciparum* antigen preparations for quality, integrity, and purity is an important collaborative component of this project. Periodic monitoring by immunocytochemical probes for change (antigenic variation) in long-term cultural material and for inter-strain differences will also involve network collaboration.
10. **Isolation and Evaluation of Malaria Immunogens**

Principal Investigator: Dr. Theodore Green

Institution: University of Missouri

The primary focus of this project is to develop a merozoite-based vaccine against *P. falciparum*. It is expected that for reasons of purity and safety, this immunogen will be a merozoite subunit antigen.

a. **Antigen Isolation**

The isolation and purification of merozoite antigens is progressing by two related approaches: the direct fractionation of merozoite antigens and the separation of dissociated immune complexes. These procedures employ electrophoretic, chromatographic and salt precipitation techniques. A merozoite lipoprotein antigen has been purified and is being studied. Activities ancillary to this goal have included the refinement of the project-developed growth-and-reinvasion in vitro test, with emphasis on the occurrence of inhibited schizonts.

b. **Strain Cloning**

By use of the critical dilution technique, it has been possible to isolate three cloned populations of the FCR-3/FMG African strain of *P. falciparum*. These clones are characterized by differences in morphology, growth in culture and pathogenicity in Aotus monkeys and are therefore of great scientific interest. An immunization study employing one of these clones in Aotus monkeys was conducted and demonstrated protection by vaccination. The animals were grouped by sex, age, weight and karotype with three animals per group. One group was immunized with $2.6 \times 10^9$ merozoites from culture in Freund's complete adjuvant I.M., followed by $1.3 \times 10^9$ merozoites in Freund's incomplete adjuvant two weeks later. The control group received an aqueous emulsion containing only the adjuvant on the same schedule. Challenge was made six weeks after the final immunization, and consisted of $10^6$ Aotus erythrocytes infected with the homologous parasites administered intravenously. One immunized animal remained negative following challenge while the other two showed a 10 day delay in onset and early cure of parasitemia with milder parasitemias than controls. There were no deaths, and no drug intervention was required.
11. **Primate Investigations in Central and South America Contributing to Development of a Malaria Vaccine**

Principal Investigator: Dr. Carlos A. Espinal

Institution: PAHO/Instituto Nacional de Salud, Bogota, Colombia

The Malaria Immunity Research Project at INS is a subproject under the New Mexico project (Sec. III-B-6, above). The project, although subject to many initial delays, is now making progress on six of its seven objectives, and has established active collaboration with many of the other working groups at INS. Close working relationships have also been established with the Colombian National Malaria Control Program, to which the project entomologist has been loaned.

Progress toward project objectives:

a. **Cryopreservation**

Cryopreservation feasibility studies for the transport and preservation of malaria infected blood are being carried out.

b. **Serology Studies**

Available tests such as the enzyme linked immunosorbent assay (ELISA), indirect immunofluorescence and the CARl assay are being studied with both cryopreserved material from New Mexico and fresh material from continuous cultures established in Bogota.

c. **Aotus Susceptibility to P. falciparum Infection**

Studies of the susceptibility of different Aotus subspecies to P. falciparum infection are well under way. Some 15 strains of P. falciparum have already been adapted to culture and an additional five strains are being adapted to culture. Some of the strains are drug resistant and some are susceptible. The resistant strains grow better in culture.

d. **Aotus Breeding**

The establishment of a breeding colony of Aotus monkeys for use in vaccination studies is a key objective of the project. Some 94 monkeys are on hand with another 100 scheduled for delivery in January 1981. The project has the capacity for 350 monkeys, but would need additional cages to reach that capacity. Only one Aotus has been born to date, but another five are pregnant. Concern was expressed over the methods used in collecting the monkeys.

The project is prohibited from performing its own collecting since that is the responsibility of INDERENA, the government organization responsible for natural resources. It is hoped that INDERENA will permit the assignment of INS men to assist the monkey catching teams, in the future. The problem is that the teams catching monkeys mix different
species together and overcrowd the temporary cages to the point where many monkeys die and/or contract disease and die after they have been received by INS. The entire first shipment of 50 monkeys received from INDERENA died from Herpes.

e. Vaccination Studies

The conduct of vaccination studies to explore various regimens and dosage schedules to achieve maximum protection with the lowest quantities of antigen is underway. The most recent immunization trial involves 8 experimental monkeys and 3 controls, to be challenged soon. Lyophilized culture material from New Mexico will be used as the antigen source.

f. Vector Culture

Adaptation of various malaria mosquito vectors to laboratory culture will allow studies of the parasite, the vector and host relationships. Colonies of two anopheline species are well established and in use.

g. Field Studies of Malaria Prevalence

Field studies to determine the prevalence of malaria are the development (or course) of immune responses in 'non-immune' and 'immune' individuals. This is the only objective in which the project is unable to report any progress. This study was planned in collaboration with the military hospital. Because of changing assignments there has been no continuity on the hospital staff and project staff was too limited to enable assumption of the responsibility. However, an epidemiologist sent to Mexico for training will be returning soon and will be assigned this responsibility.
C. Program Evaluation and Review Technique (PERT)

Dr. Peter Contacos, formerly of the Center for Disease Control, and Dr. Charles Miller, formerly of the Parke-Davis organization, described Dr. Contacos's analysis of the steps necessary to achieve the ultimate goal of producing viable anti-malaria vaccine(s), and Dr. Miller's application of the Critical Path Method (CPM) to determine the most time-efficient sequence of investigatory activity in reaching the ultimate goal.

The CPM was defined as the logical arrangement of required activities from initiation to completion; many of these activities may and should overlap or coincide in time. To this arrangement is then applied an anticipated time-table for each activity and a designation of the individuals responsible for the achievement of sub-goals along the chain. The CPM also provides identification of those places in the chain where there will be sufficient information in the hands of AID managers to make critical decisions with respect to efforts to assure adequate fund availability, the timing of (and the precise working of) future Requests for Proposals, the identification of both investigators and institutions and, in the final analysis, the determination as to whether the probability of success warrants continued pursuit of the goal.

Dr. Miller presented two PERT charts, still tentative in nature and not timed to completion, showing the sequential steps and decisions necessary for the development of viable vaccines within the AID Collaborative Network of investigators. Significantly, both incorporate genetic engineering as a key factor in translating laboratory results into vaccine availability in volume.

The PERT chart is a management tool, a guide to present (and potential) participants, but neither a straitjacket nor an immutable projection of activity. It can and in all probability will require modification to take account of breakthroughs or possible dead-end investigations, both within the AID network and without.

The attached charts (Annexes II and III), segmented to permit reproduction at a nominal cost, may be taped to produce a continuous start-to-finish network plan. It must be reemphasized that they represent an initial attempt to apply the PERT approach to the development of sporozoite and merozoite vaccines, and are not considered definitive or even complete.
### D. Mini - Workshops

A new feature in the pattern of workshop conduct was the mini-workshop, a forum which allowed younger investigators to present their current research results and, in some instances, provided opportunity for principal investigators to offer supplementary detail of activities which had perforce been touched on briefly in their earlier reports. The mineworkshops were conducted simultaneously in different locations.

#### 1. Hybridoma Technology and Genetic Engineering

**Moderator:** Dr. Gary Campbell

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<th>Speaker</th>
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<td>Monoclonal antibodies against sporozoites of <em>Plasmodium knowlesi</em>.</td>
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<tr>
<td>b. Dr. Nobuko Yoshida</td>
<td>Monoclonal antibodies against sporozoites of <em>Plasmodium nigeriensis</em>.</td>
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<td>c. Dr. Joan Rener</td>
<td>Use of monoclonal antibodies to identify antigen targets of transmission-blocking immunity.</td>
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<td>d. Dr. Marcel Hommel</td>
<td>Assays for monoclonal antibodies directed against surface antigens of erythrocytic stages of <em>P. knowlesi</em> and <em>P. falciparum</em>.</td>
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<td>e. Dr. Joan Rener</td>
<td>Production of monoclonal antibodies which block merozoite invasion of erythrocytes.</td>
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<td>f. Dr. Diane Taylor</td>
<td>Monoclonal antibodies to stage and species specific antigens of the rodent malaria parasite, <em>Plasmodium yoelii</em>.</td>
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<tr>
<td>g. Dr. Gary Campbell</td>
<td>Production of monoclonal antibodies against <em>Plasmodium falciparum</em>.</td>
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<td>h. Dr. Carter Diggs</td>
<td>Production of monoclonal antibodies against <em>Plasmodium falciparum</em> at WRAIR.</td>
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<td>i. Dr. Ken Hunter</td>
<td>Human monoclonal antibodies to malarial antigens.</td>
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<td>j. Dr. Araxie Kilejian</td>
<td>Prospects and problems in the use of recombinant DNA for the production of a malaria vaccine.</td>
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33.
2. In Vitro Cultivation Systems

Moderator: Dr. James Jensen

Speaker

a. Dr. James Jensen
b. Dr. Wasim Siddiqui
c. Dr. Phuc N-Dinh
d. Mr. Titus Ifediba
e. Dr. Burkhard Enders
f. Dr. Michael Hollingdale
g. Dr. June Whaun
   Dr. Kyle Webster

Subject

Human Serum Replacement, Erythrocyte Storage, Laboratory Large Scale Production
Laboratory Large Scale Production
Field Application of Culture Techniques
Development of Infective Gametocytes
Automated Large Scale Production
Details of Exo-Erythrocytic Culture Techniques
Purine Metabolism in Cultural Parasites
3. Monkey Models for Falciparum Malaria

Moderator: Dr. Karl H. Rieckmann

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<td>a. Dr. Karl Rieckmann</td>
<td>Breeding experience in a small colony of <em>Aotus</em> monkeys</td>
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<tr>
<td>b. Dr. Francisco López-Antunano</td>
<td>Program of primatology sponsored by the Pan American Health Organization.</td>
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<td>c. Dr. William Cole</td>
<td>Experiences in a large <em>Aotus</em> colony.</td>
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<td>d. Dr. Wasim Siddiqui</td>
<td><em>P. falciparum</em> - <em>Aotus</em> model system: some experiences at the U. of Hawaii.</td>
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<td>e. Dr. William Trager</td>
<td>Infectivity of cultured parasites to <em>Aotus</em> monkeys.</td>
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<td>f. Dr. Theodore Green</td>
<td>Cloning and characterization of <em>P. falciparum</em>.</td>
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<td>g. Dr. Karl Rieckmann</td>
<td>Development of immunity to falciparum malaria in adult <em>Aotus</em> monkeys.</td>
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<td>h. Dr. William Collins</td>
<td><em>Aotus</em> monkeys as models for vaccine studies - advantages and disadvantages.</td>
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<td>i. Dr. C. Kent Campbell</td>
<td>Adaptation of <em>P. falciparum</em> into the squirrel monkey (<em>Saimiri sciureus</em>).</td>
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<td>j. Dr. Marcel Hommel</td>
<td><em>P. falciparum</em> in the squirrel monkey: variation of circulating parasites in normal and splenectomized animals.</td>
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<td>k. Dr. John Barnwell</td>
<td>Phenotypic changes of <em>P. knowlesi</em> after passage through intact and splenectomized Rhesus monkeys.</td>
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<tr>
<td>l. Dr. L.L. Pereira da Silva</td>
<td>Protective antibodies in <em>P. falciparum</em> - infected <em>Saimiri</em> monkeys.</td>
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4. **Antigen Isolation (Merozoites, Sporozoites, Culture Supernatants), Purification and Characterization**

**Moderator:** Dr. Hans-G. Heidrich

<table>
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<tr>
<th>Speaker</th>
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<td><strong>a. Release, isolation and purification procedures for intracellular parasites. Isolation of spontaneously released merozoites</strong></td>
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<tr>
<td>(1) Dr. S.C. Kan</td>
<td>The use of purified saponin for isolation and purification of <em>Plasmodium falciparum.</em></td>
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<tr>
<td>(2) Dr. Hans-G. Heidrich</td>
<td>Lectin fragmentation of <em>P. falciparum</em> infected erythrocytes from continuous cultures and free-flow electrophoresis separation of free parasite stages.</td>
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<td>(3) Dr. John E.K. Mrema</td>
<td>Isolation of spontaneously released merozoites from continuous cultures of <em>P. falciparum.</em></td>
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<td>(4) Dr. Harold Stanley</td>
<td>Isolation of <em>P. falciparum</em> merozoites using Percoll gradients.</td>
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<td><strong>b. Isolation and purification of sporozoites</strong></td>
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<tr>
<td>(1) Dr. Hans-G. Heidrich</td>
<td>Isolation of <em>P. berghei</em> sporozoites using free-flow electrophoresis.</td>
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<tr>
<td><strong>c. Characterization of antigens from intracellular parasites, spontaneously released merozoites and from sporozoites</strong></td>
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<tr>
<td>(1) Dr. Diane W. Taylor</td>
<td>Two-dimensional gel-analysis of <em>P. yoelii</em> proteins.</td>
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<td>(2) Dr. Robert T. Reese</td>
<td>Protective antigens from the asexual exo-erythrocytic stages of <em>P. falciparum.</em></td>
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<tr>
<td>(3) Dr. Martin Weiss</td>
<td>Affinity-binding of merozoite proteins to erythrocyte receptors.</td>
</tr>
<tr>
<td>(4) Dr. Seymour Schulman</td>
<td>Affinity-binding of merozoite proteins to erythrocyte receptors.</td>
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<tr>
<td>(5) Dr. Theodore Green</td>
<td>Isolation and characterization of the lipoprotein antigen from <em>P. falciparum.</em></td>
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<td>(6) Dr. Nobuku Yoshida</td>
<td>Biosynthesis of protective antigens by <em>P. berghei</em> sporozoites.</td>
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<td><strong>d. Antigens from culture supernatants</strong></td>
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<tr>
<td>(1) Dr. John E.K. Mrema</td>
<td>Is there only junk in the culture supernatants?</td>
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</tbody>
</table>
(2) Dr. James B. Jensen  Soluble exo-antigens from exhausted culture media.

e. Antigen isolation and characterization

(1) Dr. George Cain  Techniques to be used in the antigen isolation and characterization strategy.

(The following report on presentations in the Antigen Isolation, Purification and Characterization Mini-Workshop, provided by the session moderator, Dr. Heidrich, typifies the presentations and discussions which characterized the mini-workshops. Interested parties may contact speakers at the other Mini-workshops for elaboration of their presentations. Addresses of all speakers appear in Annex I.)

The purpose of the session was to discuss the most recent results on the isolation, purification, and characterization of malaria "antigens". It was made clear in the introductory remarks that the word "antigen" is being abused. It should be used only when single molecules (proteins, sugars, glycoproteins, lipids, lipoproteins) are being described. It should not be used when discussing cells, such as merozoites, or membrane systems. Here the terms "cell-associated antigens", "membrane-associated antigens" are correct.

The session was organized under five main topics. In order to give the audience as complete information as possible, introductory slides were shown and discussed at the beginning of each topic; speakers described experiences in their own laboratories, and also listed techniques used and results obtained by other groups not represented in the meeting. In the following text, the investigations presented in the session are underscored and abstracts are given.
Topic 1: Release, Isolation and Purification Procedures for Intracellular Parasites. Isolation of Spontaneously Released Merozoites


b. Techniques for release of parasites from host cells such as: Pressure procedures (Wallach), SCP-sonication procedure (Kreier), immune-lysis (Trigg, Trager), saponin-lysis (Zuckerman, Siddiqui), lectin-fragmentation and free-flow electrophoresis, centrifugation techniques, cell sorting.

c. Isolation of spontaneously released merozoites with continuous filtration techniques (Cohen), affinity (Con-A) and ion-exchange columns (Pasteur Institute, Sterling, Kilejian), membrane sieving technique, Percoll gradient purification, albumin gradient purification (Siddiqui, Eisen).

Abstract: The use of purified saponin for isolation and purification of Plasmodium falciparum (S.C. Kan and W.A. Siddiqui)

Commercial saponins were purified by Sephadex G-25 gel chromatography. Purified saponins, which increased hemolytic potency 3-4 fold, were used to release P. falciparum from infected erythrocytes. The bulk of erythrocytic membrane debris was removed from the parasite preparation as demonstrated by SDS-PAGE and by Laurell's rocket immunoelectrophoresis. Results on the purity and quality of the isolated material (parasites) were not discussed.


Intracellular parasites of Plasmodium falciparum obtained from continuous cultures were freed and separated from their human host erythrocytes using a method similar to that described for separating Plasmodium vinckei from rodent erythrocytes. Infected erythrocytes, concentrated by the Plasmagel (3% gelatin) technique, were agglutinated by phytohemagglutinin and disrupted gently by successive passage through 100, 40, 20, 10, and 5 μm nylon filters. The mixture of free parasites and small erythrocyte membrane vesicles was suspended in a medium containing 11 mM triethanolamine acetate, .015 mM MgCl2, 5.5 mM glucose and 0.25 M sucrose, pH 7.4 (IN KOH), and passed through a Free-Flow Electrophoresis Apparatus FFV (Bender & Hobein, Munchen) at 135 V/cm and 170 mA, t=5°C. About 10 mg of total protein were processed per hour. Most of
the fractions containing free parasites did not show contamination with erythrocyte constituents as determined by light and electron microscopy, polyacrylamide gel electrophoresis and enzymatic analysis. Using these analytical procedures, 0.1-0.2% host cell material contamination could be picked up. In addition, the various stages of free parasites of Plasmodium falciparum exhibited different electrical surface charges. Rings and trophozoites were highly negatively charged whereas schizonts and, in particular, merozoites showed low negative charges. Thus, the various states could be isolated separate from each other.


Spontaneously released merozoites were isolated from pre-washed cultures that contained "fresh" erythrocytes and first cycle parasites. The simple, fast isolation procedure involved a combination of centrifugation and membrane sieves (5 or 3 microns and 1.2 microns). The merozoites were free from host components as determined by electron microscopy and by polyacrylamide gel electrophoresis (PAGE). Merozoite proteins were demonstrated using stained and autoradiographed gels (PAGE).

Abstract: Isolation of Plasmodium falciparum merozoites. Preliminary results on the use of Percoll gradients (Harold Stanley, Susan G. Langreth, and William Trager)

In order to undertake immunization experiments with Plasmodium falciparum merozoites, this parasite stage must first be separated from the host erythrocyte material. Preliminary experiments using density gradients prepared with Percoll indicate that this technique is useful in isolating the merozoites. The procedure currently being used is as follows: 1) Erythrocytes from synchronous (schizont-rich) cultures were diluted 1:10 with RPMI 1640 containing 10% human serum and 10% RBC extract (RSE) and incubated 4 hrs at 37°C; 2) The material was centrifuged twice at 500 g for 10 min to remove most of the RBCs; 3) The supernatant from the last centrifugation was layered over 60% Percoll in RSE and centrifuged at 1800 g for 30 min. Most of the intact RBCs and parasite residual bodies formed a pellet at the bottom of the centrifuge tube; the merozoites and contaminating membrane material banded on top of the Percoll; 4) This band was removed, mixed 1:1 with fresh RSE, layered over 40% Percoll in RSE and centrifuged at 1800 g for 20 min. The membrane contaminants banded at the top of the Percoll while the merozoites were centrifuged to the bottom of the tube.
Although the merozoite preparation was contaminated with 9% residual bodies, it contained less than 0.1% contamination with either other parasite stages or intact RBCs. Analysis of these merozoite preparations by electron microscopy showed that they had normal ultrastructure and had retained their surface coat. In addition, when gently fixed with 0.1% glutaraldehyde, they still reacted with ferritin-labeled antibodies from sera of monkeys immune to falciparum malaria. Further purification of the merozoites is in progress.

Topic 2: Isolation and Purification of Sporozoites

Ion-exchange and affinity column procedures (Nussenzweig, Vanderberg), filtration techniques and Hypaque gradient centrifugation (Biomedical Research Institute, Naval Medical Research Institute, Bawden, Pacheco, Leef, Beaudoin), Free-flow Electrophoresis procedure.


Sporozoites of rodent malaria Plasmodium berghei were separated from ground mosquito debris by use of continuous free-flow electrophoresis technique. The material for sporozoite separation was prepared by grinding infected mosquitoes (Anopheles stephensi) in the presence of 0.05% DNAse in Medium 199 and filtering with 100, 40, and 20 μm nylon filters. It was further cleaned by use of a two-step discontinuous Hypaque gradient (40% and 60% v/v) and resuspended in an 11 mM triethanolamine acetate buffer, containing 0.5 mM MgCl₂, 5.5 mM glucose and 0.25 M sucrose, pH 7.4 (1N KOH), prior to electrophoresis. Electrophoresis was carried out in the same buffer in an FFV Apparatus (Bender & Hobein, Munchen) at 135 V/cm and 170 mA, t= 5°C. The material from 400 mosquitoes in 1.5 ml of buffer was separated within 70 minutes, and two distinct bands were observed. The smaller, less prominent band seen more toward the anode was found by both light microscopy and transmission electron microscopy to contain the separated sporozoites. The larger, more visible band contained mosquito debris and bacteria and only a small number of sporozoites. Repeated separation with whole body sporozoites from 400 mosquitoes showed a recovery rate of approximately 75%-90% of the parasites, i.e., 6-8x10⁶ sporozoites per 400 mosquitoes. These separated sporozoites showed a positive immunofluorescent response when exposed to immune serum. After surface iodination aside from other components, a 43,000-45,000 molecular weight constituent was found to be a major surface component. Morphological analysis demonstrated (using tannin stain) a polysaccharide-rich layer on the surface of the sporozoites. Bacterial contamination was almost completely removed electrophoretically from the isolated sporozoites.
Topic 3: Characterization of Antigens from Intracellular Parasites, Spontaneously Released Merozoites and Sporozoites

a. Extraction procedures (freeze-thawing, Triton X-100, Nonidet NP-40; protease blockers).

b. Isolation procedures (chromatography)

c. Tests for absence of host cell material.

d. Characterization: tests for antigenicity (CSP, IFA, growth inhibition assay, immunodiffusion, immunoelectrophoresis, cross-immunoelectrophoresis), antigen-antibody analysis (Protein A), gel electrophoresis, surface analysis using 125-iodine, Na-boro(3H)hydride (Miller, Kilejian), hybridoma technology and using monoclonal antibodies (Nussenzweig, Perrin, Wellcome Research Laboratories), immunoelectronmicroscopy using labelled antibodies (Langreth, Reese), biochemical assays (Sherman), metabolic studies.


Extracts of erythrocytic-stage parasites of P. yoelii are routinely used in Ag-induced proliferation studies. These extracts were analyzed by 2-dimensional electrophoresis using the silver staining technique described by Switzer, et al. 1979. Over 100 non-RBC "spots" of presumed parasite origin were visualized. Monoclonal Abs are being used to identify which of the polypeptides are malarial Ags.

Abstract: Protective antigens from the asexual erythrocytic stages of P. falciparum (Robert Reese)

Sera from humans and Aotus monkeys which had the ability to inhibit parasite growth to varying degrees were used to identify proteins from late stage or surface labeled merozoites which appeared to correlate with the inhibitory capacity of the sera. One protein had a molecular weight of ~78,000, the other was ~45,000.

Abstract: Affinity-binding of merozoite proteins to erythrocyte receptors (Martin Weiss, Seymour Schulman).

An inhibition assay was developed to study red blood cell receptor and merozoite recognition protein. N-acetylglucosamine, was found to inhibit penetration with no toxic effect. Glycophosphit A was shown to have a non-specific effect. The receptor on the erythrocyte may be useful as a ligand to separate out merozoite recognition proteins by means of affinity binding and subsequent disruption of the binding.
Abstract: Erythrocyte and Merozoite Receptors (Margaret Perkins)

Merozoites were isolated from in vitro cultures and separated from red cell membrane by passing over a sepharose column linked to anti-RBC. The merozoites invade red blood cells. The invasion is inhibited by 10 mg trypsin which also removes 2-3 high molecular weight bands (MW 250,000, 130,000). Treatment of erythrocyte surface with protease or neuraminidase blocks reinvansion, suggesting that a sialoglyco-protein is involved in the binding/invasion of merozoites. Purified glycophorin A at 50 µg/ml will completely inhibit reinvansion of merozoites. Conclusion: Erythrocyte receptor is at least in part glycoprotein A. Other sialoglycoproteins and glycolipids have to be tested.

Abstract: Isolation and characterization of the lipo-protein antigen from P. falciparum (Ted Green)

Antigens were isolated from solubilized naturally released merozoites and from sorbitol-lysed schizonts. Semi-purified antigens were obtained by ammonium sulfate precipitation of these preparations. Final purification was accomplished by PAGE. A lipo-protein complex antigen was isolated, purified and characterized. The antigen is of parasite origin, is free of red cell contaminants and is relatively poorly antigenic. The antigenicity resides with the protein component. The antigen has not been demonstrated to produce antibodies in rabbits.

Abstract: Biosynthesis of protective antigens by P. berghei sporozoites. (Nobuko Yoshida, Ruth Nussenzweig, Victor Nussenzweig)

P. berghei sporozoites synthesize in vitro the protective surface antigen Pb44 and two other internal antigens Pb54 and Pb52 that are also recognized by protective monoclonal antibodies. There is indication that Pb52 is the precursor to Pb44.
Topic 4: Antigens from Culture Supernatants

"Wash-off" antigens from Plasmodium berghei (Kreier, Israel group), culture supernatants from Plasmodium knowlesi (Cohen, Trigg), culture supernatants from Plasmodium falciparum.

Abstract: Is there only junk in the culture supernatants?
(J.E.K. Mrema, Hans-G. Heidrich, B. Avner, S. Tokuda, G. Campbell, K. Rieckmann)

Using G-35-methionine incorporation and PAGE autoradiographic studies of P. falciparum cultures, the 30,000 g supernates contained parasite antigens. These antigens were released into the culture medium during maturation and collection of P. falciparum. Antisera raised in mice against these supernate antigens showed inhibitory effect on the maturing parasite stages of the second erythrocytic cycle (after exposure of merozoites to antisera).

Abstract: Soluble exo-antigens from exhausted culture media
(James B. Jensen)

Exoantigens from culture medium are mitogenic, producing lymphocyte blast transformation in previously uninfected individuals. They are also pyrogenic when injected into rabbits.

Topic 5: Antigen Isolation and Characterization

Abstract: Techniques to be used in the antigen isolation and characterization strategy (Dr. George Cain)

Methods are described for the quantitative transfer of parasite antigens, separated by SDS-polyacrylamide gel electrophoresis, to nitrocellulose sheets or to diazobenzyl-oxymethyl cellulose. These techniques are more rapid and simple than conventional Staphylococcus Protein A precipitation or immune complexes, and they circumvent the possibilities of proteolysis or antigen aggregation during immune complex formation.
IV. AID Malaria Vaccine Research Strategy Review

In a concluding session which was limited to AID malaria strategy consultants who had participated in the workshop proceedings, a detailed review was undertaken of the strategy, established in 1978, which has underlain the selection of the 11 projects which are currently receiving AID support. The consultants took account of the progress achieved by the principal investigators and their staffs, as outlined in the reports of the principal investigators and detailed in the mini-workshops, as well as progress by scientists outside the AID collaborative network.

In particular, they concluded that the goal of what had been designated as Priority 1 -- improvement in continuous culture systems -- has been largely achieved, at least with respect to *P. falciparum*.

Set forth below are the preliminary conclusions of the expert consultants with respect to the priorities which should govern AID in formulating requests for proposals (RFP) and making decisions concerning fields of investigation warranting AID support. AID has requested the consultants to give further consideration to the points set forth and to submit comments, additions, deletions and clarifications of the draft document. Upon receipt of responses, AID will prepare a final strategy statement on support of research in the field of malaria immunology.
1. Characterization and purification of antigenic material. Identification of protective antigenic molecules related to protective immunity was considered to be the essential starting point. The development of protocols to standardize experimental design techniques and the production of a few standard cloned parasite strains for use by all were considered absolutely necessary at this time in order to expedite progress and insure comparability of results.

2. All other activities were considered to be subordinate to the number one priority as listed above. Secondary objectives (not listed in priority sequence) were considered to be as follows:

a. Development of in vitro correlates based primarily on humoral responses which would provide a test for protective immunity.

b. Independent comparison and evaluation of currently available in vitro assays to develop a standardized assay.

c. Development of continuous culture methods for species of human malaria other than P. falciparum (on which network research has to date been concentrated) with emphasis on P. vivax. Research should not, however, be restricted to human malarias. Study of biological and biochemical characteristics of other species of malaria both in vivo and in vitro may also yield insight into mechanisms of protective immunity.

d. Development of an alternative model system to permit use of the large numbers of animals needed for greater statistical validity in certain studies.

e. Support of planned replication and independent verification of significant findings and publication of confirmatory studies, whether results are positive or negative.

f. Promotion of a program of information exchange through observation visits and/or work visits between project personnel of appropriate laboratories, including some outside the AID network.

g. Increase in the present level of effort of sporozoite immunity research.
h. Deferral (for the present) of any expansion of effort in the development of a suitable adjuvant.

i. Improvement of the supply of monkeys, including the development of breeding facilities.

j. Continued support of research of the exo-erythrocytic stage of malaria parasites with emphasis on *P. falciparum*.

k. Initiation of feasibility studies in the biogenetic engineering area.
V. Summary Conclusions: Results of Malaria Immunology and Vaccine Research Workshop, January 14 - 16, 1981

Summary: Results of recent Malaria Immunology and Vaccine Research Workshop - January 14 - 16, 1981. The recent APHA-convened workshop to review progress toward the goals of the AID-supported projects in the field of malaria immunology and vaccine research and to update AID's strategy for the support of further research programs in malaria immunology was well attended by scientists from various laboratories both within and outside the AID Collaborative Network (see detailed participant list). The general consensus of the attendees was that the workshop was timely, informative and well executed. Mini-workshops, a very successful new addition to the overall workshop plan, allowed younger investigators to present their up-to-date research results. One of the most important results of the mini-workshops was the development of communication links between investigators. Many possible collaborative projects were discussed by the various scientists present.

As a result of the workshop, and the supplemental meeting with the RAC Malaria Subcommittee, several follow-up activities by AID management are indicated in order to expedite progress towards the goal of a safe, effective vaccine against human malaria. These actions include:

A. Reviewing the consultant's recommendations for updating AID's current strategy in this area and preparing an appropriate new strategy document.

B. Organizing a meeting in the near future with the principal investigators and other consultants, as appropriate, to begin work on standardizing research protocols, data recording and analysis procedures, identifying and selecting specific cloned strains of P. falciparum for use by network laboratories, and standardizing other procedures as necessary to maximize data reliability.
C. Organizing a committee to review all experimental protocols which will involve the utilization of Aotus monkeys.

D. Continuing to collaborate closely with other funding organizations/agencies, through the Inter-Agency Coordinating Committee on Malaria Immunology and Vaccination Research.

E. Continuing to collaborate with the Inter-Agency Coordinating Committee to address the potential future problems involving patent rights for experimental malaria vaccines. A meeting of funding agency representatives is tentatively scheduled for June of 1981 to discuss this subject.

F. Cooperating with W.H.O. in supporting a Joint W.H.O. - A.I.D. workshop, in connection with the TDR Scientific Working Group Meeting, to be held in Washington sometime during March or April, 1982.
Annex I

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