PD-AAT-378 4-096

AGENCY FOR INTERNATIONAL DEVELOPMENT

PROJECT DATA SHEET

1. TRANSACTION CODE: A (A = Add, C = Change, D = Delete) — Amendment Number: ____

DOCUMENT CODE: 3

2. COUNTRY/ENTITY: Worldwide

3. PROJECT NUMBER: 931-0453.12

4. BUREAU/OFFICE: S&T/H — 10

5. PROJECT TITLE (maximum 40 characters): Malaria Immunology - BRI

6. PROJECT ASSISTANCE COMPLETION DATE (PACD): MM 06 DD 15 YY 89

7. ESTIMATED DATE OF OBLIGATION (Under "B." below, enter 1, 2, 3, or 4)
A. Initial FY 86 B. Quarter 3 C. Final FY 88

8. COSTS ($000 OR EQUIVALENT $1 =)

A. FUNDING SOURCE	FIRST FY .86 B. FX	C. L/C	D. Total	LIFE OF PROJECT E. FX	F. L/C	G. Total
AID Appropriated Total	1184		1184	3735		3735
(Grant)	(1184)	( )	(1184)	(3735)	( )	(3735)
(Loan)	( )	( )	( )	( )	( )	( )
Other U.S. 1.						
Other U.S. 2.						
Host Country						
Other Donor(s)						
TOTALS	1184		1184	3735		3735

9. SCHEDULE OF AID FUNDING ($000)

A. APPROPRIATION	B. PRIMARY PURPOSE CODE	C. PRIMARY TECH. CODE 1. Grant	2. Loan	D. OBLIGATIONS TO DATE 1. Grant	2. Loan	E. AMOUNT APPROVED THIS ACTION 1. Grant	2. Loan	F. LIFE OF PROJECT 1. Grant	2. Loan
(1) ST/H	540	542				3735		3735	
(2)									
(3)									
(4)									
TOTALS						3735		3735	

10. SECONDARY TECHNICAL CODES (maximum 6 codes of 3 positions each)

11. SECONDARY PURPOSE CODE

12. SPECIAL CONCERNS CODES (maximum 7 codes of 4 positions each)
A. Code
B. Amount

13. PROJECT PURPOSE (maximum 480 characters)

The purpose of this project is to develop malaria vaccines against the liver stages of the human malaria parasites, to complete the development of the ISI assay, and to provide infected mosquitoes for malaria vaccine clinical trials.

14. SCHEDULED EVALUATIONS
Interim MM 11 YY 87 MM 11 YY 88 Final MM 06 YY 89

15. SOURCE/ORIGIN OF GOODS AND SERVICES
☐ 000 ☐ 941 ☒ Local ☐ Other (Specify) ____

16. AMENDMENTS/NATURE OF CHANGE PROPOSED (This is page 1 of a ____ page PP Amendment.)

17. APPROVED BY
Signature: Kenneth J. Bart, M.D.
Title: Agency Director for Health
Date Signed: MM 01 DD 15 YY 87

18. DATE DOCUMENT RECEIVED IN AID/W, OR FOR AID/W DOCUMENTS, DATE OF DISTRIBUTION
MM DD YY

PROJECT AUTHORIZATION

Name of Entity: Interregional

Project Title: The Exoerythrocytic Stage of Human Malaria Parasite

Project No.: 931-0453.12

Grantee: Biomedical Research Institute
Rockville, Maryland

1. Pursuant to Section 104 of the Foreign Assistance Act of 1961, as amended, I hereby authorize the centrally-funded research project entitled "The Exoerythrocytic Stage of Human Malaria Parasite" involving not to exceed $3,735,000 of S&T Bureau grant funds for fiscal year 1986 over a three-year period from the date of authorization subject to the availability of funds in accordance with the A.I.D. OYB/Allotment process, to help in financing the costs of the project.

2. The project will conduct research to develop vaccine(s) against the several species of human malaria and against the several stages of the malaria parasite, specifically the exoerythrocytic stages. The project will, as well, become a vital component of the clinical trials through the development of sporozoite invasion assay and the production of malaria infected mosquito for human challenge studies.

3. The agreement(s) which may be negotiated and executed by the officer(s) to whom such authority is delegated in accordance with A.I.D. regulations and delegations of authority shall be subject to the following terms and conditions, together with such other terms and conditions as A.I.D. may deem appropriate.

4. Source and Origin of Commodities, Nationality of Services

a. Commodities financed by A.I.D. under the project shall have their source and origin in the cooperating country* or the United States except as A.I.D. may otherwise agree in

*Each cooperating country where research, training, technical or other assistance takes place under the project shall be deemed to be a cooperating country for the purpose of permitting local cost financing.

writing. Except for ocean shipping, the suppliers of commodities or services shall have the cooperating country or the United States as their place of nationality, except as A.I.D. may otherwise agree in writing.

b. Ocean shipping financed by A.I.D. under the project shall, except as A.I.D. may otherwise agree in writing, be financed only on flag vessels of the United States.

Clearances:
S&T/H, GShivers GS Date 4/11/86
S&T/H, AVanDusen Date 4/25/86
S&T/PO, GGower Date 7 MAY 86
GC/CP, STisa Date 4/23/86

Kenneth J. Bart, M.D.
Agency Director for Health

5/8/86
(Date)

S&T/H/CD:JErickson:tns:3/26/86:2698t
Revised:4/11/86

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

ACTION MEMORANDUM FOR THE AGENCY DIRECTOR FOR HEALTH 11 APR 1986

FROM: S&T/H, Ann Van Dusen Ann Van Dusen

SUBJECT: Malaria Vaccine Development -- Biomedical Research Institute, Rockville, Maryland

Action: Your approval is required for a grant of $3,735,000 from Section 104 of the Foreign Assistance Act of 1961 as amended for project 931-0453.12, Malaria Immunity and Vaccination Research, the Biomedical Research Institute, Rockville, Maryland.

We have reviewed an unsolicited research proposal from the Biomedical Research Institute, proposing a three-year program primarily in the area of the exoerythrocytic stages of human malaria species. This project is a fundamental component of the Agency's malaria vaccine program, and is the only major project working on the exoerythrocytic stages of malaria. The specific project objectives include: (1) the production of Anopheline mosquitoes infected with P. falciparum for use in the natural challenge component of the human volunteer clinical trials, (2) the completion of the development of the inhibition of sporozoite invasion (ISI) assay for direct use in the A.I.D. and U.S. Army human clinical trials as well as in the endemic area field trials against P. falciparum and P. vivax, (3) the study of biochemical synthesis of EE antigen that may be involved in the initial invasion of the red blood cells, (4) the interactions between the sporozoite ligand-hepatic receptors, and (5) the immunoelectron microscopic and biochemical characterization of sporozoite invasion of hepatocytes and EE development.

In the past two years, researchers at BRI have made significant advances to achieve the full in-vitro cycle of development of the exoerythrocytic stage (EE) of P. falciparum and P. vivax in cloned human hepatoma (Hep G2-A16) and embryonic human lung (WI38) cells. When the sporozoite invades a liver cell, both the internalized sporozoite and its surrounding parasitophorous vacuole membrane (PVM) strongly stain for the sporozoite CS protein. During attachment and invasion of P. vivax CS protein is apparently shed onto the surface of the host cell, retained at varying levels and detectable throughout the complete EE cycle. However, CS protein reactivity on EE schizonts for P. falciparum declines rapidly and may be absent from EE merozoites. Further elaboration of this cellular level phenomenon may have significant importance for the development and utility of sporozoite-based P. vivax and P. falciparum vaccines. The key,

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therefore, is that this project is focused on a new method to attack the sporozoite stage of the malaria parasite, not with the circulating blood stream, but as the parasite attempts to penetrate the liver cells where it goes through the exoerythrocytic stage before emerging and beginning the asexual blood cell stage of the disease. This project is of fundamental importance to the A.I.D. malaria program because it provides the only assay for protection to be used in the human trials and, as well, provides the required malaria infected mosquitoes for the human trials.

The unsolicited research proposal is attached for further information and details of the specific experimental design.

Justification to the Congress: Project Funding is included in the FY 1986 Congressional Presentation, Annex V, Centrally Funded Programs, page 53.

Clearances Obtained: This project was approved by the A.I.D. external expert panel on the basis of scientific methodology, qualifications and experience of the proposed investigators, and the institutional capability in accordance with FPR 1-4.909, FPR 1-4.910 (b) and (c), and FPR 1-4.911.

Recommendation: That you sign the attached authorization.

Attachments:
1. Project Authorization
2. BRI proposal

Clearances:
S&T/H, GShivers GS Date 4/11/86
S&T/PO, GGower Date 7 May 86
GC/CP, STisa ST Date 4/23/86

S&T/H:JEErickson:tns:3/31/86:2698t
Revised:4/11/36

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May 5, 1986

MEMORANDUM:

TO: S&T/H, Kenneth J. Bart, M.D.

FROM: S&T/H/VWBDC, James M. Erickson

SUBJECT: Status - B.R.I. Proposal - Malaria Vaccine Research

Per our discussion after the receipt of Dr. Hollingdale's letter of April 28, 1986, we plan to go forward with the project. We are satisfied that the primaquine studies will be useful to the immunology of malaria field since primaquine is being used as a tool to help locate the specific hepatic receptor which could, when identified, lead to a sporozoite vaccine with a different mode of action from the prototype of New York University. The budget will be reduced during the negotiation phase by the contract negotiators.

We would move the PAF forward ASAP subject to the PIO/T reflecting budget issues to be addresssed by SER/AAM/ST/HP.

Concurrence: KJBart _____ Date _____

Research Contract Proposal

entitled

The exoerythrocytic stage of human malaria parasites

Submitted to

Office of Health
Room 709C, RPC SA-18
United States Agency for
International Development
Washington, D.C. 20523

Submitted by

Biomedical Research Institute
12111 Parklawn Drive
Rockville, Maryland 20852

Michael R. Hollingdale, Ph.D.
Principal Investigator

James L. Leef, Ph.D.
Director

A. PROGRESS ACHIEVED ON CURRENT CONTRACT

The current contract (June 16, 1983 to June 15, 1986) contains two principal objectives. The first, to achieve the full cycle of development of the exoerythrocytic (EE) stage of *Plasmodium vivax* and *P. falciparum* in a suitable cell culture system, has been fully achieved. The full cycle of development of *P. vivax* was demonstrated in the human hepatoma (HepG2-A16) cell line. Of interest was the observation that *P. vivax* sporozoites differentiated *in vitro* into primary schizonts and persisting non-dividing parasites resembling hypnozoites previously described *in vivo*. *P. falciparum* sporozoites also invaded HepG2-A16 cells, and inhibition of sporozoite invasion (ISI) by monoclonal antibodies to the circumsporozoite (CS) protein was correlated with *in vivo* protection. The ISI activity of antibodies raised in rabbits by the synthetic CS peptide vaccine, or of antibodies raised in mice by the recombinant CS protein vaccine predicted their *in vivo* protection. The complete cycle of EE development of *P. falciparum* has been achieved in cultured human hepatocytes through the subcontract to the University of Nijmegen.

The second objective was molecular characterization of the human hepatocyte receptor for plasmodial species, and this has been characterized as protein, probably associated with the hepatic cytoskeleton, and is possibly similar for *P. falciparum* and *P. berghei*. This project is in progress and will receive the major emphasis for the remainder of the contract period.

The results from these two objectives are summarized below.

Summary of results

A. To achieve the full *in vitro* cycle of development of the EE stage of *P. vivax* and P. *falciparum* in a suitable cell culture system.

1. Selected cell lines

Both primary cell cultures and continuous cell lines have been examined for susceptibility to *P. vivax* and *P. falciparum* sporozoite invasion, and for subsequent EE development. Primary cultures included cells derived from rodent, simian and human tissues, and cell lines were derived from rodent liver, monkey kidney and Hela cells. However, although occasional invasion of some cell types was observed, especially *P. falciparum* of VERO cells, and *P. vivax* of rat hepatoma cells (1), infections were either low or negative. Only the cloned human hepatoma (HepG2-A16) cell line showed significant infection with either species (2), although low levels of invasion was observed in embryonic human lung (WI38) cells, previously used for *P. berghei* studies (2).

The full cycle of EE development of dividing and non-dividing parasites of *P. vivax* was observed in the HepG2-A16 cell line (3). *P. vivax* sporozoites invaded HepG2-A16 cells, and transformed into trophozoites by 25 hr, and measured about 5-6μm in diameter. After day 3, nuclear division was initiated and by day 5 small multinucleated schizonts were seen, which rapidly grew to an average diameter of 37μm by day 9, with some parasites as large as 80μm. Merozoites were seen by phase microscopy at days 9 to 12. In contrast to these rapidly growing EE schizonts, small 5-6μm parasites, were seen at days 5 to 9 when reacted in an immunoperoxidase antibody test (3) using a monoclonal antibody (Mab) (4) to the *P. vivax* CS protein. Approximately similar numbers of schizonts and non-dividing parasites were seen at each time interval. These small parasites closely resembled EE trophozoites seen at 24 hr, with a central round body surrounded by a membrane, both of which strongly reacted with the *P. vivax* Mab. This strong reactivity was quite different from the weaker reactivity of the larger schizonts, and closely resembled *in vivo* parasites (3). A necessary adaption of the culture technique was irradiation of the HepG2-A16 cell monolayer prior to sporozoite inoculation to prevent cell division (5). Such monolayers remained stable for at least 5 weeks, and may allow for long term observations of these hypnozoite-like parasites, which thus far have been shown to persist at least 15 days *in vitro* (3).

The strains used for these culture studies were ONG and Chesson, of South Asian origin, in which relapses occur shortly following the primary infection. The effects of inoculation into human volunteers of graded doses of the Chesson strain of *P. vivax* had suggested that the proportion of sporozoites that give rise to primary EE schizonts was similar to those giving rise to hypnozoites. This was supported by *in vivo* studies in chimpanzees (6), and the occurence of similar numbers of primary EE schizonts and non-dividing small parasites in the HepG2-A16 cells suggested that the *in vitro* culture system likely represented the *in vivo* infection. In a second series of experiments (7), a strain isolated from North Korea was used and only a single primary EE schizont, but many hypnozoites, were observed. This again confirmed dilution experiments in volunteers which had predicted that sporozoites of this strain, from temperate areas and characterized by a long period between infection and relapse, would differentiate predominantly into hypnozoites.

Earlier work suggested that *P. vivax* sporozoites inoculated into cultures of human hepatocytes would develop into EE parasites (8). This was not confirmed until 1984, when Mazier and her colleagues demonstrated primary *P. vivax* EE schizonts in cultured human hepatocytes (9). EE development was only detected using Giemsa staining and hypnozoites were not described. Although it was claimed that *in vitro* invasion of red blood cells by EE merozoites also occurred, the evidence remains unconvincing. A major obstacle to using cultured human hepatocytes is the restriction on their availability. Secondly, such cultures are non-dividing and are viable for short periods of time, whereas the HepG2-A16 cell line grows in continuous culture. Despite these problems, use of

cultured human hepatocytes may be essential for *in vitro* culture of *P. falciparum*. Although *P. falciparum* sporozoites invaded the HepG2-A16 cells, only a small proportion (10%) showed transformation into EE trophozoites, and further development into schizonts was never seen. Only human hepatocytes support the growth of the EE stages of *P. falciparum*. This was first achieved by Meis and his collaborators in 1984 who used immunofluorescent antibody tests to detect developing *P. falciparum* EE schizonts immediately adjacent to the host cell nucleus (10). Again this was confirmed by Mazier's group in 1985 (11).

2. Serologic characterization.

The serological properties of *P. vivax* and *P. falciparum*, as well as further studies of *P. berghei*, EE stages have been examined. *P. vivax* and *P. berghei* were grown in the HepG2-A16 cell line, and *P. falciparum* was grown in human hepatocytes. We have used monoclonal antibodies to sporozoites and erythrocytic merozoites, and hyperimmune human sera from endemic areas, in both immunoperoxidase and immunofluorescent antibody assays.

Immediately following sporozoite invasion, both the internalized sporozoite and its surrounding parasitophorous vacuole membrane (PVM) strongly stain for the sporozoite CS protein (12). During attachment and invasion, CS protein is apparently shed onto the surface of the host cell, and can still be detected after invasion; the significance of CS - hepatic interactions will be fully discussed in section 3 describing receptor characterization. Primary EE schizonts of *P. vivax* (3), like those of *P. berghei*, retain CS protein antigenicity throughout the complete EE cycle, and the protein is apparently present in EE merozoites. However, quantitatively the CS protein appears to decline throughout the *P. berghei* cycle using a radioimmunoassay, and this appears to be true for *P. vivax*. Interestingly, the CS protein of *P. berghei* is in the fully processed form following invasion (13). In contrast, it appears that CS protein reactivity declines rapidly in *P. falciparum* and may be absent in EE merozoites (10).

We have previously shown that immune sera to erythrocytic stages of *P. berghei* weakly react with *P. berghei* EE trophozoites, but strongly react with EE schizonts and merozoites. This has been extended using *P. vivax*. A monoclonal antibody to the major 210K erythrocytic merozoite surface glycoprotein antigen of *P. vivax* also reacts with *P. vivax* EE schizonts, but this antigen is synthesized only during the final 24 hr preceeding EE merozoite formation (14). Thus, *P. vivax* EE schizonts were negative at day 7, but became strongly positive at day 8, using immunoperoxidase staining. A similar result was obtained for *P. berghei*. A monoclonal antibody to the similar glycoprotein antigen of erythrocytic merozoites of *P. yoelii* (15) reacted in immunofluorescence tests with *P.*

berghei erythrocytic and EE parasites.

Current experiments have shown the feasibility of detecting EE antigens using immune sera applied to Western blots of highly infected cultures of HepG2-A16 cells. Preliminary results suggest that *P. berghei* schizonts at day 3 contain an immunodominant antigen possibly common to erythrocytic parasites.

3. Electron microscopy.

The ultrastructure of EE parasites of *P. berghei*, *P. vivax* and *P. falciparum* have been examined by electron microscopy (16, 17, 18). The entire sequence of development of *P. berghei* from invasion to merozoite formation was described (16). Invasion and transformation of *P. berghei* sporozoites in HepG2-A16 cells was identical to that described *in vivo* in rat liver. Of interest was the observation that as the sporozoite enters, a narrow electron dense band, which appears to originate from the ductule of the rhoptry, connects the apical end of the sporozoite and the invaginating plasmalemma of the HepG2-A16 cell. The ultrastructure of transformation into trophozoites, nuclear division, and merozoite formation corresponded to our earlier light microscopic observations. Occasionally, intranuclear sporozoites were seen *in vivo* in rat hepatocytes, and Giemsa-stained cultures of *P. berghei* in HepG2-A16 cells suggested that such sporozoites develop normally into mature EE parasites (19).

HepG2-A16 cell cultures of *P. vivax* were examined by electron microscopy to initially study the ultrastructure of large primary EE schizonts. Since the infectivity of *P. vivax* sporozoites is lower than *P. berghei*, confirmation of the complete development of schizonts was thought to be a necessary prerequesite for studying the small hypnozoite-like parasites. This was the first ultrastructural study of the EE parasites of *P. vivax* (17) and demonstrated that at 8 days, the EE parasites were multinuclear, surrounded by a distinct PVM, and were similar to EE stages of various species of *Plasmodium* grown *in vivo*.

The ultrastructure of the EE parasites of *P. falciparum* was studied in cultures of human hepatocytes (18). The EE parasites were found at densities of up to $100/cm^2$ and grew at rates comparable to that *in vivo* in the chimpanzee. The 5 and 7 day schizonts were very similar to other mammalian EE parasites, eg. the secretory activity and distribution of the peripheral vacuole system, but also resembled avain parasites in their nucleolar structure and mitochondrial form and may thus be distinct from *P. vivax*. An unexpected observation was the presence of microtubules together with a thick plasmalemma in the late EE forms, structures that are typically found in the sporozoite pellicle. In one section cut transversely, 15 microtubules were localized to a section of the parasite plasmalemma only $0.9\mu m$ wide. As was found with *P. berghei*, *P. falciparum* sporozoites also penetrate the hepatocyte nucleus *in vivo*, and appeared to be surrounded by a PVM which originated from either the hepatocyte cell membrane or nuclear membrane.

4. Inhibition of sporozoite invasion (ISI) by antibodies as an *in vitro* assay of protective immunity.

Our earlier experiments (2,20) had suggested that monoclonal antibodies reactive with the CS protein block sporozoite invasion of cultured cells. That this inhibition was predictive of protective immunity was suggested by the findings that such monoclonal antibodies neutralized sporozoite infectivity to chimpanzees and that sera from human volunteers immunized with and resistant to challenge with *P. vivax* and *P. falciparum* sporozoites also completely blocked attachment and invasion. Therefore inhibition of sporozoite invasion (ISI) is a potential *in vitro* assay of protective antibodies.

The need for such an *in vitro* assay has been given considerable impetus by the recent cloning of the *P. falciparum* CS protein gene and its expression in *Escherichia coli* (21,22). Analysis of its nucleotide sequence revealed that the CS protein contains a repeat epitope region of 37 tandem asn-ala-asn-pro tetrapeptides and 4 asn-val-asn-pro tetrapeptides. Candidate malarial vaccines have been proposed that are either synthetic (NYU) or recombinant derived (WRAIR) (21,22). In the synthetic approach, a synthetic dodecapeptide asn-ala-asn-pro-asn-ala-asn-pro-asn-ala-asn-pro or $(NANP)_3$ has been tested for immunogenicity in rabbits (23), or mice (24). In the recombinant approach, proteins composed of 16,32, or 48 tandem copies of the tetrapeptide sequence were expressed in *E. coli*, and their immunogenicity tested in mice (25). Antibody responses in vaccinated animals were assayed for reactivity with synthetic peptides or intact sporozoites. Sera were then tested in the ISI assay to predict protective activity (23, 24, 25).

Significant ISI activity of rabbit immunoglobulin to the $(NANP)_3$ synthetic peptide was demonstrated, and ISI activity was directly related to concentration of antibody. A strong effect was observed with IgG concentrations as low as $2\mu g/ml$. When the antibodies to $(NANP)_3$ were removed by absorption with peptide bound to Sepharose beads, the inhibitory effect was abolished (23). In these immunization studies the synthetic peptide was coupled to tetanus toxoid using gluteraldehyde and inoculated in complete Freunds adjuvant.

In studies using recombinant proteins, complete Freunds adjuvant, alum or no adjuvant were used, and the pooled mouse antisera were tested for ISI activity. Three ISI values were calculated: ISI_{max} was the highest ISI value at any serum concentration, and ISI_{100} or ISI_{50} was the reciprocal of that serum concentration that inhibited invasion by 100% or 50%. Both sera to the synthetic peptides and to the recombinant proteins showed a prozone effect in which inhibition was actually reduced at high immunoglobulin or antibody concentrations. This effect is probably due to low levels of antibodies which bind to the repeat epitope region without causing neutralization and thus block the binding of inhibitory antibodies; as the serum or immunoglobulin is diluted these blocking antibodies are diluted out. Thus ISI_{max} represents the true maximum ISI activity. In the study with

synthetic peptide $(NANP)_3$, up to 95% ISI was demonstrated. With recombinant proteins, ISI_{max} values of 95-100% were achieved using complete Freund's adjuvant or alum, and 74-100% without adjuvant. ISI_{100} values were 100-200. ISI_{50} values varied from 85,000 with complete Freunds adjuvant to 19,000 with alum and 8,200 with no adjuvant. These values were for the R32 recombinant protein given as a primary inoculation followed by 2 boosts. ISI_{50} values were lower after 1 boost (30,000; 3,400;1250 respectively). These results, together with antibody levels to repeat epitope sequence peptides, suggest that a similar sequence of inoculations using alum in the human volunteer Phase 1 and 2 trials might be expected to elicit significant levels of protective antibodies.

In these immunogenicity trials, although both antibody levels to the repeat epitope sequence and ISI levels were measured, no direct correlation with protection is possible. This will be possible during Phase 2 human studies, when vaccinated volunteers will be challenged with *P. falciparum* sporozoites, and resistance measured. Since the *in vitro* assay measures only the inhibitory effect of antibody upon sporozoite invasion, other antibody mediated mechanisms are not tested. These include antibody mediated opsonization by macrophages via the Fc receptor. Since liver sinusoids are lined with Kupffer cells, this mechanism is likely to be at least as important as direct antibody inhibitions of invasion. Phase 2 trials will begin to allow analysis of ISI data in an attempt to predict the ISI_{max}, ISI_{100} or ISI_{50} values at which vaccinated humans can be expected to be completely resistant to sporozoite challenge.

A second question not answered by the immunogenicity trials is the correlation between anti-repeat epitope antibody titers by ELISA and ISI activity, as all animals were immunized by procedures which induce maximum antibody levels. A correlation between each technique is essential, however, both in predicting actual resistance to challenge, and for developing assays suitable for testing pre-existing anti-sporozoite immunity in malaria endemic areas. Use of both a serological and functional assay together would provide valuable information on the acquisition and persistence of anti-sporozoite immunity.

Preliminary results using endemic sera from the Gambia (26) suggested that adult sera had significant ISI activity. In one case a patient had negative ISI and CSP activity during the dry season of low transmission and converted to positive ISI and positive CSP by the end of the rainy season. Examination of sera from Indonesia (27) by ELISA and ISI suggested that very young babies lacked significant antibody levels. However, beginning at 2-3 yrs, ISI activity became evident and peaked by 16 yrs. ELISA titers did not show direct correlation to ISI activity, and further experiments are in progress to determine which antibody class is significant in determining ISI activity.

5. To study *in vitro* infectivity of cryopreserved sporozoites

It is of advantage to be able to freeze and store sporozoites when they are available in quantity and to use a standardized batch for a series of experiments. Methods of cryopreservation were assayed using *P. berghei* sporozoites, and infectivity measured by invasion of HepG2-A16 cells. Optimum preservation of infectivity was obtained if sporozoites were suspended in mouse serum (50%) and hydroxyethyl starch (50%) and frozen at 30 to 60°C per minute. Infectivity before freezing was 8%, and after freezing and thawing was 0.5%. Cryopreservation of sporozoites may be of practical use for storage of those species such as *P. vivax*, which are not available continuously.

B. Molecular characterization of the human hepatocyte receptor for Plasmodial sporozoites.

1. Characterization.

Of considerable interest is whether sporozoites of different species recognize similar or different hepatic receptors. Species specificity of infection exists *in vivo* but how it operates on a cellular level is unknown. Certainly, rodent sporozoites do not infect humans, and *vice versa*. However this could result either from species specificity at the hepatic level influencing sporozoite infectivity and development, or at the erythrocytic level. *In vitro* sporozoite infectivity is not strictly species specific. *P. berghei* sporozoites equally develop EE parasites in rodent hepatocytes, human embryonic (WI38) lung fibroblasts, and human hepatoma (HepG2-A16) cells. However, human sporozoites *P. vivax* and *P. falciparum* appear only to develop EE parasites within human cells, whether hepatocytes or hepatoma cells. Hepatoma HepG2-A16 offer several advantages for the study of sporozoite receptor(s) because they are susceptible to attachment and invasion by rodent, *P. berghei* and *P. yoelii*, and human *P. vivax* and *P. falciparum* sporozoites.

The chemical nature of the putative *P. berghei* sporozoite receptor has been identified as protein (29). Attachment and invasion of the sporozoites was completely abolished after treatment of the cells with papain or chymotrypsin, though not with trypsin. Earlier unpublished results had shown that WI38 cells pretreated with chymotrypsin were also rendered insusceptible to *P. berghei* sporozoite attachment and invasion. Treatments of HepG2-A16 cells with agents that might be expected to abolish carbohydrate and lipid reactivity had no effect on *P. berghei* sporozoite attachment or invasion. The sporozoite receptor is distinct from three specific hepatic receptors, the galactose-terminal glycoprotein receptor, the fucose receptor , and the mannose/N-acetyl-glucosamine receptor, as their respective ligands used at concentrations that completely block receptor function had no effect on *P. berghei* sporozoite attachment and invasion.

Attachment of sporozoites to HepG2-A16 (and other selected cell types) involves the

apparent shedding of CS protein onto the surface of the target cell (12). This is followed by invagination of the target cell membrane and interiorization of the sporozoite. The CS protein released onto the cell surface has an apparent pattern of immunoperoxidase staining which might reflect a specific distribution of CS receptors on the HepG2-A16 cell surface. Treatment of HepG2-A16 cells with drugs (colchicine or cytochalasin B) that disrupt cytoskeletal structure, particularly intermediate filaments, also disrupt the pattern of CS staining. This suggests that the CS receptor is linked through the cell membrane into underlying cytoskeletal filaments.

Cytoskeletons prepared from HepG2-A16 cells, but not from cell types insusceptible to sporozoite attachment, retained reactivity with sporozoites. Analysis by polyacrylamide gel electrophoresis revealed that such preparations contained multiple proteins. These proteins were transferred by Western blotting to nitrocellulose strips which were then incubated with *P. falciparum* or *P. berghei* sporozoites. Both *P. falciparum* and *P. berghei* sporozoites bound to a similar region on the strips (45-55,000 daltons), with minor binding to other regions. The specificity of this binding data was suggested by the finding that monoclonal antibodies to the CS protein blocked binding.

Anti-cytoskeletal antisera raised in rabbits reacted with the surface of HepG2-A16 cells by immunofluorescence, suggesting that the cytoskeletal preparations retained surface components. Antisera raised in mice to proteins of the region to which sporozoites bound also reacted with the HepG2-A16 cell surface. Both anti-cytoskeletal antiserum, and antiserum to one band of the 45-55,000 dalton region, also partially inhibited sporozoite attachment to HepG2-A16 cells. These results suggest that *P. falciparum* and *P. berghei* sporozoites recognize a similar or identical HepG2-A16 cell receptor.

The occurence of a common receptor for both *P. falciparum* and *P. berghei* sporozoites on HepG2-A16 cells suggests that the CS ligand is common to both species. This would argue against the species-specific repeat epitope regions acting as ligands. Thus antibody-medieated anti-sporozoite protective immunity may function indirectly via the repeat epitope region rather than directly by blocking ligand-receptor recognition. Experiments were designed to test this hypothesis using recombinant and synthetic sequences as potential sporozoite ligands.

A series of synthetic peptides were synthesized, containing from 4 to 24 amino acids of the NANP *P. falciparum* repeat epitope sequence, as described for the immunogenicity studies in mice (24). These were compared with the recombinant proteins $R16tet_{32}$, $R32tet_{32}$, $R48tet_{32}$ (25) for binding to HepG2-A16 cells. At concentrations of 1000-0.1 μg/ml, none of the synthetic peptides either bound to the HepG2-A16 cells, or inhibited *P. falciparum* sporozoite invasion. Initial studies with the recombinant proteins showed that each apparently bound to the HepG2-A16 cell surface. Binding was detected by immunoperoxidase staining of HepG2-A16 cells using a monoclonal antibody reactive

with the NANP repetitive sequence. However, inhibition of *P. falciparum* sporozoite invasion by the recombinant proteins was impossible to assay by the immunoperoxidase assay. The occurence of the NANP sequence on both the recombinant protein and sporozoite surface masked visualization of invading sporozoites. This was partially overcome by using a monoclonal antibody directed to the minority NVDP repeat sequence which occurs 4 times compared to 37 NANP repeats in each CS protein molecule. In contrast, $R32tet_{32}$ contained 30 NANP and 2 NVDP sequences. This allowed *P. falciparum* sporozoites to be visualized at recombinant protein concentrations up to 5μg/ml, and an apparent dose response relationship between $R32tet_{32}$ concentration and inhibition of sporozoite invasion was demonstrated. The discrepancy between the lack of activity of synthetic peptides of up to 24 amino acids, or $(NANP)_6$, as compared to the apparent activity of the $R32tet_{32}$, or $(NANP)_{30}$ $(NVDP)_2tet_{32}$, recombinant protein remained to be resolved.

This might be explained by differences in sizes and morphology of synthetic peptides and recombinant proteins. However, examination of the amino acid sequence of the plasmid vector derived tet_{32} tail revealed multiple sequences of 12 of the 32 amino acids were arginine molecules which raised the isoelectric point to above 12.0. The possibility thus arose that the activity of $R32tet_{32}$ was due to the highly charged plasmid-derived 32 amino acid tail non-specifically binding to the HepG2-A16 cells. Binding experiments using ^{125}I-labelled $R32tet_{32}$ demonstrated that it bound to cells as well as glass or plastic surfaces. The involvement of the tet_{32} tail was confirmed by removing the tail from the R32 repeat sequence. This was done by trypsin digestion of $R32tet_{32}$, which was converted to R32-leucine-arginine. The R32-leucine-arginine molecule did not bind to HepG2-A16 cells nor inhibit *P. falciparum* sporozoite invasion. The lack of activity of the repeat sequence as a ligand recognizing the HepG2-A16 cell surface was further confirmed using synthetic peptides which had been polymerized after synthesis to represent either $(NANP)_{10}$, $(NANP)_{15}$, $(NANP)_{35}$ and $(NANP)_{47}$. Again, none of these synthetic molecules bound to HepG2-A16 cells.

The conclusion from these experiments was in agreement with the cytoskeleton experiments which predicted that the sporozoite ligand could not be the species-specific repeat epitope regions. Therefore, the ligand region had to be contained within other regions of the CS protein. Comparison of the amino acid sequence of *P. knowlesi* and *P. falciparum* showed that the CS protein contained two conserved regions of homologous sequence (30). Region 1 of *P. falciparum* (22) was similar but not identical to the N region of *P. knowlesi* (30). It has been suggested (30) that these regions were conserved for a specific ligand function of recognition of hepatocytes. This would be in agreement with the concept of a similar or identical receptor on HepG2-A16 cells and human hepatocytes, since both *P. knowlesi* and *P. falciparum* invade HepG2-A16 cells and human hepatocytes.

Publications from Current Contract Period

Hollingdale, M. R., Leland, P., Schwartz, A. L.: 1983. *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line. Am. J. Trop. Med. Hyg., 32: 682-684.

Hollingdale, M. R., Leland, P., Leef, J. L., Schwartz, A. L.: 1983. Entry of *Plasmodium berghei* sporozoites into cultured cells, and their transformation into trophozoites. Am. J. Trop. Med. Hyg., 32: 685-690.

Cogswell, F. B., Krotoski, W. A., Hollingdale, M. R., Gwadz, R. W.: 1983. Identification of hypnozoites and tissue schizonts of *Plasmodium vivax* and *P. cynomolgi* by the immunoperoxidase method. Am. J. Trop. Med. Hyg., 32: 1454-1455.

Alvares, A. P., Ueng, T., Scheibel, L. W., Hollingdale, M. R.: 1984. Impairment of hepatic cytochrome P-450-dependent monooxegenases by the malaria parasite *Plasmodium berghei*. Mol. Biochem. Parasitol., 13: 277-282.

Meis J. F. G. M., Hollingdale, M. R. Verhave J. P., Aikawa, M.: 1984, Intranuclear localization of *Plasmodium berghei* sporozoites. Cell. Bio. Int. Rep., 8: 1016.

Leland, P., Sigler, C. I., Danforth, H. D., Hollingdale, M. R.: 1984. Inhibition of *Plasmodium berghei* sporozoite invasion of cultured hepatoma cells. Trans. R. Soc. Trop. Med. Hyg., 78: 639-640.

Aikawa, M., Schwartz, A. L., Uni, S., Nussenzweig, R. S., Hollingdale, M. R.: 1984. Ultrastructure of *in vitro* cultured exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line. Am. J. Trop. Med. Hyg., 33: 792-799.

Sigler, C. I., Leland, P., Hollingdale, M. R.: 1984. *In vitro* infectivity of irradiated *Plasmodium berghei* sporozoites to cultured hepatoma cells. Am. J. Trop. Med. Hyg., 33: 544-547.

Hollingdale, M. R., Nardin, E. H., Tharavanij, S., Schwartz, A. L., Nussenzweig, R. S.: 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells; an *in vitro* assay of protective antibodies. J. Immunol., 132: 909-913.

Zavala, F., Hollingdale, M. R., Schwartz, A. L., Nussenzweig, R. S., Nussenzweig, V.: 1985. Immunoradiometric assay to measure the *in vitro* penetration of sporozoites of malarial parasites into hepatoma cells. J. Immunol., 134: 1202-1205.

Schwartz, A. L., Hollingdale, M. R.: 1985. Primaquine and lysomotropic amines inhibit malaria sporozoite entry into human liver cells. Mol. Biochem. Parasitol., 14: 305-311.

Hollingdale, M. R., Collins, W. E., Campbell, C. C., Schwartz, A. L.: 1985. *In vitro* culture of two populations (dividing and nondividing) of exoerythrocytic parasites of *Plasmodium vivax*. Am. J. Trop. Med. Hyg., 34: 216-222.

Hollingdale, M. R.: 1985. Malaria and the liver. Hepatol., 5: 327-335.

Hollingdale, M. R., Leland, P., Sigler, C. I., Leef, J. L.: 1985. *In vitro* infectivity of cryopreserved *Plasmodium berghei* sporozoites to cultured cells. Trans. R. Soc. Trop. Med. Hyg., 79: 206-208.

Hollingdale, M. R., Leland, P., Sigler, C. I.: 1985. *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* in irradiated hepatoma cells. Am. J. Trop. Med. Hyg., 34: 21-23.

Hollingdale, M. R., McCann, P. P., Sjoerdsma, A.: 1985. *Plasmodium berghei*: effects of inhibitors of ornithine decarboxylase on the exoerythrocytic stage. Exp. Parasitol., in press.

Hollingdale, M. R.: 1985. *In vitro* testing of anti-malarial schizonticides. In; Primaquine: Pharmacokinetics, metabolism, toxicity and activity, in press.

Uni, S., Aikawa, M., Collins, W. E., Campbell, C. C., Hollingdale, M. R. Electron microscopy of *Plasmodium vivax* exoerythrocytic stages grown *in vitro* in a hepatoma cell line. Am. J. Trop. Med. Hyg., in press.

Hollingdale, M. R., Leland, P.: 1985. *In vitro* cultivation of the exoerythrocytic stage of malarial parasites. In; Atomic energy applications in parasitology, IAEA, Vienna: in press.

Beaudoin, R. L., Hollingdale, M. R.: Parasite biology - *In vitro* cultivation of the exoerythrocytic stages. In; Textbook of malaria, ed Wernsdorfer & McGregor, in press.

Ballou, W. R., Rothbard, J., Wirtz, R. A., Gordon, D. M., Williams, J. S., Gore, R. A., Schneider, I., Hollingdale, M. R., Beaudoin, R. L., Maloy, W. L., Miller, L. H., Hockmeyer, W. T.: 1985. Immunogenicity and biologic activity of antibodies to synthetic peptides of the circumsporozoite protein of *Plasmodium falciparum*. Science, 228: 996-999.

Young, J. F., Hockmeyer W. T., Gross, M., Ballou, W. R., Wirtz, R. A., Trosper, J. H., Beaudoin, R. L., Hollingdale, M. R., Miller, L. H., Diggs, C. L., Rosenberg, M.: 1985. Expression of *P. falciparum* circumsporozoite protein derivatives in *E. coli* for development of a human malaria vaccine. Science. 228: 958-962.

Zavala, F., Tam, J. P., Hollingdale, M. R., Cochrane, A. H., Quakyi, I., Nussenzweig, R. S., Nussenzweig, V.: 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. Science, 228: 1436-1440.

Meis, F. G. M., Verhave, J-P., Ponnudurai, T., Hollingdale, M. R., Rijntjes, J. M., Smith, J. E., Sinden, R. E., Jap, P. H. K., Meuwissen, J. H. E. Th., Yap, S. H.: Fine structure of the liver forms of the malaria parasite *Plasmodium falciparum* in human hepatocytes *in vitro*. Cell Tissue Res. In press.

Meis, J. F. G. M., Rijntjes, J. M., Verhave, J-P., Ponnvdurai, T., Hollingdale, M. R., Yap, S. H.: Infection of cryopreserved adult human hepatocytes with *Plasmodium falciparum* sporozoites. Cell Biol. Intl. Reports, in press.

Pancake, S. J., Hollingdale, M. R.: Characterization of the *Plasmodium berghei* sporozoite receptor on human hepatoma cells. Ex. Parasitol., submitted.

Krotoski, W. A., Garnham, P., C.C., Cogswell, F. B., Collins, W. E., Bray, R. S., Gwadz, R. W., Killick-Kendrick, R., Wolf, R., Sinden, R., Hollingdale, M. R., Lowrie, R. C., Koontz, L. C., Stanfill, P. S.: Observations on early and late post-sporozoite tissue stages in primate malaria. IV. Pre-erythrocytic schizonts and/or hypnozoites of Chesson and North Korean strains of *Plasmodium vivax* in the chimpanzee. Am. J. Trop. Med. Hyg. submitted.

Hollingdale, M. R., Collins, W. E., Campbell, C. C.: *In vitro* culture of exoerythrocytic parasites of the North Korean strain of *Plasmodium vivax* in hepatoma cells. Am. J. Trop. Med. Hyg. submitted.

Hollingdale, M. R., Ballou, W. R., Aley, S. B., Pancake, S. J., Miller, L. H., Hockmeyer, W. T. *Plasmodium falciparum* recombinant CS proteins elicit protective anti-sporozoite antibodies in mice that inhibit sporozoite invasion (ISI) of hepatoma cells. J. Immunol. submitted.

Hollingdale, M. R., Sigler, C. I., Leland, P. : *Plasmodium berghei*: *in vitro* effects of primaquine on exoerythrocytic parasite development. Ex. Parasitol., submitted.

Hollingdale, M. R. *In vitro* culture of exoerythrocytic malaria parasites. In: Culture of parasites *In vitro*, eds Baker and Taylor, Academic Press (London). in preparation

Pancake, S. J., Cooper, J. A., Hollingdale, M. R. Reaction of *Plasmodium berghei* sporozoites with cytoskeletal-associated target cells sites. Cell Biol. Intl. Reports, in preparation.

Aley, S. B., Barnwell, J. W., Bates, M. D., Collins, W. E., Hollingdale, M. R. Common Antigens of *Plasmodium vivax* merozoites from blood or liver stages demonstrated by specific monoclonal antibody, in preparation.

Abstracts

Hollingdale, M. R., Schwartz, A. L. 1983. Primaquine and other lysomotropic amines inhibit malaria sporozoite entry into human liver cells. Ann. Mtg. Am. Soc. Trop. Med. Hyg., San Antonio, Tx.

Hollingdale, M. R. 1983. *In vitro* invasion of cultured cells by sporozoites of primate and human species. 2nd Int. Conf. Malaria Babesiosis. Annecy, France.

Hollingdale M. R., Leland, P., Sigler, C. I., Leef, J. L. 1983. *In vitro* infectivity of cryopreserved malarial sporozoites. Ann. Mtg. Soc. Cryobiol., Cambridge, U. K.

Hollingdale, M. R., Schwartz, A. L. 1983. Sporozoites recognize specific hepatocyte receptors. Symp. Mol. Cell. Biol., Mol. Biol. Host-Parasite Int., Park City, Utah

Hollingdale, M. R., Campbell, C. C., Collins, W. E., Schwartz, A. L. 1984. *In vitro* culture of two populations (mitotically active and non-active) of exoerythrocytic parasites of *Plasmodium vivax*. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Baltimore, Md.

Hollingdale, M. R., Sigler, C. I., Leland, P., Leef, J. L., Aikawa, M. 1984. *In vitro* effects of primaquine on exoerythrocytic parasites of *Plasmodium berghei*. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Baltimore, Md.

Hollingdale, M. R., 1984. The exoerythrocytic stage of malarial parasites. Mtg. Nuc. Mag. Res. Intracell. Parasit. Woods Hole, Mass.

Hollingdale, M. R., 1984. *In vitro* culture of exoerythrocytic parasites of *Plasmodium vivax*. XI Int. Cong. Trop. Med. Malaria, Calgary, Canada.

Pancake, S. J., Hollingdale, M. R., Schwartz, A. L. 1985. Characteristics of target cell receptors for malaria sporozoite surface protein (*Plasmodium berghei* Pb44 protein). Symp. Mol. Cell. Biol., Mol. Biol. Host-Parasite Int., Park City, Utah.

Pancake, S. J., Aley, S., Hollingdale, M. R. 1985. Cytoskeletal association of cellular receptors for malarial circumsporozoite proteins. Fed. Proc., Annaheim, Ca.

Meis, J. F. G. M., Verhave, J. P., Ponnudurai T., Smith, J. E., Hollingdale, M. R., Rijntjes, P. J. M., Yap, S. H., Meuwissen, J. H. E. Th. 1985. Culture of *Plasmodium falciparum* exoerythrocytic forms in primary human hepatocytes. Ann. Mtg. Belgian Dutch Soc. Parasitol., Brussels, Belgium.

Hollingdale, M. R. 1985. Malarial sporozoites recognize specific hepatocyte receptors. Ann. Mtg. Belgian Dutch Soc. Parasitol., Brussels, Belgium.

Meis, J. F. G. M., Verhave, J. P., Ponnudurai, T., Smith, J. E., Hollingdale, M. R., Rijntjes, P. J. M., Yap, S. H., Meuwissen, J. H. E. Th. 1985. Culture of *Plasmodium falciparum* exoerythrocytic forms in primary human hepatocytes. Int. Cong. Protozool. Nairobi, Kenya.

Hollingdale, M. R. Hockmeyer, W. T., Miller, L. H., Beaudoin, R. L., Young, J. F. 1985. *Plasmodium falciparum* CS proteins elicit protective antibodies in mice which block sporozoite invasion of hepatoma cells. Int. Cong. Protozool., Nairobi, Kenya.

Hollingdale, M. R. 1985. Inhibition of polyamines in malarial species. Gordon Research Conference. Polyamines, N. H.

Hollingdale, M. R., Pancake, S. 1985. Malaria sporzoite invasion is mediated by specific CS protein - hepatic cell interactions. Joint Mtg. Roy. Soc. Trop. Med. Hyg. and Scan. Soc. Parasitol. Copenhagen, Denmark.

Aley, S. B., Bates, M. D., Barnwell, J. W. Taylor, D. W., Hollingdale, M. R. 1985. Common antigens on hepatic and blood stage schizonts of human and rodent malarias. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Miami, Fla.

Hollingdale, M. R. 1985. Quantitative *in vitro* assay for tissue stage anti-malarial drugs. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Miami, Fla.

Meis, J. F. G. M., Verhave, J. P., Ponnudurai, T., Meuwissen, J. H. E. Th., Hollingdale, M. R., Rijntjes, P. J. M., Yap. S. H. 1985. Ultrastructure of *Plasmodium falciparum* in primary human hepatocytes *in vitro*. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Miami, Fla.

Hoffman, S. L., Wirtz, R., Ballou, W. R., Schneider, I., Hollingdale, M., Cole, C., Trosper, J. H., Campbell, J., Marwoto, H., Wistar, R., Hockmeyer, W. T. 1985. Correlation between antibodies to repeat region of circumsporozoite protein ($R32tet_{32}$), CS precipitation, inhibition of sporozoite invasion of hepatoma cells, and age in sera from a hyperendemic area. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Miami, Fla.

Pancake, S. J., Cooper, J. A., Hollingdale, M. R. 1985. Cytoskeletal association of target cell receptors for malarial circumsporozoite protiens. Ann. Mtg. Am . Soc. Trop. Med. Hyg., Miami, Fla.

B. SIGNIFICANCE

The progress made in this laboratory to investigate the exoerythrocytic stage of malaria has led to major investigations being initiated in several other laboratories. This extension of interest in the EE stage in recent years reflects the importance of this stage to current efforts to control malaria. Whereas six years ago, little was known of EE parasites except from limited studies *in vivo*, now, the *in vitro* culture techniques developed here have led to considerable advances in our understanding of this stage.

The significance of the EE stage may be considered in the context of development of an anti-malarial vaccine based on three of its four cell cycle stages. Firstly, success of an anti-sporozoite vaccine is dependent upon blocking sporozoite invasion of the target hepatocyte. The development by us (3, 31, 32), and others (9, 10, 11), of *in vitro* culture systems for rodent and human malarias, has allowed a more precise investigation of the mechanism by which anti-sporozoite antibodies confer protection. Thus, the *in vitro* ISI assay (2) represents a functional assay possibly predictive of protection. Our investigations demonstrating that the repeat epitope sequence, though eliciting strongly protective antibodies, is probably not itself functionally involved in hepatic invasion, suggest that identification of regions of the CS protein that act as ligands may present new targets for vaccine development. Secondly, antigens of the EE parasite itself may be possible vaccine(s). Although the serological development of the EE parasite has been investigated (3,33), EE antigens expressed on the surface of infected hepatocytes might represent an ideal vaccine target. Thirdly, EE merozoites invade the same target cell, the erythrocyte, as blood stage merozoites and thus must share common functional components essential to invasion. Identification of these components may be of considerable benefit to developing a merozoite vaccine.

This research is being proposed within the context of malarial vaccine research being undertaken within the AID network. The rapidly developing and imminent testing of the sporozoite vaccine involves our collaboration with both New York University and the University of Maryland. We are proposing both to maintain a colony of *P. falciparum* infected mosquitoes for the challenge studies and to continue to test sera by the ISI assay. The recent cloning of the *P. vivax* CS protein gene (34, 35) indicates that similar systems for *P. vivax* will need to be developed, and a source of *P. vivax* infected mosquitoes becomes of vital importance. Application of the ISI assay to epidemiological surveys is proposed in collaboration with CDC and New York University. Development of a vaccine based on sporozoite antigens includes identification of the ligand-receptor interactions, studies in which we closely collaborate with New York University. Serological investigation of the developing EE parasites and merozoites requires use of monoclonal antibodies, and these are available to us from New York University, and the CDC. Since successful vaccine antigens may usually, but not always, have an identifiable function, collaborative immunoelectron microscopic investigations with Case Western University are proposed. The success of the

one year subcontract at the University of Nijmegen in developing the human hepatocyte system has led to a unique resource available to the network. It is proposed to strengthen and extend this collaboration to include the University of Utrecht where the immunelectron microscopic investigations of hepatic receptors have been largely performed. Finally, identification of EE merozoite antigens will allow their comparison with the blood stage merozoite investigations underway at the University of Hawaii and the Scripp's Institute.

This proposal is thus envisaged as containing two major components. Firstly, significant component of support for current or future vaccine trials involving sporozoite challenge and serum characterization (sections 1 and 2). Secondly, basic research to further identify new vaccine targets (Sections 3, 4, 5).

C. SCIENTIFIC OBJECTIVES

1. Production of Anopheline mosquitoes infected with *P. falciparum*

2. Development of the inhibition of sporozoite invasion (ISI) assay for use in clinical trials and in areas endemic for *P. falciparum* and *P. vivax*.

3. Study of the biochemical synthesis of EE antigens that may be involved in the initial invasion of red blood cells.

4. Sporozoite ligand-hepatic receptor interactions.

5. Immunoelectron microscopic and biochemical characterization of sporozoite invasion of hepatocytes and EE development.

1. Production of Anopheline mosquitoes infected with *P. falciparum*.

During the current contract period, the insectary facility at the Institute has been expanded to six environmental rooms. This facility will allow the production of rodent and *P. falciparum* infected mosquitoes. In addition, two tissue culture rooms for the production of *P. falciparum* gametocyte cultures have been equipped with hoods and incubators and support equipment.

a) Production of mosquitoes.

Currently, a colony of *A. freeborni* mosquitoes has been raised and is housed in one insectary at 27°C, 80% humidity. After pupation, the newly emerged mosquitoes are housed for 5-7 days before being transferred to either of two infecting insectaries at 26°C, 80% humidity. The infecting rooms are contained within a high security facility, which has improved screening precautions to prevent escape of infected mosquitoes. A second colony of *A. stephensi* mosquitoes derived from the KAZUR strain from Pakistan has been obtained from

the University of Nijmegen, and is raised in a second rearing room at 25°C, 80% humidity. This strain has been shown to be more susceptible to *P. falciparum* infection than *A. freeborni* and is currently being tested by us. It is likely that either *A. freeborni* or *A. stephensi* (KAZUR) will be used routinely, rather than both species.

Challenge studies are completely dependent upon infected mosquitoes being available exactly when required. Therefore we have decided to run parallel systems to help ensure a continuous production. A major problem is the chance of mechanical failure of equipment. Each insectary has been connected to an alarm system, which when sounded will both summon an engineer from the Institute staff retained for insectary maintenance, and close down all equipment within that room until repaired. Voltage surges have been controlled by fitting control systems to each room. Periodic monitoring during the night is performed by Institute personnel.

The remaining rooms are used for rearing *A. stephensi* mosquitoes at 25°C, 80% humidity, adjacent to the high security *P. falciparum* rooms, for infection with *P. berghei*. The susceptibility of *A. stephensi* (KAZUR) to *P. berghei* is also being investigated. It is anticipated that only two species or strains of *Anopheles* mosquitoes will be eventually raised, for infection with *P. falciparum* or rodent malarias.

b) *P. falciparum* gametocyte cultures.

Under the direction of a new investigator, Dr. V. do Rosario, we have successfully cultured *P. falciparum* gametocytes that are infective to *A. freeborni* mosquitoes, and produce significant salivary gland sporozoite infections (2,000 - 5,000/mosquito). For this, both erythrocytes and human serum are tested for suitability for gametocyte production. Currently blood is obtained weekly from the American Red Cross (Bethesda, Maryland), and human serum from Plasma Supplies. Both are tested for hepatitis B and HTLV-III viruses before use. The strain of *P. falciparum* is NF54 originally from the University of Nijmegen and is of unknown geographic origin. It is anticipated that this culture system will be maintained for production of *P. falciparum* mosquitoes for non-human *in vitro* research projects here and, by agreement, will also be sent to NYU.

For human challenge studies, we are developing a completely separate culture system collaboratively with the University of Maryland, to exclude the possibility of hepatitis B or HTLV-III viral infection. A panel of human volunteers will be created in the University of Maryland, and screened for hepatitis B or HTLV-III infection. Such volunteers are expected to be medical students who probably will be available for several years. Suitable volunteers, of blood group A+ or O+, will be bled for erythrocytes and serum, which will be tested at this Institute for suitability for gametocyte culture. It is expected that approximately 1/3 of all donors will not support infections of *P. falciparum* gametocytes *in vitro*. Suitability will be tested by infection to *A. freeborni* mosquitoes and production of sporozoites. Initially, at least 50 volunteers will be surveyed, of whom about 20 are hoped to be available for testing. Suitable donors will be bled at 8 week intervals to maintain *P. falciparum* gametocyte cultures.

The University of Maryland has agreed to provide these samples for screening, and will maintain the panel, including periodic testing for hepatitis B and HTLV-III viruses, and bear the costs on our behalf. Since such culture systems are necessarily expensive and provide limited amounts of infected mosquitoes, they will be used primarily for challenge studies. The University of Maryland will also test and supply the NF54 strain to be used to ensure that it is free from hepatitis B and HTLV-III viruses.

P. falciparum gametocyte cultures will be maintained separately, in each of two tissue culture rooms. Human blood and serum will be stored in separate refrigeraters and -70°C freezers. Each system will be color coded throughout to ensure that blood, serum cultures and mosquitoes to be used in the human challenge studies are not confused with our routine cultures.

P. falciparum NF54 cultures will be maintained in plastic $25cm^2$ or $75cm^2$ flasks at 37°C for 14-20 days, until the presence of mature stage V gametocytes has been confirmed by Giemsa staining. Exflagellation will also be tested, though gametocyte morphology is a more accurate indicator of successful infectivity. When suitable, *P. falciparum* gametocyte cultures will be transferred to the high security infecting insectaries, and fed through membranes to *A. freeborni* or *A. stephensi* (KAZUR) mosquitoes. Infected mosquitoes will be housed until day 14, when the presence of salivary gland sporozoites will be determined.

All mosquito dissections will be performed only by experienced laboratory technical personnel within a laboratory attached to the high security insectaries. Only after removal of salivary glands will sporozoites be removed from the high security area. Entrance to the high security laboratory and insectaries is restricted to approved investigators or technicians. Mosquitoes for challenge studies will be placed in sealed polystyrene containers for shipment to the University of Baltimore.

c) Rodent malaria infected mosquitoes.

Infection of *A. stephensi* or *A. stephensi* (KAZUR) mosquitoes will be maintained using *P. berghei* infected mice or hamsters. In addition, infection of *A. freeborni* mosquitoes with *P. yoelii* will be initiated using infected mice or rats. Such mosquitoes will be housed in a non-high security insectary for 18-21 days until used.

d) *P. vivax* infected mosquitoes.

A supply of *P. vivax* infected mosquitoes is essential both for likely vaccine studies with the cloned *P. vivax* CS protein, or derived synthetic peptides, and for basic studies, particularly to extend our serological investigations (section 3). The CDC have continued to supply significant numbers of highly infected *A. stephensi* mosquitoes, using infections in chimpanzees, and have allowed collaborative studies between CDC and us on *P. vivax* serology. Future supplies of *P. vivax* infected mosquitoes will be continued to further these collaborative studies. However, there is some evidence that *P. vivax* sporozoites, produced from feeding mosquitoes on human infected blood, are more infectious *in vitro* to the human hepatoma HepG2-A16 cell line, than feeding from chimpanzees. The infectivity rates were 5% compared

to 0.1%.

It is proposed, therefore, that consideration be given to developing use of an insectary in areas endemic for *P. vivax* infections, where human volunteers can provide infected blood for membrane feeding. Two facilities are likely to fullfill this requirement: SUCEN (superintendencia de Controle de Endemias, Director Dr. A. G. de Souza, Principal Investigator Dr. M. Boulos), Sao Paolo, Brazil and Hospital General SSA (Director Dr. J. Mendez) Tapachula, Mexico. It is proposed that either, or both, facilities be encouraged to produce quantities of *P. vivax* infected mosquitoes for shipment to this Institute.

2) Development of the inhibition of sporozoite invasion (ISI) assay for use in clinical trials and areas endemic for *P. falciparum* and *P. vivax*.

The ISI assay has been proposed as a potential *in vitro* assay predictive of protective antibodies. Inhibition of sporozoite invasion of HepG2-A16 cells was correlated with protective immunity *in vivo* either by passive transfer or resistance of vaccinated volunteers to sporozoite challenge. The ISI assay demonstrated that the *P. falciparum* synthetic CS vaccine (23), like the recombinant CS protein vaccine (25), induced high levels of antibodies that block sporozoite invasion. Adsorption of these antibodies by immobilized CS peptides completely abolished ISI activity (23). While ISI is a functional assay, binding assays that measure the overall anti-CS response are also used, including IFA, ELISA, and RIA. Little is known of the correlation between ISI and these binding assays or between either type of assay and protective immunity upon challenge. It is proposed to further develop and evaluate the ISI assay, and to study its correlation both with binding assays and with protection.

The current ISI assay (2) is performed by adding *P. falciparum* or *P. vivax* sporozoites to HepG2-A16 cells in the presence or absence of immune serum, or control serum. After 3 hr incubation at 37°C, in 5% CO_2 in air, invaded sporozoites are visualized by an immunoperoxidase antibody assay using monoclonal antibodies to the relevent sporozoite species. Three ISI values may be obtained: ISI_{max} - the maximum inhibition of invasion at any serum dilution; ISI_{100} - the highest serum dilution that completely (100%) blocked invasion; ISI_{50} - the highest serum dilution that reduced invasion by 50%. ISI_{max} is often higher as the serum is diluted due to a significant prozone effect (36). Not all sera completely block invasion at any dilution, and thus do not have an ISI_{100} titer, only ISI_{50}. Generally, correlation between ISI activity and binding activities, particularly ELISA, has been good (23, 24,25), when used in the immunogenicity studies in mice or rabbits. However, less correlation is found when used with endemic sera from patients as exposure to the sporozoite induces a more variable antibody response than synthetic peptides. Three areas of research are thus proposed.

a) Improvement of the ISI assay.

The assay as described relies on microscopic evaluation of stained slides to measure activity. Care must be taken to distinguish morphologically attached surface sporozoites from interior-

ized sporozoites. However the immunoperoxidase antibody assay is much more accurate than immunofluorescent antibody studies used by others. The assay may be improved as follows:

i) Removal of attached sporozoites from the surface of the HepG2-A16 cell monolayer. Factors influencing sporozoite attachment such as cations, pH, isotonicity, and susceptibility to enzymes will be studied. Non-invaded sporozoites could then be removed after the 3hr incubation period, but before fixation, by rinsing the cells with a suitable buffer. Preliminary results suggest that trypsin at 10-30μg/ml will remove almost all surface sporozoites.

ii) DNA probes. Recently (37) a DNA probe has been developed at New York University that detected developing *P. berghei* EE stages within rat hepatocytes *in vivo*. This probe was developed using *P. berghei* infected red blood cells, and it is likely that similar probes for *P. falciparum* or *P. vivax* can also be developed. Thus, invaded sporozoites could be detected within the HepG2-A16 cells in a fast one-step procedure similar to the use of DNA probes for detecting *P. falciparum* infections of erythrocytes.

b) Development of binding assays.

Comparison of ISI and binding assays such as IRMA, IFA, or ELISA using sera from the human challenge studies or endemic areas will give a better understanding of the correlation of each with protection. However, many factors that influence either functional assays such as ISI, and the binding assays, may result in no correlation. For example, the affinity of binding of individual subclasses of antibodies may be important in determining ISI activity, but be less important in binding assays. The situation could arise where only high affinity antibodies act functionally to block sporozoite invasion, whereas high and low affinity antibodies bind equally to synthetic peptides in IRMA or ELISA or sporozoites in IFA. An adaptation of binding assays so that only high affinity antibodies bind to synthetic peptides might lead to better correlation of binding assays with protection. Such high affinity ELISA assays have been developed, and with Dr. J. Martin (LAC-USC Medical Center, Los Angeles) discussions are taking place to possibly adapt the ELISA assay.

c) Immunogenicity and human challenge studies.

It is proposed to use the ISI assay in future immunogenicity studies, either with *P. falciparum*, or with *P. vivax*. The ISI assay suggested that protective antibodies are elicited by synthetic *P. falciparum* CS peptides or recombinant proteins, and distinguished the immune responses induced by different antigenic preparations used with or without adjuvants. From this, and the binding assay data, it was possible to predict that both vaccines may be successful when used with adjuvants suitable for human use, such as alum.

i) Further immunogenicity studies will be undertaken to study the protective response to *P. falciparum* CS antigens. For example, the actual vaccine formulation to be approved for human use will likely be assayed in immunogenicity studies in non-human primates or rodents. Similarly, the recent cloning and sequencing of the *P. vivax* CS gene strongly suggests that immunogenicity studies of *P. vivax* antigens are imminent. After approval, Phase 1 and 2

studies at the University of Maryland are likely to generate significant number of sera taken after both vaccination (Phase1), and before and after sporozoite challenge (Phase2). It has been agreed between the University of Maryland, New York University, and us, that selected sera will be tested by the ISI assay, as well as binding assays at those institutions. Of considerable interest will be comparison of ISI activities *in vitro* with the *in vivo* human volunteer Phase 2 challenge studies.

ii) Evaluation of ISI activity of sera from malaria-endemic areas. The development of new assays, such as the Zavala assay for determining mosquito infectivity and malarial speciation (38), and the probability of using DNA probes for diagnosis of infection, will be extremely useful for surveying the epidemiology of current malaria infection. With the advent of the sporozoite CS vaccine, it is now essential that the current immunological profiles of people within such areas regarding naturally-acquired immunity to the CS protein be evaluated before such a vaccine is introduced. At the recent The Americas Conference on Malaria Vaccine(s) and Clinical Trials it was proposed that serological assays including binding assays such as ELISA, and functional assays as ISI, be used to characterize naturally acquired immunity.

Clearly, use of the ISI assay requires that it be fully integrated into an epidemiological situation where all factors regarding transmission of malaria are considered. This requires that such a project be meticulously planned in advance to consider the data that is required. As described earlier, the ISI assay is more time consuming and tedious to perform than automated binding assays. However, as a functional assay it will provide information directly related to protective immunity rather than exposure to the CS protein. Certain logistical problems that arise in using the assay in an epidemiological survey restrict its use to certain specified target groups. It is proposed that this investigation be closely collaborative with the CDC, who have initiated an epidemiological investigation in agreement with AID in western Kenya. With CDC, target groups for evaluation by ISI will be selected as part of their overall project. Sera will be collected by CDC personel and provided to us for ISI testing. Two types of investigation will be undertaken to determine a) ISI activity longitudinally in adults, b) acquisition of ISI activity related to age.

It will be necessary to first determine whether serum samples can be collected by the filter paper technique, or whether actual blood samples will have to be drawn by syringe or collection in capillary tubes. In the first, a small volume of blood, from finger or ear lobe prick, is adsorbed on to filter paper of known diameter, and dried. Later, the filter paper discs are hydrated in 1ml buffer, and the dried serum antibodies are thus reconstituted to a 1/10 dilution. Such reconstituted serum samples will be compared for ISI activity with serum samples drawn by syringe.

i) Longitudinal studies. Earlier studies (26) suggested that adults in The Gambia had significant levels of anti-*P. falciparum* antibodies measured by IFA. Our studies suggested that ISI activity substantially increased during the transmission season, presumably in response to an increase in the number of bites of *P. falciparum* infected mosquitoes. This study is

designed to further understand the influence of transmission measured by the infection rate of mosquitoes using the Zavala assay, appearance of infection measured by blood films, exposure to CS antigen measured by ELISA assay using (NANP) peptide, and the appearance of ISI activity. Serum samples from adults will be taken in different locations where transmission and exposure may be expected to vary, comparing areas close to the edge of the lake with higher elevations, for instance. Seasonal variations will be tested by taking sera at different times throughout the year.

ii) Age studies. Binding assays have shown that neonate have maternal anti-malarial antibodies that decline by 6-12 months. Thereafter, natural immunity to malaria takes several years to acquire. It is proposed to study the acquisition of natural immunity in selected individuals in Kenya. CDC have agreed that it would be feasible to take small blood samples from young babies and to take follow-up samples from the same individuals over several years. For this an initial group of young babies will be tested, and then followed with the expectation of an attrition rate of volunteers. However, it is anticipated that about 20 children could be continuously followed from an initial group of 50-80 babies. Although these numbers are relatively small compared to numbers that can be assayed by binding assays, the value of the ISI data that can be generated, particularly in the context of the overall program, is significant.

iii) Other studies. The study with CDC is likely to represent a unique opportunity to evaluate ISI activity as part of an integrated program. However, sera from other endemic areas of *P. falciparum* infection are available. It is proposed to continue the evaluation of Indonesian sera (Dr. S. Hoffman, NMRI), as well as sera from other locations as they become available. It is also proposed to measure ISI activity against *P. vivax* CS protein, especially with CDC and New York University, using sera from Guatemala. Finally, if either or both of the proposed collaborative *P. vivax* insectaries in Tapachula, Mexico or Sao Paolo, Brazil become operative, they would be centers from which sera could be collected.

3. Study of the biochemical synthesis of exoerythrocytic antigens.

Because of the difficulties in obtaining sufficient material, research on the vaccine potential of the EE stages of human malaria has been largely neglected. The hepatic stages offer a number of potential advantages for vaccine research, both in concert with potential blood stage vaccines and as an independent vaccine target. Development of vaccines based on blood stages of malaria have been hindered by rapid and seemingly unlimited changes in antigenicity. Hope remains that receptor/ligand interactions such as endothelial sequestration for *P. falciparum* and merozoite invasion of erythrocytes for all malarias will require at least a constant active region of some exposed protein, but experiments thus far have shown substantial antigenic diversity. Antigens on the surface of infected erythrocytes in human and simian systems have demonstrated the ability to completely change within one cycle of invasion. The merozoite of the EE stage, while somewhat different in composition from the blood

stage merozoite, invades the same target cell (the erythrocyte) and probably uses a similar mechanism. Analysis of common proteins between EE and blood stage merozoites could help to identify antigens essential in the invasion process. Moreover, EE merozoites are found at the start of the host infective cycle and are therefore under significantly less immune pressure than their blood stage equivalents. This fact could translate in at least two different ways. Firstly, EE merozoites may contain less irrelevant antigen, or chaff, intended mainly to confuse the host immune system during the rupture/invasion process. Secondly, surface proteins on EE merozoites may demonstrate less antigenic diversity than blood stage merozoites. This more limited repertoire on early host stages has been suggested in the case of another family of blood parasites, the African trypanosomes. A vaccine against such a limited repertoire would result in a second barrier against infection, complementary to a sporozoite vaccine.

We propose to initiate studies on the human pathogen *P. vivax* grown in HepG2-A16 cells in our laboratory and on *P. falciparum* parasites grown in primary human hepatocyte cultures in collaboration with the University of Nijmegen (section 5). Initial studies will use existing immune reagents developed against other stages of the malaria life cycle and cross reacting with EE stages. Monoclonal antibodies against *P. vivax* blood stages have been provided us by Drs. John Barnwell (New York University) and Gary Campbell (Center for Disease Control). Similar reagents against *P. falciparum* have been provided by Drs. Diane Taylor (Georgetown University) and Jeff Lyon (WRAIR). To complement these immune reagents, we will immunize mice with EE stage schizonts and merozoites in an attempt to make EE stage specific monoclonal antibodies. Hybridoma fusion and cloning are established procedures in this laboratory, having been already used for making monoclonal antibodies against HepG2-A16 cells and *P. berghei* blood stages. Assays of antigenic development will be of two general categories:

a) Microscopic assays.

Antibodies will be screened for activity on developing EE stages by both fluorescence and histochemical techniques. Positive reactions will be compared with reactivity on blood stage parasites for identification of similarities or differences in antigen distribution. Distribution of key antigens will be determined by immunoelectron microscopy in collaboration with Dr. Aikawa (Case Western University) and Dr. H. J. Geuze (University of Utrecht).

b) Immunochemical assays.

All antibodies positive by microscopic techniques will be screened by a variety of separation techniques in an effort to identify the protein or glycoconjugate in question. Possible screens will include immunoprecipitation of metabolically labeled EE stage parasites followed by SDS-PAGE and immuno detection on lateral transfers of SDS-PAGE (Western blots). Should the background of normal hepatic proteins conceal expected parasite proteins, more sensitive methods of separation, specifically Isoelectric focussing/SDS-PAGE two dimensional gels, will be used in place of normal SDS-PAGE both for precipitation and for Western blots.

In both microscopic and immunochemical assays a special effort will be made to detect parasite dependent antigens on the surface of infected liver cells. This can be readily accom-

plished in our *in vitro* culture system by testing the immune reagents on intact, viable infected cells. The existence of any such parasite marker would be an ideal vaccine candidate, stimulating an immune response against a stage of the parasite which is low in number and slow to produce new infections. Parasite modifications of the host cell surface have been well characterized in blood stage asexuals, including morphologic (39), antigenic (40), and functional (41) changes.

Concomitant with these studies we will develop methods for the enrichment of EE schizonts and merozoites. There are several potential physical methods whereby this may be accomplished. Simplest is the procedure used by us with cultures of EE stages of *P. berghei* where schizonts are allowed to mature and rupture, and the freed merozoites collected in the culture medium and concentrated by centrifugation. This procedure may be enhanced and the yield increased by applying osmotic stress to the mature cultures. In blood cultures, schizont-swollen infected cells are substantially more sensitive to osmotic stress. Finally we will try density gradient separation of infected cells. This procedure has been shown to work well with plasmodium blood stages under both natural (42) and osmoticly stressed conditions (43). Enriched parasites would increase the sensitivity of immunochemical assays facilitate the development of monoclonal antibodies against EE stages.

In addition to the blood stage antigens present in EE stages, we will also look for the persistence or synthesis of antigens from other stages of the parasite life cycle, including gametes, ookinetes, and sporozoites. The CS protein of sporozoites has long been known to persist throughout EE parasite development (33), but little is known of its localization or form. Immunoelectron microscopy can reveal the localization of this major vaccine target after internalization. Immunoprecipitation of metabolically labeled parasites will reveal whether the antigen is synthesized by EE parasites or merely residual protein derived from the transformed sporozoite. Similarly, Western blot analysis will indicate if the protein is maintained intact or if it is substantially degraded within the EE parasite. An understanding of the localization and fate of this protein may provide an insight into possible effects of the sporozoite vaccine on development of the EE parasite.

With a firm understanding of the antigenic makeup of the EE stages, potential target antigens localized on accessible surfaces of the parasite or infected cells or related to known targets in other stages of the parasite life cycle, could be selected for direct testing in the *in vitro* culture system. To test for EE merozoite and infectivity, an assay system for the *in vitro* invasion of erythrocytes from EE stages will be developed. Conditions for the co-culturing of EE and blood stage parasites will be optimized so as to provide an easily manipulated *in vitro* system for testing potential vaccine sera. Direct inoculation of blood cultures from EE stages will also facilitate the comparison of antigenic repertoires of EE and blood stages from the same malarial isolate.

4. Sporozoite - Hepatocyte Receptor interactions

The sporozoite vaccines now in development depend upon the induction of a protective humoral response to the repeat epitope region of the CS proteins, an ideal target in terms of its immunogenicity and efficacy in model systems and apparent intra-species constancy. Evidence from this laboratory indicates that while antibodies against the repeat region clearly block sporozoite invasion of hepatic cells, the repeat region is not itself directly responsible for cell attachment. Recent evidence showing variability of the repeat region within a species, e.g. *P. cynomolgi* (44), *P. knowlesi* (45), and even *P. falciparum* (46), and the lack of known function of the repeat region, suggest that the repeat region may vary under the immune pressure generated by use of the vaccine. Concomitant with the development and testing of the sporozoite vaccine, it is imperative that we better understand the sporozoite - hepatic invasion process that we are attempting to prevent. Identification of the several steps of the invasion process, the receptors implicated and the sporozoite protein or parts of proteins involved could identify alternative vaccine targets that are under evolutionary pressure to retain functionality.

Steady progress on the characterization of the hepatic receptor has been maintained in this laboratory by characterizing chemical and immune treatments which can inhibit or enhance the sporozoite invasion process (see progress section). While these general studies will continue, we propose to greatly expand our efforts in development of immune reagents that interfere with invasion and in the demonstration of actual binding interaction of sporozoite protein or parts of proteins with the putative hepatic receptor.

Having demonstrated that a concentrated rabbit antiserum against the hepatic plasma membrane can inhibit invasion, we have begun a program of manufacturing monoclonal antibodies against specific hepatic plasma membrane proteins. These monoclonal antibodies will be characterized in terms of their surface availability, cellular distribution, and peptide recognition. They will be tested directly for inhibition of invasion using the ISI assay, but with the realization that lack of inhibition would only relate to the epitope involved, not the protein recognized. More important is the creation of a library of immune reagents to permit the rapid affinity purification of potential receptors indentified by crosslinking or other studies. Affinity purified hepatocyte proteins could then be used to make monospecific antibodies against the entire protein. A general immune reagent such as a monospecific antiserum against a putative receptor would be much more likely to inhibit receptor activity.

In addition to our efforts with immune reagents we are actively investigating the possibility of direct binding to hepatic cells of isolated or constructed sporozoite proteins and peptide sequences. We anticipate using three sources of potential sporozoite ligand.

1. Native CS proteins. Limited crosslinking studies have been performed in collaboration with this laboratory using intact sporozoites. We plan to extend these studies to use isolated,

partially purified CS protein derived by detergent partitioning from sporozoites. These studies have the advantage that the fully processed forms of the CS protein from any available species of malaria may be used. Quantities of this material will, however, be limited.

2. Molecular constructs. The entire gene for the CS protein of *P. falciparum*, and more recently *P. vivax*, has been isolated, making possible expression clones in bacteria or yeast. Relatively large fragments of CS protein can be prepared in milligram amounts, facilitating the testing of major regions of the protein for binding activity. In this laboratory we have already tested the repeat region of *P. falciparum* for binding activity using a molecular construct consisting of 16 repetitions of the *P. falciparum* repeat, made and isolated by Smith, Klein, and French. Other constructs will be tested as they are made available. Of special interest would be a molecular construct of the entire CS protein but with the repeat region deleted.

3. Synthetic peptides. Synthetic peptides based on specific amino acid sequences of the CS protein are readily constructed to include areas of the CS protein that appear interesting either because of clustering of specific amino acids or because of strong interspecies conservation of sequence. Because of their limited size, synthetic peptides might be considered the least likely of the three forms of the CS protein to retain sufficient secondary structure to act as strong ligands. Nevertheless, initial studies in this laboratory have produced promising results. Small peptides from similar regions of the N terminal part of the CS proteins of both *P. falciparum* and *P. knowlesi* have demonstrated substantial affinity for HepG2-A16 cells and are currently being tested as described below.

Potential sporozoite ligands will be radioactively labeled with ^{125}I, either directly on tyrosine (47) or via protein binding vehicles such as Bolton-Hunter (48) or Wood's (49) reagents, and screened by equilibrium binding to intact, viable HepG2-A16 cells. Saturable reactions will be further characterized by kinetic parameters as well as specificity to hepatic cells and inhibitory effect in ISI assay. Inhibitory effects of antibodies or biochemical treatments on cell binding will be contrasted with known effects on ISI. EM autoradiography, in collaboration with Drs. M. Aikawa (Case Western University) and Geuze (University of Utrecht), will be used to identify the subcellular localization of the bound receptor.

Crosslinking studies will be used to identify putative hepatic receptors involved in ligand binding. Small molecular weight ligands will be radiolabeled as before and crosslinked permanently to unlabeled hepatic cells using reagents such as di-succinyl suberimidate (30). Crosslinked hepatic proteins can then be easily identified by autoradiography of total protein separated by PAGE. High molecular weight ligands (e.g. whole CS protein) would be crosslinked to radiolabeled hepatic cells (either ^{125}I-lactoperoxidase surface label (51) or metabolic incorporation of radiolabeled amino acids) using a reversible crosslinking agent such as dimethyl-3,3'-dithiobis propionimidate (52). Total proteins will be solubilized in detergent and the ligand precipitated by specific antisera. Precipitated samples will then be separated by PAGE under conditions that either maintain or reverse the inter-molecular crosslinks.

Putative receptor proteins crosslinked to the ligands should both co-precipitate with the ligand and show substantial size differences under reversed or non-reversed conditions. Hepatic proteins identified in this manner will be enriched by normal protein isolation procedures or, if possible, purified by affinity chromatography, and monospecific antisera raised. Likewise, each of the potential ligands will be used to make specific immune serum for use in immunochemical procedures and ISI testing.

Because of the known inhibitory action of even Fab fragments of monoclonal antibodies against the CS protein in ISI, our initial focus is strongly on the CS protein in its various forms. However, we recognize the lack of information on the nature of the sporozoite ligand and will obviously incorporate into our study other sporozoite surface or rhoptry proteins as they are characterized.

5. Immuno-electron microscopic and biochemical characterization of sporozoite-hepatocyte invasion and exoerythrocytic development.

Central to our understanding of the mechanism by which the potential CS protein anti-malarial vaccine confers protective immunity is elucidation of sporozoite-hepatocyte interactions. Determination of the ligand domain of the CS protein that recognizes specific hepatic receptor(s) will be further pursued using *P. falciparum* and *P. vivax* and the hepatoma HepG2-A16 cell system (Section 4). However, isolated and cultured human hepatocytes also provide an essential system for study of such interactions. While HepG2-A16 cells have been shown to possess closely similar or identical surface receptors to those found on freshly isolated hepatocytes (53), our studies would clearly benefit by comparison of HepG2-A16 cells with human hepatocytes. Secondly, although *P. vivax* sporozoites invade and develop through the complete EE cycle in both HepG2-A16 cells (3) and hepatocytes (9), full EE development of *P. falciparum* only occurs in hepatocytes (10,11). One possible explanation may be that *P. falciparum* sporozoite invasion of HepG2-A16 cells may ultimately lead to their internalization in compartments not compatible with EE development.

Studies *in vitro* using human hepatocytes are still scarce, mainly due to their limited availability and the lack of suitable methods for preservation when viable hepatocytes are available. These difficulties have been overcome at the Department of Gasteroenterology (Nijmegen University, head Dr. S. H. Yap) by developing an enzymatic perfusion method to obtain high yields of adult human hepatocytes and by developing a cryopreservation technique to store the isolated hepatocytes for a long period (54). Electron microscopy of these hepatocytes in primary cultures showed typical fine structures with well developed desmosomes and structures similar to bile caniculi (18). The cell polarity was also evident. In cultures using extracellular matrix prepared from human liver, these typical structures were maintained for at least 4 weeks and the cell survival was extended to at least 3 months. In addition, these hepatocytes retained the ability to secrete high levels of albumin for at least 3 weeks. In the collaborative effort started in 1984,

these facilities have been used for the infection of cultured hepatocytes with *P. falciparum* (10), and for the study of the ultrastructure of the intracellular parasite (18). Furthermore, cryopreserved adult human hepatocytes have been infected with *P. falciparum* (55).

The basic mechanism of the interaction of the sporozoite with the hepatocyte surface is not yet understood. Therefore, we propose a further extension of the present collaboration with a third research group, the Department of Cell Biology of the University of Utrecht, head Prof. Dr. H. J. Geuze, who is a widely recognized expert on membrane receptors of human hepatoma cells. It was in their laboratory that immuno-electron microscopy on ultrathin cryosections was developed.

In their laboratory, the lysosomal pathway was detected by using ultrathin cryosections obtained from mildly fixed, cryoprotected tissue, and antibodies were raised against lysosomal enzymes and the receptor for newly synthesized enzymes (56,57,58). Antibody binding sites were visualized with protein A-gold (59,60, 61). It is proposed to apply these techniques for the study of the interactions of sporozoites with cultured human hepatocytes.

Only recently, with the introduction of the immuno-electron microscopy on ultrathin cryosections, has it been possible to describe morphologically the organelles that play an important role in receptor - mediated endocytosis. Receptor-ligand complexes were found to be internalized by coated pits and then delivered to the Compartment of Uncoupling Receptors and Ligands (CURL) (62, 63, 64). It is likely that the invasion of the sporozoite proceeds by receptor-mediated endocytosis, and using antibodies that recognize the sporozoite receptor, or monoclonal antibodies to the CS protein which binds to the receptor, it can be demonstrated whether the sporozoites make use of CURL. Furthermore the nature of the parasitophorous vacuole can be investigated using antibodies raised against intracellular marker enzymes.

A widely used schizonticide, primaquine, has besides its schizonticidal properties also the ability to raise the internal pH in acidic organelles into which the sporozoites are probably transferred. The acidity of CURL was demonstrated recently with immunoelectron microscopy and antibodies to primaquine (65). Furthermore, preliminary results showed that receptor distribution is also affected by this drug. Biochemical methods have demonstrated that after administration of primaquine to the medium of the hepatoma cell line (HepG2-A16), the receptors rapidly disappeared from the cell surface and moved to an intracellular pool (66). Since primaquine also affected the invasion of sporozoites (67) it is clear that the entry of sporozoites is mediated by receptors that are functionally similar. To what extent this effect of primaquine affects the internalization of sporozoites could be investigated with electron microscopy of HepG2-A16 cells treated with different primaquine concentrations before or after infection with sporozoites. On the other hand primaquine is a schizonticide and the site of action in the schizont is poorly understood. The use of an antibody raised against primaquine in combination with ultrathin cryosections might elucidate the target organelles in the schizont.

The three groups involved will work closely together on these projects, and Dr. J. P. Verhave of the Department of Medical Parasitology, University of Nijmegen, will act as the central co-

ordinator and person responsible for all administration of the project.

The three departments involved are:

Department of Medical Parasitology, University of Nijmegen: Prof. Dr. J. H. E. T. Meuwissen

Department of Cell Biology and Electron Microscopy, University of Utrecht: Prof. Dr. H. J. Geuze

Department of Gasteroenterology, University of Nijmegen: Dr. S. H. Yap

Objectives:

a. To study the molecular basis of sporozoite receptor(s) on hepatocytes, and to produce a monoclonal antibody to the receptor(s).

b. To characterize the CS protein ligand-hepatocyte receptor(s) interactions involved in sporozoite attachment and invasion by immuno-electron microscopy.

c. To investigate by immuno-electron microscopy the antigenicity of the developing EE parasite within hepatocytes, particularly the EE merozoite.

d. To establish *P. falciparum* red blood cell cultures by *in vitro* EE merozoite invasion.

a. Sporozoite receptor characterization and function will also be studied in cultured human hepatocytes, in close collaboration with similar studies using HepG2-A16 cells at BRI (Section 4).

Monoclonal antibodies will be prepared which are reactive with the surface of HepG2-A16 cells (Section 3) and with human hepatocytes. Those antibodies which are surface-reactive will also be tested for their ability to block sporozoite attachment to HepG2-A16 cells or hepatocytes. Although *P. falciparum* sporozoites invade both cell types similarly, suggesting the CS protein receptor is either closely similar or identical, comparative studies of both cell types are necessary to determine if this is true. Those monoclonal antibodies which inhibit sporozoite invasion by binding to the hepatocyte surface will be further tested to determine the hepatocyte component they recognize, and by immuno-electron microscopy. To determine the surface component, either whole hepatocytes or hepatocyte membranes will be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) and the monoclonal antibodies reacted in Western blots. Alternatively, whole cells or membranes will be solubilized and the receptor immunoprecipitated with the monoclonal antibodies, dissociated and examined by PAGE.

An alternative approach to characterization of the sporozoite receptor is based on the assumption that domains of the *P. falciparum* CS protein recognize specific hepatic receptor(s). Thus either the complete CS protein or its reactive domains prepared as synthetic peptides, can be used

as probes to react with the receptor. However, the complete CS protein is presently unavailable, and it is therefore proposed to prepare it directly from *P. falciparum* sporozoites using monoclonal antibodies (CSP2) to the CS protein (68). This is possible because of the availability of large numbers of highly infected mosquitoes produced by the Department of Medical Parasitology. Isolated CS protein will be covalently bound to CNB activated Sepharose 4B to prepare an affinity column. ^{125}I-labelled hepatocyte membranes will be applied to the affinity column, and the putative receptor eluted. The pooled material containing the receptor will be mixed with specific monoclonal or polyclonal anti-CS antibodies and protein-A Sepharose to remove cold CS proteins, followed by lyophilization. The receptor preparation will be examined by PAGE techniques, and will also be used to prepare specific antibodies in rabbits for use in immuno-electron microscopy.

b. The ligand-receptor interactions involved in sporozoite attachment and invasion will be studied using *P. falciparum* sporozoites and human hepatocytes as described earlier. *P. falciparum* sporozoites will be added to cultures of hepatocytes, and the cultures will be fixed at various time intervals (5min - 60min), using fixation conditions similar to those used for HepG2-A16 cells (56). Subsequent to fixation the cells will be embedded in gelatin and ultrathin sections prepared for electron microscopy.

The interaction of *P. falciparum* CS protein with the hepatic surface will be studied using monoclonal antibodies to the CS protein repeat epitope region, or antibodies to other CS protein domains as they become available. While receptor characterizations leading to receptor isolation and preparation of specific antibodies are in progress (section 4), rabbit antibodies to known human hepatocyte components will also be used. These include: asialoglycoprotein receptor (62) IgA receptor (63), cathepsin D (56), and clathrin (62). In all cases, these antibodies will be visualized with protein A coupled to different sizes of gold particles (60,61). By this procedure, the localization of more than one antibody can be determined from the distribution of the different sized gold particles.

As our studies continue to characterize both the ligand and hepatic receptor biochemically, further polyclonal and/or monoclonal antibodies will be tested by immuno-electron microscopy.

c. Using the *P. falciparum* - hepatocyte culture system, the antigenicity of the developing EE parasite, and particularly EE merozoites, will be studied ultrastructurally. Our earlier results have suggested that the antigenicity of developing EE parasites changes during schizogony. This will be investigated in *P. falciparum*, and specific antigens will be localized by immuno-electron microscopy.

The distribution of the *P. falciparum* CS antigen will be studied following invasion and transformation into trophozoites and subsequently during schizogony and merozoite formation, using specific monoclonal antibodies. Similarly, blood stage antigens, particularly those located

on the merozoite surface, will be studied using monoclonal antibodies. As described in Section 3, certain merozoite surface antigens of *P. vivax* and rodent malarias are synthesized late in the EE cycle. Similar antigens present on the *P. falciparum* merozoite surface will also be studied.

Primaquine will be used in two separate studies; a) to determine the relative acidity of endocytic structures which may be associated with sporozoite invasion and EE development; b) to study its localization with EE parasites when acting as an EE schizonticide.

a) The specificity of receptor involvement in sporozoite attachment and invasion will be studied following our earlier observations (67) that the sporozoite receptor is internalized in HepG2-A16 cells pre-treated with pharmacological concentrations of primaquine. Hepatocytes will be similarly treated with primaquine, or other lysomotropic amines, and the effect on *P. falciparum* sporozoite attachment and invasion studied by immunoperoxidase staining as for HepG2-A16 cells. Ultrastructurally, the effect of primaquine pretreatment on the distribution of *P. falciparum* CS protein associated with its hepatic receptor will be studied using monoclonal antibodies to the CS protein and gold labelling.

Primaquine will also be used to study the acidity of endocytic compartments during invasion. *P. falciparum* sporozoites will be allowed to attach to and invade hepatocytes *in vitro* in the presence of primaquine. Ultrathin sections will then be treated with antibodies to primaquine (65) to study its distribution in CURL multivesicular bodies, lysosomes and Golgi cisternae.

The EE schizonticide activity of primaquine will be investigated, by firstly determining the *in vitro* effects of primaquine on developing *P. falciparum* EE parasites using the techniques developed with *P. berghei* EE parasites in HepG2-A16 cells. Then, those concentrations of primaquine which inhibit *P. falciparum* EE development will be added to *P. falciparum* EE cultures in hepatocytes, and the cultures cryosectioned and examined by electron microscopy using anti-primaquine antibodies. Since primaquine is used principally to cure *P. vivax* infections, it is anticipated that similar experiments will be performed using *P. vivax* EE parasites developing either in HepG2-A16 cells, or in human hepatocytes which had been cryopreserved and transported to BRI. After infection, cells will be frozen and shipped to the University of Utrecht for electron microscopy.

d. Although *P. falciparum* EE parasites appear to fully develop within cultured hepatocytes, definitive evidence of merozoite formation is still lacking. Further attempts by Dr. Meis to study *P. falciparum* EE merozoite formation are continuing. The *in vitro* infectivity of such merozoites can be tested by adding human red blood cells to hepatocyte cultures and then examining the erythrocytes for merozoite invasion. Claims (11) that *in vitro* invasion of erythrocytes occured form EE merozoites released by cultured hepatocytes relied on immunofluorescent antibody techniques, and since the red blood cells were not further cultured successfully, whether or not infectious EE merozoites were produced remains in doubt. Therefore, further attempts will be made to initiate *in vitro* red blood cell cultures from *P. falciparum* EE merozoites. If

successful, these cultures will be subsequently maintained *in vitro*, and both asexual and sexual parasitic development studied. Such studies have particular relevence in studying the genetics of *P. falciparum*, avoiding the need for infection of monkeys following genetic recombination of gametocyte infections in mosquitoes.

SECTION D REFERENCES

1. Hollingdale, M. R., Sigler, C. I., Leland, P. in preparation

2. Hollingdale, M. R., Nardin, E. H., Tharavanij, S., Schwartz, A. L., Nussenzweig, R. S. 1984. J. Immunol., 132: 909.

3. Hollingdale, M. R., Collins, W. E., Campbell, C. C., Schwartz, A. L. 1985. Am. J. Trop. Med. Hyg., 34: 216.

4. Nardin, E. H., Nussenzweig, V., Nussenzweig, R. S. 1982. J. Exp. Med., 156: 20.

5. Sigler, C. I., Leland, P., Hollingdale, M. R. 1984. Am. J. Trop. Med. Hyg., 33: 544.

6. Krotoski, W. A., Garnham, P. C. C., Cogswell, F. B., Collins, W. E., Bray, R. S., Gwadz, R. W., Killick-Kendrick, R., Wolf, R., Sinden, R., Hollingdale, M. R., Lowrie, R. C., Koontz, L. C., Starfill, P. S. 1985. Am. J. Trop. Med. Hyg. in press.

7. Hollingdale, M. R., Collins, W. E., Campbell, C. C. 1985. Am. J. Trop. Med. Hyg., in press.

8. Doby, J. M., Barker, R. 1976. C. R. Seances Soc. Biol., 170: 661.

9. Mazier, D., Landau, I., Drulhe, P., Milther, F., Gugen Guillouzo, C., Baccam, D., Baxter, J., Chigot, J. P., Gentilini, M. 1984. Nature, 307: 367.

10. Smith, J. E., Meis, J. F. G. M., Ponnudurai, T., Verhave, J-P., Moshage, H. J., Lancet, i: 757.

11. Mazier, D., Beaudoin, R. L., Mellouk, S., Druilhe, P., Texier, B., Trosper, J., Mittgen, F., Landau, I., Paul, C., Brandicourt, O., Gugen Guillouzo, C., Langlois, P. 1985. Science, 227: 440.

12. Hollingdale, M. R., Leland, P., Leef, J. L., Schwartz, A. L. 1983. Am. J. Trop. Med. Hyg., 32: 685.

13. Zavala, F., Hollingdale, M. R., Schwartz, A. L., Nussenzweig, R. S., Nussenzweig, V., 1985. J. Immunol., 134: 1202.

14. Aley, S. B., et al., in preparation.

15. Taylor, S. W. personal communication.

16. Aikawa, M., et al, 1984. Am. J. Trop. Med. Hyg., in press.

17. Uni, S., et al, 1985. Am. J. Trop. Med. Hyg., in press.

18. Meis, J. F. H. M., et al, Cell Tissue Res., in press.

19. Meis, J. F. H. M., Hollingdale, M. R., Verhave, J-P., Aikawa, M. 1984. Cell. Biol. Int. Rep., 8: 1016.

20. Hollingdale, M. R., Zavala, F., Nussenzweig, R. S., Nussenzweig, V. 1982. J. Immunol. 128: 1929.

21. Enea, V., Ellis, J., Zavala, F., Arnot, D. E., Asanavich, A., Masuda, A., Quakyi, I., Nussenzweig, R. S. Science, 225: 628.

22. Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L., Wirtz, R. A., Hockmeyer, W. T., Meloy, W. L., Haunes, J. P., Schneider, I,. Roberts, D., Sanders, G. S., Reddy, E. P., Diggs, C. L., Miller, L. H. 1984. Science, 225: 593.

23. Zavala, F., Tam, J. P., Hollingdale, M. R., Cochrane, A. H., Quakyi, I., Nussenzweig, R. S., Nussenzweig, V., 1985. Science, 228: 1436.

24. Ballou, W. R., Rothbard, J., Wirtz, R. A., Gordon, D. M., Williams, J. S., Gore, R. W., Schneider, I., Hollingdale, M. R., Beaudoin, R. L., Meloy, W. L., Miller, L. H., Hockmeyer, W. T. 1985. Science, 228: 996.

25. Young, J. F., Hockmeyer, W. T., Grosss, M., Ballou, W. R., Wirtz, R. A., Trosper, J. H., Beaudoin, R. L., Hollingdale, M. R., Miller, L. H., Diggs, C. L., Rosenberg, M., Science, 228: 958.

26. Nardin, E. H., Nussenzweig, R. S., McGregor, I. A., Bryan, J. H. 1979. Science, 106: 597.

27. Hoffman, S. L., Wirtz, R., Ballou, W. R., Schneider, I., Hollingdale, M. R., Cole, C., Trosper, J. H., Campbell, J., Marwoto, H., Wistar, R., Hockmeyer, W. T. 1985. Ann. Mtg. Am. Soc. Trop. Med. Hyg., Miami, Fla., Abstract 367.

28. Hollingdale, M. R., Leland, P., Sigler, C. I., Leef, J. L. 1985. Trans. R. Soc. Trop. Med. Hyg., 79: 206.

29. Pancake, S. J., Hollingdale, M. R. Exp. Parasitol., Submitted.

30. Vegara, U., et al., 1985. J. Immunol. 134: 1345-3445.

31. Hollingdale, M. R., McCyllough, M., Leef, J. L., Beaudoin, R. L. 1981. Science, 213: 1021.

32. Hollingdale, M. R., Leland, P., Schwartz, A. L. 1983. Am. J. Trop. Med. Hyg., 32: 682.

33. Hollingdale, M. R., Leland, P., Leef, J. L., Leef, M. F., Beaudoin, R. L. 1983. Am. J. Trop. Med. hyg., 32: 24.

34. Arnot, D. E. et al, 1985. Science, 230: 815.

35. McCutchan, T. F. et al., 1985. Science, 230: 1381.

36. Hollingdale, M. R., Ballou, W. R., Aley, S. B., Pancake, S. J., Miller, L. H., Hockmeyer, W. J. J. Immunol. submitted.

37. Ferreira, A., Morimoto, J., Enea, V., Nussenzweig, V. 1985. Ann Mth. Am. Soc. Trop. Med. Hyg., Miami, Fla., Abstract 372.

38. Zavala, F. et al., 1982. Nature, 299: 737-738.

39. Luse, S. A. and Miller, l. H., 1971. Am. J. Trop. Med. Hyg. 20: 655-660.

40. Leech, V. H., et al., 1984. J. Exp. Med. 1567-1575.

41. Ginsburg, H. et al., 1983. Mol. Biochem. Parasitol. 8: 177-190.

42. Saul, A., Myler, P. E., and Kidson, C. T. 1982. Bull. Wld. Hlth. Org. 60: 755-759.

43. Aley, S. B., Sherwood, J. A., and Howard, R. J. 1984. J. Exp. Med. 160: 1585-1590.

44. Cochrane, A. H. et al., 1985. MBP 14: 111.

45 Sharma, S., Svec, P., Mitchell, G. H., Godson, G. N. 1985. Science, 229: 779.

46. Weber, J. L., and Hockmeyer, W. T. 1985. MBP, 15: 305.

47. Fraker, P. J. and Speck, J. C. 1978. Biochem. Biophys. Res. Commun. 80: 849-857.

48. Bolton, A. E. and Hunter, W. M. 1973. Biochem. J. 133: 529-539.

49. Wood, F. T. et al., 1975, Anul. Biochem. 69: 339.

50. Hill, M., Beechet, J. and dAlbis, A. 1979. FEBS Lett 102: 282-286.

51. Hubbard, A. L. and Cohn, Z. A. 1972. J. Cell Biol. 55: 390.

52. Wang, K. and Richards, F. 1975. J. Biol. Chem. 250: 6622.

53. Knowles, B. B., Howe, C. C. and Aden D. P. 1980. Science, 209: 497.

54. Rijntjes, P. J. M., Moshage, H. J., Van Gemert, P. J. L., de Waal, R., Yap, S. H. submitted.

55. Meis, J. F. G. M., Rijntjes, P. J. M, Verhave, J-P., Ponnudurai, T., Hollingdale, M. R., Yap, S. H. Cell. Biol. Intl. Reports, in press.

56. Geuze, H. J., Slot, J. W., Strous, G. J., Halsilik, A., Von Figura, K. 1985. J. Cell. Biol. in press.

57. Geuze, H. J., Slot, J. W., Strous, G. J., Luzio, J. P., Schwartz, A. L. 1984. EMBO J., 3: 2677.

58. Geuze, H. J., Slot, J. W., Strous, G. J., Hasilik, A., Von Figura, K. 1984. J. Cell. Biol., 98: 2047.

59. Slot, J. W., Geuze, H. J. 1983. Immunohistochemistry, ed. Guello, A. C., Wiley and Sons, Chicester, UK, 323.

60. Slot, J. W., Geuze, H. J. 1985. Eur. J. Cell. Biol., 38: 87.

61. Slot, J. W., Geuze, H. J. 1981. J. Cell. Biol., 90: 533.

62. Geuze, H. J., Slot, J. W., Strous, G. J., Lodish, H. F., Schwartz, A. L. 1983. Cell, 32: 277.

63. Geuze, H. J., Slot, J. W., Strous, G. J., Peppard, J., Von Figura, K., Schwartz, A. L. 1984. EMBO J., 3: 2677.

64. Geuze, H. J., Slot, J. W., Strous, H. J., Schwartz, A. L. 1983. Eur. J. Cell. Biol., 32: 38.

65. Schwartz, A. L., Strous, G. J., Slot, J. W., Geuze, H. J. 1985. EMBO J., 4: 899.

66. Schwartz, A. L., Bolognesi, A., Fridovitch, S. E. 1984. J. Cell. Biol., 90: 732.

67. Schwartz, A. L., Hollingdale, M. R. 1985. Mol. Biochem. Parasitol., 14: 305.

68. Meuwissen, J. H. E. T. and Meis, J. F. G. M. Personal communication.

Collaborative studies will be performed at the following Institutions

1. Case Western University: Dr. M. Aikawa
2. Centers for Disease Control: Drs. C. Campbell and W. E. Collins
3. Georgetown University: Dr. D. Taylor
4. Naval Medical Research Institute: Dr. S. Hoffman
5. New York University: Drs. R. S. and V. Nussenzweig
6. University of Nijmegen (Medical Parasitology): Drs. J. H. E. T. Meuwissen and J. P. Verhave
7. University of Nijmegen (Gastroenterology): Dr. S. H. Yap
8. Rockeffeller University: Dr. J. Tam
9. Sao Paulo (SUCEM): Dr. Boulos
10. Tapachula: Dr. Mendez
11. University of Utrecht: Dr. H. J. Geuze
12. Walter Reed Army Institute of Research: Dr. W. T. Hockmeyer

E. JUSTIFICATION FOR BUDGET

1. Personnel

To achieve the goals of the proposed contract, it is considered essential that a proper balance of Investigators and technicians be established, with Dr. M. Hollingdale as Principal Investigator. Both Dr. do Rosario and Dr. Aley are working on the current project. Dr. do Rosario is directing the production of *P. falciparum* gametocyte cultures, and their infection into Anopheline mosquitoes. His considerable prior experience of *P. falciparum* blood stage cultures in the U. K., Brazil and North Carolina make him well qualified for such a responsibility. Further, his experience of working for three years in Brazil has enabled him to become completely familiar with the scientific community both in Brazil, and throughout Central and South America. Through him, a facility is being established in Sao Paolo, and he will closely work with the projects to be established there and with investigators who will come to us. As he is a consultant consultant to WHO and PAHO, the proposed collaborations in Tapachula, Mexico, and Sao Paolo should be greatly facilitated by these organizations.

Dr. Aley is a renowned biochemist from NIH who joined this group in 1984. His background was the biochemical analysis of malarial antigens inserted into the red blood cell membrane or on the merozoite surface. This has led to development and application of techniques to identify the ligand-receptor interactions of sporozoite invasion of hepatocytes, which he is proposed to direct on the new contract. Further, his biochemistry experience has enabled him to now identify specific EE antigens and to investigate the biochemistry of their synthesis and expression. It is proposed that Dr. Kumar, also currently at NIH, will join Dr. Aley to pursue these investigations. Dr. Kumar has had seven years experience at NIH studying the biochemistry of gametocyte antigens, and will add considerable breadth to our expertise. This group is also joined by Ms. Michelle Bates, who is finishing her M. Sc degree while here, and again will be involved in the biochemistry of malarial antigens.

This group has been fortunate in that it has retained several technicians for several years, and who are completely familiar with our laboratory procedures. Ms. Leland has been here eight years and directs the technical services. Ms. Sigler and Ms. Cooper have been here three and one half and one and one half years respectively. The expansion of the group to establish *P. falciparum* gametocyte cultures and additional Anopheline colonies necessarily requires additional technical positions, especially to maintain the research effort. Two more positions are requested full time, and a part-time laboratory aide. Finally, maintenance of our six insectaries requires the part time services of an Institute engineer, Mr. J. Puckett, who helped install each room.

2. Supplies

The establishment of the new *P. falciparum* facility has led to a large increase in the requirements of supplies for culture including disposable plastic ware, media, sera, and biochemical reagents. The insectaries also require expanded support for pans, food and animals to maintain the colonies. The development of new biochemical and molecular biological technologies greatly facilitate research, but are also expensive. However, to maintain competitive research, it is desirable that these increased costs be met.

3. Equipment

This group has been extremely fortunate in being able to acquire a significant amount of equipment during 1980-1985. We now have fully equipped tissue culture and biochemistry laboratories. The requested equipment to be purchased during Year 1 is to complete our biochemistry laboratories. It is anticipated that our progress will require DNA electrophoresis, and modification of the ISI assay to use ELISA techniques will require an ELISA reader to accurately test the increased numbers of samples expected to be generated from the various field operations. As we integrate our research into the context of field studies, it is anticipated that a computer will be required to analyse and store all the data.

4. Communications

Our publication record suggests that a significant volume of future manuscripts will be maintained. Many journals now require fees per page of text, and the increased biochemical nature of our research requires improved graphics and illustration.

5. Travel

It is requested that each investigator attend an annual meeting of the ASTMH each year. Important meetings with large malaria components are planned in 1986, including Rio de Janeiro, Brisbane and Munich. It is anticipated that our research data will be presented at each meeting. The advent of an insectary and research facility in Sao Paolo means that 1 or 2 investigators each year are required to visit Brazil. Similarly, the practical use of the facility at Tapachula, Mexico also requires yearly vists. The proposed collaboration with CDC to study the immunological responses to *P. falciparum* sporozoites in Kenya will require at least one visit by an investigator, during year 2.

6. Subcontracts

1. The justification for the subcontract to the Netherlands is fully described in section 5. The budget proposed is an extremely cost-effective way of pursuing important fundamental research using systems that are unavailable within the USA.

Subcontracts

1. Enclosed

2. BRI has at its disposal a large collection of botanical specimens collected in malaria endemic areas throughout the world by the department of Agriculture. These samples were collected because of their use as fever depressents (potential anti-malarial drugs) in the various areas. We plan to prepare extracts from these samples and test them for anti-malarial activity *in vitro* and *in vivo*.

3. This subcontract with Anne Goodman Ph. D. is funding for a second year for literature search and periodic reports on "The Relative Effects of Concomitant Vaccinations or Immunicological Phenomena such as Immunosuppression." This subcontract was initially funded on AID Cooperative Agreement No. DPE-4530-A-00-4036-00 for a period of one year. It has proven very successful in light of the four progress reports thus far completed and one more year of support is required for its completion.

BUDGET

PERSONNEL		YR 1	YR2	YR3
Principal Investigator M. R. Hollingdale		54,450	58,534	62,924
Investigator V. do Rosario		36,000	38,700	41,603
Investigator S. Aley		36,000	38,700	41,603
Investigator N. Kumar		36,000	38,700	41,603
Assistant Investigator M. Bates		22,000	23,650	25,424
Technical Supervisor P. Leland		24,567	26,410	28,390
Technicians	C. Sigler	20,517	22,056	23,710
	J. Cooper	18,190	19,554	21,021
	S. Leath	16,800	18,060	19,415
	D. St. Jules	16,800	18,060	19,415
	Vacant	16,800	18,060	19,415
	Vacant	16,800	18,060	19,415
	Vacant (50%)	8,000	8,600	9,245
Engineer J. Pucket (25%)		6,250	6,719	7,223
	TOTAL	329,174	353,863	380,406
OVERHEAD at 119,53% of salaries		393,462	422,972	454,699
SUPPLIES		175,000	188,125	202,234
EQUIPMENT				
DNA electrophoresis		8,000		
Fluoremeter		7,000		
ELISA reader and plate washer		20,000		
Airfuge		5,000		
Computer		10,000		
HPLC columns		1,200	1,200	1,200
Maintenance		5,000	5,000	5,000
		56,200	6,200	6,200
COMMUNICATIONS		10,000	10,750	11,550

TRAVEL	YR1	YR2	YR3
National meetings x 4	5,000	5,375	5,778
VI ICOPA Brisbant x 1	3,000		
Int. Sym. Malaria Brazil x 1	2,000		
IX Int. Cong. Inf. Parasit. Dis. Germany x 1	2,000		
Laveran Society, France x 2		4,300	
Sao Paolo - investigator visits	3,000	3,225	3,467
Tapachula - investigator visits	3,000	3,225	3,225
Kenya - investigator visits		3,225	
	18,000	19,350	12,470

SUBCONTRACTS			
1. University of Nijmegen (Enclosed)	101,000	99,000	97,000
2. J. L. Leef P. I.		50,000	
3. S. A. Goodman P. I.		40,000	
TOTAL	1,082,836	1,190,260	1,164,559

TOTAL 3 YRS 3,437,655

UNIVERSITY OF NIJMEGEN

Budget proposal for the Sporozoite-receptor studies on human hepatocytes

		1st year	2nd year	3rd year
Postdoctoral fellow Gastroenterology (Yap)				
Dr. P.J.M. Rijntjes (Nijmegen)	100%	$ 29,500	$ 29,500	$ 29,500
Technician Gastroenterology Nijmegen	50%	10,000	10,000	10,000
Postdoctoral fellow Cell Biology (Geuze)				
Dr. G. Posthuma (Utrecht)	100%	$ 29,500	29,500	29,500
Technician Cell Biology (Utrecht)	50%	10,000	10,000	10,000
Technician Parasitology (Verhave,Nijmegen)	50%	10,000	10,000	10,000
Supplies for tissue culture, serology, cryo-EM, biochemistry		12,000	8,000	8,000
Travel: 1 roundtrip Netherlands-WashingtonDC for each fellow			2,000	
Total		$ 101,000	$ 99,000	$ 97,000

Grand total $ 297,000

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CURRICULUM VITAE

Name:	Michael Richard H ''ingdale
Date of Birth:	
Place of Birth:	
S. S. Number	
Education:	
1967 B. Sc.	University of Liverpool, (Honors) Bacteriology
1971 Ph. D.	Lister Institute of Preventive Medicine, University of London, Microbiology
Postdoctoral Training:	
1970-1974	London School of Hygiene and Tropical Medicine
Research Fellowships:	
1974-1976	Department of Microbiology, Harvard School of Public Health, Boston, Massachusetts
1976-1978	Department of Pathology, The John Hopkins University School of Medicine, Baltimore, Maryland
1978-1979	Research Associate, Department of Parasitology, The Rockefeller University, New York
Current Position:	
1979-	Head, Malaria Department, and Principal Investigator, Biomedical Research Institute, Rockville, Maryland
Current Research:	Principal Investigator, US A. I. D. Contract No. DPE-0453-C-00-3051-00 The exoerythrocytic stage of human malarial parasites.
	Principal Investigator, WHO grant, *In vitro* testing of anti-malarial tissue schizonticides.
	Co-Investigator, NIH grant, Surface properties and intracellular antigens of plasmodial sporozoites.

Professional Societies:

American Society of Tropical Medicine and Hygiene

Royal Society of Tropical Medicine and Hygiene

Tissue Culture Association

Publications:

1. Hollingdale, M. R.: The antigens of *Mycoplasma hominis*. Ph. D. Thesis, University of London, 1971.

2. Hollingdale, M. R., Lemcke, R. M.: The antigens of *Mycoplasma hominis*. J. Hygiene, Camb. 67: 585-602, 1969.

3. Hollingdale, M. R., Lemcke, R. M.: The antigens of *Mycolplasma hominis*. Proc. Fifth Intl. Cong. Infect. Dis. (Vienna), 403-406, 1970.

4. Hollingdale, M. R., Lemcke, R. M.: Antigenic differences within the species *Mycoplasma hominis*. J. Hygiene, Camb. 68: 466-477, 1970,

5. Hollingdale, M. R., Lemcke, R. M.: Membrane antigens of *Mycoplasma hominis*. J. Hygiene Camb. 70: 85-97, 1972.

6. Hollindale, M. R., Manchee, R. J.: The role of mycoplasma membrane proteins in the absorption of animal cells to mycoplasma hominis colonies. J. Gen Microbiol. 70: 391-393, 1972.

7. Hollingdale, M. R., Murray, I. G.: Antibody responses in Farmer's Lung Disease. Proc. Fourth Intl. Cong. Aspergillosis and Farmer's Lung. (Basel) 280-287, 1974.

8. Hollingdale, M. R.: Antibody responses in patients with Farmer's Lung Disease to antigens from *Micropolyspora faeni*. J. Hygiene, Camb. 72: 79-89, 1974.

9. Hollingdale, M. R.: Antibody responses in patients with Farmer's Lung Disease to antigens from *Thermoactinomyces vulgaris*. J. Hygiene, Camb. 74: 35-41, 1975.

10. Hollingdale, M. R.: Isolation of lipopolysaccharide from the walls of *Micropolyspora faeni*: chemical composition and serological reactivity. J. Gen. Microbiol. 86: 250-258, 1975.

11. Cooper, M. D., Hollingdale, M. R., Vinson, J. W., Costa, J.: A passive hemagglutination test for diagnosis of Trench Fever due to *Rochalimaea quintana*. J. Infect. Dis. 134: 605-609, 1976.

12. Herrmann, J. E., Hollingdale, M. R., Collins, M. F., Vinson, J. W.: Enzyme immunoassay and radioprecipitation test for the detection of antibodies to *Rochalimaea* (Rickettsia) *quitana*. Soc. Exp. Biol. Med. 154: 285-288, 1977.

13. Hollingdale, M. R., Herrmann, J. E., Vinson, J. W.: Enzyme immunoassay of antibody to *Rochalimaea quintana*: diagnosis of Trench Fever and serologic cross-reactions among other Rickettsiae. J. Infect. Dis. 137: 578-582, 1978.

14. Hollingdale, M. R.: Lipopolysaccharides in *Micropolyspora faeni*. J. Allergy Clin. Immunol. 61: 236-237, 1978.

15. Hollingdale, M. R., Kilejian, A.: Purification of *Plasmodium lophurae* exoerythrocytic merozoites by an ion exchange column. J. Protozool. 26: 616-619, 1979.

16. Shenker, B. J., Willoughby, W. F., Hollingdale, M. R., Mann, T. N.: *In vivo* response to inhaled proteins. III. Inhibition of experimental immune complex pneumonitis after suppression of peripheral blood lymphocytes. J. Immunol. 124: 1763-1772, 1980.

17. Hollingdale, M. R., Vinson, J. W., Herrmann, J. E.: Immunochemical and biological properties of the outer membrane associated lipopolysaccharide and protein of *Rochalimeau quintana*. J. Infect. Dis. 141: 672-679, 1980.

18. Hollingdale M. R., Leef J. L., McCullough M., Beaudoin R. L., 1981. *In vitro* culture of the exoerythrocytic stage of *Plasmodium berghei* from sporozoites. Science, 213: 1021-1022.

19. Hollingdale, M. R., Leland, P,. 1982. Detection of exoerythrocytic stages of *Plasmodium berghei* in fixed liver tissue and cultured cells by an immunoperoxidase antibody technique. Trans. R. Soc. Trop. Med. Hyg., 76: 624-626.

20. Hollingdale, M. R., Zavala, F., Nussenzweig, R. S., Nussenzweig, V.: 1982. Antibodies to the protective antigen of *Plasmodium berghei* sporozoites prevent entry into cultured cells. J. Immunol, 128: 1929-1930.

21. Ramsey, J. M., Beaudoin R. L., Hollingdale, M. R.: 1982. Infection of tissue-culture cells with ^{60}Co gamma irradiated malaria sporozoites. In; Nuclear techniques in the study of parasitic infections, IAEA: 19-25.

22. Hollingdale, M. R., Leland P., Leef J. L., Leef, M. F., Beaudoin R.L.: 1983. Serological reactivity of *in vitro* cultured exoerythrocytic stages of *Plasmodium berghei* in indirect immunofluorescent or immunoperoxidase antibody tests. Am. J. Trop. Med. Hyg., 32: 24-30.

23. Hollingdale, M. R., Leland, P., Leef, J. L., Beaudoin, R. L.: 1983. The influence of cell type and culture medium on the *in vitro* cultivation of exoerythrocytic stages of *Plasmodium berghei*. J. Parasitol., 69: 346-352.

24. Hollingdale, M. R., Leland, P., Schwartz, A. L.: 1983. *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line. Am. J. Trop. Med. Hyg., 32: 682-684.

25. Hollingdale, M. R., Leland, P., Leef, J. L., Schwartz, A. L.: 1983. Entry of *Plasmodium berghei* sporozoites into cultured cells, and their transformation into trophozoites. Am. J. Trop. Med. Hyg., 32: 685-690.

26. Cogswell, F. B., Krotoski, W. A., Hollingdale, M. R., Gwadz, R. W.: 1983. Identification of hypnozoites and tissue schizonts of *Plasmodium vivax* and *P. cynomolgi* by the immunoperoxidase method. Am. J. Trop. Med. Hyg., 32: 1454-1455.

27. Alvares, A. P., Ueng, T., Scheibel, L. W., Hollingdale, M. R.: 1984. Impairment of hepatic cytochrome P-450-dependent monooxegenases by the malaria parasite *Plasmodium berghei*. Mol. Biochem. Parasitol., 13: 277-282.

28. Meis J. F. G. M., Hollingdale, M. R. Verhave J. P., Aikawa, M.: 1984, Intranuclear localization of *Plasmodium berghei* sporozoites. Cell. Bio. Int. Rep., 8: 1016.

29. Leland, P., Sigler, C. I., Danforth, H. D., Hollingdale, M. R.: 1984. Inhibition of *Plasmodium berghei* sporozoite invasion of cultured hepatoma cells. Trans. R. Soc. Trop. Med. Hyg., 78: 639-640.

30. Aikawa, M., Schwartz, A. L., Uni, S., Nussenzweig, R. S., Hollingdale, M. R.: 1984. Ultrastructure of *in vitro* cultured exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line. Am. J. Trop. Med. Hyg., 33: 792-799.

31. Sigler, C. I., Leland, P,. Hollingdale, M. R.: 1984. *In vitro* infectivity of irradiated *Plasmodium berghi* sporozoites to cultured hepatoma cells. Am. J. Trop. Med. Hyg., 33: 544-547.

32. Hollingdale, M. R., Nardin, E. H., Tharavanij, S., Schwartz, A. L., Nussenzweig, R. S.: 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells; an *in vitro* assay of protective antibodies. J. Immunol., 132: 909-913.

33. Zavala, F., Hollingdale, M. R., Schwartz, A. L., Nussenzweig, R. S., Nussenzweig, V.: 1985. Immunoradiometric assay to measure the *in vitro* penetration of sporozoites of malarial parasites into hepatoma cells. J. Immunol., 134: 1202-1205.

34. Schwartz, A. L., Hollingdale, M. R.: 1985. Primaquine and lysomotropic amines inhibit malaria sporozoite entry into human liver cells. Mol. Biochem. Parasitol., 14: 305-311.

35. Hollingdale, M. R., Collins, W. E., Campbell, C. C., Schwartz, A. L.: 1985. *In vitro* culture of two populations (dividing and nondividing) of exoerythrocytic parasites of *Plasmodium vivax*. Am. J. Trop. Med. Hyg., 34: 216-222.

36. Hollingdale, M. R.: 1985. Malaria and the liver. Hepatol., 5: 327-335.

37. Hollingdale, M. R., Leland, P., Sigler, C. I., Leef, J. L.: 1985. *In vitro* infectivity of cryopreserved *Plasmodium berghei* sporozoites to cultured cells. Trans. R. Soc. Trop. Med. Hyg., 79: 206-208.

38. Hollingdale, M. R., Leland, P., Sigler, C. I.: 1985. *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* in irradiated hepatoma cells. Am. J. Trop. Med. Hyg., 34: 21-23.

39. Hollingdale, M. R., McCann, P. P., Sjoerdsma, A.: 1985. *Plasmodium berghei*: effects of inhibitors of ornithine decarboxylase on the exoertyhrocytic stage. Exp. Parasitol., in press.

40. Hollingdale, M. R.: 1985. *In vitro* testing of anti-malarial schizonticides. WHO Publication, in press.

41. Uni, S., Aikawa, M., Collins, W. E., Campbell, C. C., Hollingdale, M. R. Electron microscopy of *Plasmodium vivax* exoerythrocytic stages grown *in vitro* in a hepatoma cell line. Am. J. Trop. Med. Hyg., in press.

42. Hollingdale, M. R., Leland.: 1985. *In vitro* cultivation of the exoerythrocytic stage of malarial parasites. In; Atomic energy applications in parasitology, IAEA, Vienna: in press.

43. Beaudoin, R. L., Hollingdale, M. R.: Parasite biology - *In vitro* cultivation of the exoerythrocytic stages. In; Textbook of malaria, ed Wernsdorfer & Mcgregor, in press.

44. Ballou, W. R., Rothbard, J., Wirtz, R. A., Gordon, D. M., Williams, J. S., Gore, R. A., Schneider, I., Hollingdale, M. R., Beaudoin, R. L., Maloy, W. L., Miller, L. H., Hockmeyer, W. T.: 1985. Immunogenicity and biologic activity of antibodies to synthetic peptides of the circumsporozoite protein of *Plasmodium falciparum*. Science, 228: 996-999.

45. Young, J. F., Hockmeyer W. T., Gross, M., Ballou, W. R., Wirtz, R. A., Trosper, J. H., Beaudoin, R. L., Hollingdale, M. R., Miller, L. H., Diggs, C. L., Rosenberg, M.: 1985. Expression of *P. falciparum* circumsporozoite protein derivatives in *E. coli* for development of a human malaria vaccine. Science. 228: 958-962.

46. Zavala, F., Tam, J. P., Hollingdale, M. R., Cochrane, A. H., Quakyi, I., Nussenzweig, R. S., Nussenzweig, V.: 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. Science, 228: 1436-1440.

47. Meis, F. G. M., Verhave, J-P., Ponnudurai, T., Hollingdale, M. R., Rijntjes, J. M., Smith, J. E., Sinden, R. E., Jap, P. H. K., Meuwissen, J. H. E. Th., Yap, S. H.: Fine structure of the liver forms of the malaria parasite *Plasmodium falciparum* in human hepatocytes *in vitro*. Cell Tissue Res. In press.

48. Meis, J. F. G. M., Rijntjes, J. M., Verhave, J-P., Ponnvdurai, T., Hollingdale, M. R., Yap, S. H.: Infection of cryopreserved adult human hepatocytes with *Plasmodium falciparum* sporozoites. Cell Biol. Intl. Reports, in press.

49. Pancake, S. J., Hollingdale, M. R.: Characterization of the *Plasmodium berghei* sporozoite receptor on human hepatoma cells. Ex. Parasitol., submitted.

50. Krotoski, W. A., Gatnham, P., C.C., Cogswell, F. B., Collins, W. E., Bray, R. S., Gwadz, R. W., Killick-Kendrick, R., Wolf, R., Sinden, R., Hollingdale, M. R., Lowrie, R. C., Koontz, L. C., Stanfill, P. S.: Observations on early and late post-sporozoite tissue stages in primate malaria. IV. Pre-erythrocytic schizonts and/or hypnozoites of Chesson and North Korean strains of *Plasmodium vivax* in the chimpanzee. Am. J. Trop. Med. Hyg. submitted.

51. Hollingdale, M. R., Collins, W. E., Campbell, C. C.: *In vtiro* culture of exoerythrocytic parasites of the North Korean strain of *Plasmodium vivax* in hepatoma cells. Am. J. Trop. Med. Hyg. submitted.

CURRICULUM VITAE

Stephen B. Aley

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SOCIAL SECURITY NUMBER:

CITIZENSHIP: United States of America

MARITAL STATUS: Married

EDUCATION:

Ph.D. in Immunology and Biochemistry The Rockefeller University	1981
Non-degree Post Graduate Student John Curtin School for Medical Research	1976
B.S. Biology (Honors) California Institute of Technology	1975

AWARDS & FELLOWSHIPS:

Rockefeller Foundation Fellowship	1981-1983
NSF Graduate Studies Fellowship	1977-1980
Rockefeller University Fellowship	1976, 1981
Thomas J. Watson Fellowship	1975-1976
ARCS Scholar	1974-1975

RESEARCH EXPERIENCE:

Biochemical characterization of exoerythrocytic stages of malaria. Michael Hollingdale, BRI.	1984-
Isolation and characterization of surface membrane from malaria infected erythrocytes. Russell Howard and Louis Miller, NIH	1981-1984

56

Compostion and dynamics of the plasma membrane of Entamoeba histolytica. William Scott and Zanvil Cohn, Rockefeller University	1978-1981
Study of macrophage cell surface proteins by immune precipitation. Celso Bianco and Zanvil Cohn, Rockefeller University	1977
Soluble regulators of the secretion of immunoglobulin by free flowing lymphoid cells during an immune response. Eric Adams and Bede Morris, JCSMR	1976
Biochemistry of bacteriophage T4 morphogenesis. William Wood, Caltech	1972-1975
Isolation and characterization of acetylcholine esterase from Torpedo californicus. Carl Johnson, Caltech	1975
Isolation and characterization of H-2 antigens. Jack Silver, Caltech	1973

BIBLIOGRAPHY:

1. Yin, H. L., S. B. Aley, C. Bianco, and Z. A. Cohn, 1980. Plasma membrane polypeptides of resident and activated mouse pertoneal macrophages. Proc. Nat. Acad. Sci. 77: 2188-2191.

2. Aley, S. B., W. A. Scott and Z. A. Cohn, 1980. Plasma membrane of *Entamoeba histolytica*. J. Exp. Med. 152: 391-404.

3. Aley, S. B., W. A. Scott and Z. A. Cohn, 1980. Isolation of the plasma membrane of *Entamoeba histolytica*. Archiv. Investigacion Medica 11 (sup 1): 41-46.

4. Murray, H. W., S. B. Aley and W. A. Scott, 1981. Susceptibility of *Entamoeba histolytica* to oxygen intermediates. Mol. Bio. Parasit. 3: 381-393.

5. Howard, R. J., J. W. Barnwell, W. A. Daniel and S. B. Aley, 1982. Radioiodination of new protein antigens on the surface of *Plasmodium knowlesi* schizont-infected erythrocytes. Mol. Bio. Parasit. 6: 331-397.

6. Howard, R. J., S. B. Aley and P. F. Lemkin, 1983. High resolution comparison of *Plasmodium knowlesi* clones of different variant antigen phenotypes by two-dimensional gel electrophoresis and computer analysis. Electrophoresis, 4: 420-427.

7. Fahey, R. C., G. L. Newton, B. Arrick, T. Overdank-Bogart and S. B. Aley, 1984. *Entamoeba histolytica:* A eukaryote without glutathione metabolism. Science, 224: 70-72.

8. Howard, R. J., J. A. Lyon, C. L. Diggs, J. D. Haynes, J. H. Leech, J. W. Barnwell, S. B. Aley, M. Aikawa and L. H. Miller, 1984. Localization of the major *Plasmodium falciparum* glycoprotein on the surface of mature intra-erythrocytic triphozoites and schizonts. Mol. Bio. Parasit., 11: 349-362.

9. Aley, S. B., J. W. Barnwell, W. Daniel and R. J. Howard, 1984. Identification of parasite proteins in a membrane preparation enriched for the surface membrane of erythrocytes infected with *Plasmodium knowlesi.* Mol. Bio. Parasit., 12: 69-84.

10. Aley, S. B., Z. A. Cohn, and W. A. Scott 1984. Endocytosis in *Entamoeba histolytica.* Evidence for a unique, non-acidified compartment. J. Exp. Med. 160: 624-37.

11. Leech, J. H., S. B. Aley, L. H. Miller and R. J. Howard, 1984. *Plasmodium falciparum* malaria: Cytoadherence of infected erythrocytes to endothelial cells and associated changes in the erythrocyte membrane. Prog. Clin. Biol. Res., 155: 63-77.

12. Aley, S. B., J. A. Sherwood and R. J. Howard, 1984. Knob positive and knob negative *Plasmodium falciparum* differ in expression of a strain-specific malarial antigen on the surface of infected erythrocytes. J. Exp. Med. 160: 1585-90.

13. McLaughlin, J. and Aley, S. B., 1985. The Biochemistry and Functional Morphology of the *Entamoeba.* J. Protozool. 32: 221-240.

14. Kutner, S., Breuer, W. V., Ginsburg, H., Aley, S. B., and Cabantchik, Z. I. 1985. Characterization of Permeation Pathways in the Plasma membrane of human erythrocytes infected with early stages of *Plasmodium falciparum*: Association with parasite development. J. Cell Physiol. in press.

15. Aley, S. B., Sherwood, J. A., Marsh, K., Eidelman, O., and Howard, R. J. 1983. Identification of *Plasmodium falciparum* isolate-Specific Proteins on sorbitol-enriched Parasitized Erythrocytes from Gambian Patients. Parasitology. in press.

Submitted:

Howard, R. J., Aley, S. A., Leech Leech, J. H., Lew, A. M., Wellems, T. E., Marsh, K., Uni, S., Aikawa, M., Rener, J., Taylor, D. W. 1985. *Plasmodium falciparum* Malaria: Identification of a Soluble Histidine-Rich Protein Released fc m Infected Erythrocytes of Knob-positive and Knob-negative parasites. J. Cell. Biol. submitted.

Sherwood, J. A., Spitalnik, S. L., Aley, S. B., Quakyi, I. A., and Howard, R. J. 1985. *Plasmodium falciparum*: The initial Identification and Characterization of Glycolipids Sunthesized by a Malaria Parasite. Exp. Parasitology. submitted.

Taylor, D. W., Evans, C. B., Hennessy, G. W., and Aley, S. B. Use of a Two-Sited Monoclonal Antibody Assay for Detecting a Heat-Stabile Malarial Antigen in the Sera of Mice Infected with *Plasmodium yoelii*. submitted.

Hollingdale, M. R., Ballou, W. R., Aley, S. B., Panckae, S. J., Miller, L. H., Hockmeyer, W. T. *Plasmodium falciparum* recombinant CS proteins elicit protective anti-sporozoite antibodies in mice that inhibit sporozoite invasion (ISI) of hepatoma cells. J. Immunol. submitted.

Curriculum Vitae

Surname:	do Rosario
Fornames:	Virgilio Estolio
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Place of birth:	[redacted]
Nationality:	Portuguese

Qualifications:

Veterinary Surgeon, University of L. Marques, Mozambique,1970
Diploma in Genetics, University of Edinburgh, 1973
PhD in Genetics, University of Edinburgh, 1976

Posts held:

Research Associate, University L. Marques, Mozambique, 1970
2nd Assist. idem, 1972
Lecturer, University of Maputo, Mozambique, 1977-1978
Research Worker, Ministry of Health, Mozambique, 1977-1978
Research Fellow, Edinburgh University, 1979-1980
Senior Technical Consultant, WHO, Oct.-Dec. 1980
Research Scientist, Instituto Evandro Chagas (F. SESP)
Belem, Brazil, 1981-1984
Visiting Research Associate Professor, University N. Carolina, Dept. Parasitology, Chapel Hill NC, 1984-1985
Staff Scientist Biomedical Research Institute, Rockville, MD 1985-present

Memberships:

Royal Society Tropical Medicine and Hygiene
Sociedade Brasileira de Genetica
American Society of Tropical Medicine and Hygiene

Publications:

Rosario, V. E. (1976) Genetics of chloroquine resistance in malaria parasites, *Nature, 261*: 585-586.

Rosario, V. E., Hall, R., Walliker, D., and Beale, G. H. (1978) Persistence of drug resistance parasites, *The Lancet, 1*: 185-187.

Rosario, V. E. (1981) Cloning of naturally occuring mixed infections of *Plasmodium falciparum*. *Science, 212*: 1037-1038.

Rosario, V. E. (1983) Caracterizacao de cepas de *Plasmodium falciparum* do Brasil. *Sep. Rev. F.SESP*, 28 2: 115-136.

Couto, A., Rosario, V. E., and Walliker, D. (1983) Analise enzimatica de 56 amostras de *Plasmodium falciparum* de Bacia Amazonica (Brazil). *Rev. Bras. Malar. D. Trop., 35*: 11-19.

Vasconcellos, M. A. e Rosario, V. E. (1983) Testes de sensibilidade *in vitro* de amostras de *Plasmodium falciparum* de Bacia Amazonica (Brazil). *Rev. Bras. Malar. D. Trop., 35*: 21-28.

Thaithong, S., Beale, G. H., Fenton, B., McBride, J., Rosario,V., Walker, A., and Walliker, D., (1984) Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. *Trans. R. S. Trop. Med. Hyg., 78*: 242-245.

Rosario, V. E. (1984) Biochemical aspects of the characterization of malaria parasites. *In* Trop. Dis. Res. Ser. *5*, Ed. Schwabe and Co. Ag. Basel (WHO Symp. 1982)

Schoefeld, L., Tharavanij, S., Saul, A., do Rosario, V., and Kidson, C. A specific S-antigen of *Plasmodium falciparum* is expressed in a proportion of primary isolates in Brazil, Thailand and Papua New Guines. *Trans R. S. Trop. Med. Hyg., 79*, 4: 493-495.

Rosario, V. E., Couto, A., Santos, M. A., de Sousa, J. M. Caraterizacao de cepas de *P. falciparum* coletadas de pacientes recrudescentes (In Press)

Rosario, V.E., Vasconcellos, M., Oliveiva, S., Assis, F. e Couto, A., Carateriza cao de 15 amostras de *P. falciparum* de 8 areas da Bacia Amazonica Brasileira, (In Press)

Abstracts:

V Congresso Medico Amazonico (5-8 Outubro 1982), Belem, Brazil.

- Respostas *in vivo* e *in vitro* do *Plasmodium falciparum* na regiao di Paragominas.
- Microtestes de sensibilidade a cloroquina.
- Resultados preliminares de classificacao de cepas de *P. falciparun*
- Selecao de cepa(s) resistentes(s) a mefloquina *in vitro*.
- Observacoes sobre diagnostico diferencial de malaria (exame microscopico).

Curso de Atualizacao em Pediatria (4-9 Agosto 1983), Belem, Brazil.

- Patologias Infeciosas Regionais.

I Congresso da Soc. Latino Americana de Medicina Tropical/XX Congresso da Soc. Brasileira de Medicina Tropical, Salvador, Brasil. (5-9 Fevereiro de 1984)

- Microteste de sensibilidade a cloroquina e mefloquina.
- Caracterizacao enzimatica de *P. falciparum*.
- Diversidade antigenica de cepas de *P. falciparum* atraves de anticorpos monoclonais.
- Tentativas de infecao de saguis da especie *Callithrix penicillata* com o *Plasmodium falciparum*.

Seminario de Pesquisa Museu E. Goeldi, Belem, Brasil (9-12 Setembro 1983)

- Caracterizacao de cepas de *Plasmodium falciparum* do Brasil.

XXI Congresso da Sociedade Brasileira de Medicina Tropical (3-8 Feveriro 1985), S. Paulo, Brasil.

- Caractérizacao de cepas de *Plasmodium falciparum* coletadas de pacientes recrudescentes.
- Clonagem de cepas de *Plasmodium falciparum* coletadas na regiao amazonica.

Other responsabilities:

Advisor for the Malaria Programme at the Instituto Evandro Chagas, Belem, Brasil, (1985-present);
Advisor for the Malaria Programme at SUCEN, Sao Paulo, Brasil, (1985-present).

May 1985

CURRICULUM VITAE

Name: Nirbhay Kumar, Ph.D.

Date and Place of Birth:

Marital Status: Married to Geetha P. Bansal; two children

Education:

1968 Graduated from High School and Intermediate (73% marks and first rank in college)

1970 B.Sc., Agra University, India
Physics, Chemistry & Math (75% Marks, First division and first position in college; 10th position in the order of merit out of about 7000 candidates)

1972 M.Sc., Banaras Hindu University, India
Biochemistry (70% marks with first division; second position in the order of merit)

1977 Ph.D., All India Institute of Medical Sciences, New Delhi, India (Biochemistry). Thesis: Hormonal Regulation of the Cartilage Growth-Studies on the Effects of Somatomedins on Cartilage from Young and Old Animals.

Brief Chronology of Employment:

July, 1983 - Present
Burroughs Wellcome Senior Research Fellow, Malaria Section, Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Maryland 20205

1982-1983 Visiting Associate, Malaria Section, Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Maryland 20205

1979-1982 Visiting Fellow, Fogarty International Center, NIH, Bethesda, Maryland 20205

1977-1979 Assistant Research officer, National Institute of Nutrition Hyderabad, India

1975-1977 Senior Research Fellow (Council of Scientific and Industrial Research), AIIMS, New Delhi, India

1972-1975 Junior Research Fellow (Council of Scientific and Industrial Research), AIIMS, New Delhi, India

Society Membership:

The American Society for Cell Biology
Indian Immunology Society

Awards and Honors:

Burroughs Wellcome Senior Research Fellowship to work at NIH (July 1983-)

Travel fellowship by the International Union of Biochemistry to attend XII International Congress of Biochemistry, Perth, Australia, 1982

Junior and Senior Research Fellowships of the Council of Scientific and Industrial Research, India (1972-1977).

National Scholarship (1970-1972)

Merit Scholarship (1968-1970)

Research Experience

1982-present: Malaria--Transmission Blocking Immunity. I have been directly involved in several projects related to identification and characterization of target antigens of antibodies blocking infectivity of malaria parasites to mosquitoes (transmission of malaria).

The target antigens of monoclonal antibodies blocking infectivity by blocking the fertilization of male and female gametes in the mosquito midgut include a set of 3 surface proteins of apparent M_r of 260 K, 59 K and 53 K. On the other hand a single protein (apparent M_r = 26K) is the target antigen for monoclonal antibodies which blocked infectivity by blocking post-fertilization development of zygotes into ookinetes.

Biochemical characterization of these target proteins was done by using a combination of various techniques including surface labeling (^{125}I), biosynthetic labeling (with ^{35}S-methionine, ^{3}H-leucine, ^{3}H-glucosamine, and ^{3}H-mannose), two-dimensional SDS-PAGE, western blotting and peptide mapping. I have found that the target antigens of fertilization blocking antibodies although present on the surface of extracellular gametes and newly fertilized zygotes, are actually synthesized by the intraerythrocytic gametocytes and the synthesis ceases during or soon after gametogenesis and fertilization. I also found that the 59 K and 53 K glycoproteins show strong hydrophobic interactions with the detergent Triton X-114 and thereby enabling the seperation of these 2 proteins from the 260 K protein in one step. Individual proteins were then further purified by Ab-affinity chromatography and hydrophobic chromatography.

My studies also revealed that during development of zygotes into ookinetes in the mosquito midgut, the parasites show a dramatic shift in their biochemical activities. The zygotes cease to synthesize and express the gamete antigens, and during their development into motile ookinetes they begin to synthesize 2 new surface proteins (26 K, 28 K)

in a developmentally regulated manner. They are glycoproteins and contain covalently linked fatty acid (^{3}H-palmitate). This is the first observation on the acylation of proteins in a parasitic system. I also found that microtubules but not microfilaments, play a crucial role in the development of zygotes into ookinetes. Inhibitors of microtubule structure and function blocked in a concentration and time-dependent manner, the in vitro and in vivo transformation of zygotes and ookinetes.

Currently, I am actively involved in several projects related to (i) identification and cloning the genes for target antigens from the genomic expression library with the help of monoclonal and polyclonal antibodies against purified antigens (ii) biochemical and immunochemical comparison of gamete antigens in the P. falciparum isolates from different parts of the world, to investigate if there is any antigenic variation and, last but not the least, (iii) attempts to characterize the components in the midgut of mosquito which might be involved in the interaction with the parasites.

1981-1982: Studies on the Receptor for Immunoglobulin E. I was involved in several projects related to the receptor for immunoglobulin E in basophilic leukemia cells. By gel permeation chromatography in guanidine hydrochloride, we obtained molecular weights of IgE binding sub-unit of the receptor and its proteolytic fragments which were helpful in suggesting a model for the receptor protein. In addition, I also studied the interaction between receptor and detergent Triton X-114 by phase separation technique.

1979-1981: Studies on Membrane-Cytoskeleton. I studied in detail the effects of post-translational covalent modifications, i.e., tyrosinolation and detyrosinolation of tubulin on the kinetics of microtubule assembly. Studies involved purification of tubulin, MAPS, tubulin tyrosine ligase, carboxypeptidase, etc. In my work on the purification and characterization of a brain carboxypeptidase we found that the enzyme preferentially removes tyrosine from polymeric (and other oligomeric forms) tubulin: suggesting thereby an important functional coupling between tyrosinolation/detyrosisolation and assembly/disassembly of microtubules. Purified tubulin from brain was enzymatically tyrosinolated or detyrosinolated and used in the studies on kinetics of microtubule assembly/disassembly, interaction between tubulin and microtubule-associated proteins and effects of phosphorylation/dephosphorylation of MAPs on the microtubule assembly.

A detailed investigation was carried out to understand the mechanism of interaction of taxol (an anti-tumor drug) with tubulin.

During the course of above studies, I also purified a new protein from brain; which interacts with tubulin and might play an important role in the regulation of microtubule organization in the cell.

Tubulin-Membrane Interactions. We looked at the possibility of interaction between tubulin and membranes. Working with neutral phosphatidylcholine lipid bilayers (vesicles), we found that tubulin interacts with these bilayers only at the endothermic phase transition temperature (38°C) and results in the formation of stable vesicle-tubulin complexes.

In subsequent studies, we found that insertion of tubulin into lipid bilayer results in their aggregation. Addition of Ca^{2+} caused fusion of the vesicles-tubulin complexes. These observations are the first of its kind where a protein mediates divalent cation-induced fusion (non-leaky in nature) of neutral phospholipid bilayers.

1977-1979: Effect of Dietary Manipulations on Aging Process in Rats. Rats (male and female) at 4 weeks of age were subjected to dietary restriction (various levels) for different time periods. Tissue respiration, lipid peroxidation, and accumulation of age pigments (lipofuscin) was monitored. Serum levels of growth hormone and modulation of growth hormone receptors by estrogen was also studied in aging rats.

1972-1977: Ph.D. Thesis. Detailed and systematic studies were carried out during graduate studies on the role of growth hormone and serum growth factors on cartilage growth in rats of various age groups. It was found that neither GH levels nor serum somatomedins-like activity are altered significantly during aging. Loss of responsiveness of cartilage to hormonal growth factors as observed for somatomedins could be an important mechanism for the cessation of cartilage growth during aging. Morphological studies demonstrated a number of chemical changes in the cartilage matrix components. Evidences were also obtained for the role of matrix in influencing the metabolic activity in the cartilage cells.

Teaching Experience

1972-1977	Undergraduate teaching at All India Institute of Medical Sciences, New Delhi, India.
1979	Eighth annual training course in Endocrinological Techniques and Their Application, Hyderabad, India.

Publications:

Kumar, Nirbhay: Hormonal regulation of the cartilage growth--studies on the effects of somatomedins on cartilage from young and old animals. Ph.D. Thesis, All India Institute of Medical Sciences, New Delhi, India, 1977.

Talwar, G.P., Kumar, Nirbhay, Pandian, M.R. and P.D. Gupta: Cellular and molecular correlates of the decline in responsiveness of cartilage and thymus to growth hormone. Mol. Cell. Endocrinol. 1: 209-226, 1974.

Talwar, G.P., Pandian, M.R., Nirbhay, Kumar, Hanjan, S.N.S., Saxena, R.K., Krishnaraj, R. and S.L. Gupta: Mechanism of action of pituitary growth hormone. Rec. Prog. Horm. Res. 31: 141-174, 1975.

Kumar, Nirbhay: Differential effects of prostaglandins on cartilage. Curr. Sci. 48: 40-41, 1979.

Kumar, Nirbhay: Unresponsiveness of cartilage to serum somatomedins; further observations. Ind. J. Exp. Biol. 17: 571-573, 1979.

Kumar, Nirbhay and P.D. Gupta: Age related changes in cartilage matrix; possible mechanism for cartilage unresponsiveness to serum growth factors. Ind. J. Exp. Biol. 18: 1238-1241, 1980.

Klausner, R.D., Kumar, Nirbhay, Weinstein, J.N., Blumenthal, R. and M. Flavin: Interaction of tubulin with phospholipid vesicles. I. Association with vesicles at the phase transition. J. Biol. Chem. 256: 5877-5885, 1981.

Kumar, Nirbhay, Klausner, R.D., Weinstein, J.N., Blumenthal, R. and M. Flavin: Interaction of tubulin with phospholipid vesicles. II. Physical changes of the protein. J. Biol. Chem. 256: 5886-5889, 1981.

Kumar, Nirbhay and M. Flavin: Preferential action of a brain detryosinolating carboxypeptidase on polymerized tubulin. J. Biol. Chem. 256: 7678-7686, 1981.

Kumar, Nirbhay: Taxol-induced polymerization of purified tubulin; mechanism of action. J. Biol. Chem. 256: 10435-10441, 1981.

Kumar, Nirbhay: Effect of matrix on the responsiveness of cartilage cells to normal human serum. Ind. J. Biochem. Biophys., 18: 378-378, 1981.

Kumar, Nirbhay and M. Flavin: Modulation of some parameters of *in vitro* microtubule assembly by tubulin tyrosinolation. Eur. J. Biochem. 128: 215-222, 1982.

Kumar, Nirbhay and M. Flavin: A new tubulin binding protein. Biochem. Biophys. Res. Comm. 106: 704-710, 1982.

Kumar, Nirbhay and H. Metzger: Gel filtration in 6M guanidine hydrochloride of the alpha subunit (and its fragments) of the receptor for immunoglobulin E. Mol. Immunol 19: 1561-1567, 1982.

Kumar, Nirbhay, Blumenthal, R., Henkart, M., Weinstein, J. and R.D. Klausher: Aggregation and calcium-induced fusion of phosphatidylcholine vesicle-tubulin complexes. J. Biol. Chem. 257: 15137-15134, 1982.

Carter, R., Miller, L.H., Rener, J., Kaushal, D., Kumar, Nirbhay, Graves, P., Grotendorst, C., Gwadz, R.W., French, C and D. Wirth: Target antigens of malaria transmission blocking immunity. Proc. R. Soc. Lond. B 307: 201-213, 1984.

Grotendorst, C., Kumar, Nirbhay, Carter, R. and D.C. Kaushal: A surface protein expressed during the transformation of zygotes of P. gallinaceum is a target of transmission blocking antibodies. Infect. Immun. 45: 775-777, 1984.

Alcaraz, G., Kinet, J.-P., Kumar, Nirbhay and H. Metzger: Phase separation of the receptor for IgE and its subunits in Triton X-114. J. Biol. Chem. 259: 14922-14927, 1984.

Kumar, Nirbhay and R. Carter: Biosynthesis of the target antigens of antibodies blocking transmission of Plasmodium falciparum. Mol. Biochem. Parasitol. 13: 332-342, 1984.

Kumar, Nirbhay and R. Carter: Biosynthesis of two stage-specific membrane proteins during transformation of Plasmodium gallinaceum zygotes into ookinetes. Mol. Biochem. Parasitol. 14: 127-139, 1985.

Kumar, Nirbhay and R. Carter: Characterization of the target antigens of transmission blocking immunity of malaria parasites. In: Vaccines 85 Approaches to Vaccines (Chanock, R. and R. Lerner, eds.), Cold Spring Harbor Laboratory Press, pp. 39-44, 1985.

Kumar, Nirbhay, Aikawa, M. and C. Grotendorst: Plasmodium gallinaceum: critical role for microtubules in the transformation of zygotes into ookinetes. Exp. Parasitol. 59: 239-247, 1985.

Kumar, Nirbhay and R. Carter: Analysis of molecular interactions and epitopes of the target antigens of transmission blocking immunity in malaria: In Vaccines-86, 1986 (In Press)

Kumar, Nirbhay Phase separation in Triton X-114 of target antigens of transmission blocking immunity in P. gallinaceum Mol. Biochem.

Abstracts and Other Professional Papers Presented:

Kumar, Nirbhay and G. P. Talwar: Responsiveness of cartilage from rats of various ages to serum factors. Proc. Abst., Soc. Biol. Chemists, India 32: 61, 1973.

Talwar, G.P., Pandian, M.R., Kumar, Nirbhay, Jailkhani, B.L., Sen, K.K., Narayanan, P.R. and P.D. Gupta: Mechanism of action of growth promoting and developmental hormones. Proc. XXII Int. Cong. Phys. Sci., New Delhi, 1975.

Kumar, Nirbhay and G.P. Talwar: Decline in the end-organ responsiveness of cartilage to somatomedins. 3rd Int. Symposium on Growth Hormone and Related Peptides, Milan, Italy, pp. 56, 1975.

Kumar, Nirbhay: Effects of serum somatomedins on cartilage from rats of various age groups. Symposium Receptors in Biol. Systems, Department of Atomic Energy, Government of India, pp. 1-20, 1978.

Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R. and Flavin, M.: Insertion of purified tubulin into phospholipid vesicles at the endothermic phase transition. Fed. Proc. 39: 829a, 1980.

Klausner, R.D., Kumar, Nirbhay, Weinstein, J.N., Blumenthal, R. and M. Flavin: 2nd Int. Cong. on Cell Biol., Berlin, 1980. Eur. J. Cell Biol. 22: 219, 1980.

Kumar, Nirbhay and M. Flavin: Enhanced MAPs binding by tyrosinolated tubulin. J. Cell Biol. 87: 255, 1980.

Kumar, Nirbhay and M. Flavin: A new tubulin binding protein. J. Cell Biol 91: 324a, 1981.

Kumar, Nirbhay and H. Metzger: Reevaluation of the molecular weights of receptor for immunoglobulin E and various fragments. Fed. Proc. 41a, 1982.

Kumar, Nirbhay, Blumenthal, R., Henkart, M. and R.D. Klausner: Aggregation and calcium-induced fusion or phosphatidylcholine vesicle-tubulin complexes. XII Int. Cong. of Biochem., Perth, Australia, 1982.

Kumar, Nirbhay and R. Carter: One step procedure for separation of one of the target antigens of transmission blocking immunity in P. gallinaceum. J. Cell. Biochem. 7A(Suppl.): 20, 1983.

Kumar, Nirbhay, Grotendorst, C. and R. Carter: Biosynthesis of antigens on the surface of the transforming zygotes and mature ookinete of Plasmodium gallinaceum. 2nd Int. Conf. on Malaria and Babesiosis, Annecy (France), September, 1983.

Kumar, Nirbhay: Development of malaria parasites in the mosquito midgut: stage specific targets of transmission-blocking antibodies. Gordon Research Conference: Immunological and Molecular Aspects of Parasitism, Plymouth, New Hampshire, August, 1983.

Kumar, Nirbhay: Target antigens of transmission-blocking immunity in malaria. The 560th meeting of the Helminthological Society of Washington, January, 1984.

Kumar, Nirbhay, Graves, P.M., Rener, J., Kaushal, D.C., Grotendorst, C., Miller, L.H. and R. Carter: Target antigens of transmission blocking immunity of malaria parasites. Meeting on Modern Approaches to Vaccines, Cold Spring Harbor Laboratory, New York, September 1984.

Current Manuscripts:

Submitted:

~~Kumar, Nirbhay: Phase separation in Triton X-114 of proteins in the sexual stages of *Plasmodium gallinaceum*: an approach for the purification of antigens of transmission-blocking antibodies.~~

Kumar, Nirbhay: Elucidation of the nature of interaction and association between three surface proteins immunoprecipitated by transmission-blocking monoclonal antibodies in *Plasmodium gallinaceum*.

In Preparation:

Kumar, Nirbhay *et al.*: Characterization of glycosylation of proteins in *Plasmodium gallinaceum* zygotes.

Invited Lectures:

1. Special Colloqium on Membrane Fusion. 12th International Congress of Biochemistry, Perth, Australia, 1982.

2. Gordon Research Conference on Parasitism, Plymouth, New Hampshire, 1983.

3. Pasteur Institute, Paris, 1983.

4. The 560th Meeting of the Helminthological Society of Washington, D.C., 1984.

5. Second Conference on Modern Approaches to Vaccines, Cold Spring Harbor Laboratory, New York, 1984.

6. The Wellcome Research Laboratories, Beckenham, United Kingdom, 1984.

7. National Institute of Immunology, New Delhi, 1984.

8. Third Conference on Modern Approaches to Vaccines, Cold Spring Harbor Laboratories, New York, 1984

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Purification of adult schistosome surface membrane, under direction of A. Simpson

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October 1981 - Oct. 1984: Department of Parasitology,
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Study of surface antigens expressed during erythrocytic [illegible]

Publications:

1. A.J.G. Simpson, M.D. Schryer, I.M. Cesari, W.H. Evans and S.R. Smithers. Isolation and partial characterization of the tegumental outer membrane of adult Schistosoma mansoni. Parasitology (1981) 83, 163-177.

2. F. Levi-Schaffer, M.D. Schryer and M. Smolarsky. The resistance of schistosomula of Schistosoma mansoni to antibodies and complement in vitro does not correlate with the binding of antibodies to the surface of the parasite. The Journal of Immunology (1982) 129(6), 2744-2751.

3. F. Levi-Schaffer, M.D. Schryer, R. Tarrab-Hazdai and M. Smolarsky. A method of introducing lipid-conjugated antigens into the surface of schistosomula. Am. J. Trop. Med. Hyg. (1983) 32(2), 343-349.

4. F. Levi-Schaffer, R. Tarrab-Hazdai, M.D. Schryer, R. Arnon and M. Smolarsky. Isolation and partial characterization of the tegumental outer membrane of schistosomula of S. mansoni. Mol. Biochem. Parasitol. (in press).

5. C.I. Newbold, M.D. Schryer, D.B. Boyle, J.S. McBride, A. McLean, R.J.M. Wilson and K.N. Brown. A possible molecular basis for strain specific immunity to malaria. Mol. Biochem. Parasitol. (1984) 11, 337-347.

6. M.D. Schryer, W. Jarra, C.I. Newbold and K.N. Brown. Specificity of immunity induced by purified 250 Kd schizont antigen and by non-replicating mature erythrocytic parasites. (In preparation).

Communications:

1. F. Levi-Schaffer, R. Tarrab-Hazdai, M.D. Schryer, R. Arnon and M. Smolarsky. Isolation of tegumental membranes of S. mansoni. Poster + abstract, 15th FEBS meeting, Brussels (1983).

2. M.D.Schryer, C.I. Newbold, W. Jarra and K.N. Brown. Strain specific immunity to malaria: vaccination studies with purified parasite antigens. Abstract of paper presented at British Society for Parasitology meeting, April 1984 (Parasitology, (1984) 89(2) xii).