PD-AAP-823

AGENCY FOR INTERNATIONAL DEVELOPMENT

PROJECT DATA SHEET

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

DOCUMENT CODE: 3

2. COUNTRY/ENTITY: S&T/Interregional

3. PROJECT NUMBER: 931-0453.05

4. BUREAU/OFFICE: S&T/Health — 10

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

6. PROJECT ASSISTANCE COMPLETION DATE (PACD): MM 06 DD 30 YY 87

7. ESTIMATED DATE OF OBLIGATION (Under 'B.' below, enter 1, 2, 3, or 4)

A. Initial FY 84 B. Quarter 3 C. Final FY 86

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

A. FUNDING SOURCE	FIRST FY 84 B. FX	C. L/C	D. Total	LIFE OF PROJECT E. FX	F. L/C	G. Total
AID Appropriated Total	620		620	1652		1652
(Grant)	(620)	( )	(620)	(1652)	( )	(1652)
(Loan)	( )	( )	( )	( )	( )	( )
Other U.S. 1.						
Other U.S. 2.						
Host Country						
Other Donor(s)						
TOTALS	620		620	1652		1652

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) S&T/H	540	542				1652		1652	
(2)									
(3)									
(4)									
TOTALS						1652		1652	

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 characterize and purify the protective antigens from the RBC stages of various species of human malaria.

14. SCHEDULED EVALUATIONS

Interim MM 02 YY 85 MM 02 YY 86 Final MM 02 YY 87

15. SOURCE/ORIGIN OF GOODS AND SERVICES

[X] 000 [ ] 941 [ ] Local [ ] Other (Specify)

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

17. APPROVED BY

Signature: George Curlin, M.D.

Title: Director, Office of Health

Date Signed: MM 03 DD 28 YY 84

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

MM DD YY

AID 1330-4 (8-79)

UNITED STATES INTERNATIONAL DEVELOPMENT COOPERATION AGENCY
AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

PROJECT AUTHORIZATION

Name of Entity: Interregional

Project Title: Malaria Immunity and Vaccination Research

Project No.: 931-0453.05

Grantee: University of Hawaii

1. Pursuant to Seciton 104 of the Foreign Assistance Act of 1961, as amended, I hereby authorize the research project entitled "Malaria Immunity and Vaccination Research" involving not to exceed $1,652,000 of S&T Bureau grant funds 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 on malaria antigens against the asexual blood stages of human malaria parasites, primarily *Plasmodium falciparum*.

Clearances:

A. ST/H: G. Curlin Date 3/28/84
B. ST/PO: G. Eaton Date 4/9/84

S&T/HP, J. Sarn, M.D.
Agency Director for
Health and Population

4-5-84
Date

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UNITED STATES INTERNATIONAL DEVELOPMENT COOPERATION AGENCY
AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

March 26, 1984

ACTION MEMORANDUM FOR THE AGENCY DIRECTOR
FOR HEALTH AND POPULATION

FROM: S&T/HP, George Curlin, M.D.

Action: Your approval is requested for a grant of $1,652,000 from Section 104 of the Foreign Assistance Act of 1961 as amended for project 931-0453.05, Malaria Immunity and Vaccination Research, the University of Hawaii.

Discussion: Attempts to find a vaccine against malaria began in the 1930's but were supplanted by new drugs developed during World War II and later followed by world-wide anti-malaria programs based upon adulticiding with DDT. Resistance to drugs and insecticides, lack of money and political instability, have reduced the prospects of malaria eradication and have stimulated renewed interest in a malaria vaccine.

This project, a continuation of research funded by AID for 6 years has made great advances in the large scale in-vitro cultivation of the RBC stages of human malarias, especially P. falciparum. Schizonts, grown in large capacity flasks have allowed the researchers to identify and characterize several P. falciparum antigens through the use of several mono-clonal antibodies. Research proposed, herein, will focus on feasibility studies on the use of recombinant DNA technology for the production of relevant antigens. The experimental design and proposed objectives are directly in line with the acceleration of the AID program recently approved by the Administator.

Justification to the Congress: Project Funding is included in the FY 1984 Congressional Presentation, Annex V, Centrally funded Programs, page 53.

Clearances Obtained: This project was approved by the AID external expert panel on the basis of scientific methodology, qualifications and experience of the proposed investigators, and the institutional capability in accordance to FPR 1-4.909.

Recommendations: 1. That you sign the attached authorization.
2. That you sign the attached justifications for non-competitive procurement.

Attachments:
1. Project Authorization
2. Non-competitive Justifications for non-competitive procurement
3. Project Paper

Clearance:
S&T/PO, G. Eaton Date 4/3/84

3

UNITED STATES INTERNATIONAL DEVELOPMENT COOPERATION AGENCY
AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

March 26, 1984

MEMORANDUM

TO: OM/COD/PE, Ms. Johni Pittnger

FROM: S&T/HP, James E. Sarn, M.D.

SUBJECT: Justification for non-competitive procurement of unsolicited proposal from the University of Hawaii entitled "Malaria Immunity and Vaccination"

The subject research proposal is an unsolicited proposal. The substance of the proposal is not available to the Government without restriction from another source, nor does it resemble any pending competitive solicitation. The substance is sufficiently unique to justify acceptance as an unsolicited proposal.

The project officer certification with reference to A.I.D. PR Notice 78-4 follows:

> I certify that neither I nor, to the best of my knowledge and belief, any other A.I.D. employee solicited the proposal or had any prior contact with the proposing institution, other than to convey interest in the field of malaria immunity and vaccination relative to the efforts described in the unsolicited proposal.

James M Erickson

Dr. James M. Erickson, Project Manager

I request that you award this contract on a non-competitive basis to the University of Hawaii without consideration of other sources.

Clearances:
S&T/PO, G. Eaton Date 4/3/84
S&T/HEA, G. Curlin Date 3/28/84

UNITED STATES INTERNATIONAL DEVELOPMENT COOPERATION AGENCY
AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

APR 5 1984

MEMORANDUM

TO: SER/CM, Johni Pittenger

FROM: S&T/HP, James E. Sarn, M.D.

SUBJECT: Justification for Non-Competitive Procurement under Project No. 931-0453.05, Malaria Immunity and Vaccination Research, the University of Hawaii.

I request that you negotiate only with the University of Hawaii for the Malaria Vaccine Research described in the PIO/T. The following justifies non-competitive procurement for this unsolicited proposal under FPR 1-4.910 (b) and (c).

1. Need Which This Procurement is Intended to Satisfy:

The latest estimates of the world wide incidence of malaria range, by WHO projection, is at between 300-400 million cases with between 2-4 million deaths due to malaria, primarily malaria caused by *Plasmodium falciparum*. The Agency's malaria vaccine program is accelerating its pace to develop, test, and market a safe, effective malaria vaccine against the several forms of human malaria. Although significant progress has been made against the mosquito stage (=sporozoite) and several prototypes are being tested, much work continues to be done to isolate "protective" antigens from the red blood cell phases (=RBC) of malaria parasites. The main reasons for the difficulties involved in research on RBC parasite forms lies in part with difficulties in "purity" involved with blood-based *in-vitro* cultures and, as well, the fact that during the RBC stages, the malaria parasites are intraerythrocytic and are therefore very difficult to attack with antibodies. This area, the RBS antigens, was specifically identified as an area for program acceleration in the Administrator's recent approval of a greatly expanded malaria vaccine

program.

2. The University of Hawaii: Qualifications to Meet the Need:

The unsolicited research proposal submitted by the University of Hawaii covers the research area to be addressed to expand our in-vitro cultivation capacity, isolate and characterize patented schizont antigens against P. falciparum, and initiate feasibility studies using MAB's to isolate the appropriate parasite DNA and initiate the use of E. coli to produce potential malaria antigens. The proposal has been reviewed and approved by A.I.D.'s external panel of expert consultants. The panel, which is composed of leading scientists in the field of malaria immunology, parasite biochemistry, vaccine development and primate biology has endorsed (in accordance with FPR 1-4.909) the scientific methodology, the exceptional experience and qualifications of the principal investigator, Dr. Siddiqui, and his staff, and the institutional capacity of the University of Hawaii to conduct the outlined scope of work. The panel concluded that the University of Hawaii is the only institute in the United States dealing with the plexi-box large scale production chambers for the in-vitro cultivation of the RBC stages of malaria parasites.

3. Prohibition Against Conducting a Market Search for this Procurement per FPR 1-4.911:

Any advertisement to find other sources to meet the objectives of this procurement would require disclosure of the design for the mass-scale in-vitro production equipment which was developed by the University of Hawaii. This plexi-box system is critical to the conduct of the reserach outline, is highly proprietary, and cannot be obtained by A.I.D. from any source other that the University of Hawaii. This exclusive availibility was confined by the expert review panel mentioned in paragraph 2 above.

Clearance:

S&T/H: G. Curlin GC Date 3/28/84
S&T/PO: K. Milow KM Date 4/2/84

6

University of Hawaii at Manoa

Department of Tropical Medicine and Medical Microbiology
John A. Burns School of Medicine • Leahi Hospital
3675 Kilauea Avenue • Honolulu, Hawaii 96816 / Cable Address: UNIHAW

December 20, 1983

Dr. James Erickson
Agency for International Development
Bureau for Science & Technology
Office of Health
Room 709C RPC SA-18
Washington, D.C. 20523

Dear Jim:

Enclosed you will find one signed and five signature copies of my research contract application together with a formal letter of submission from the Office of Research Administration, University of Hawaii.

Our research group has invested considerable time and effort in preparing this research proposal and have tried to give the progress report according to the outline presented during the AID site visit earlier this year. Intensive effort and careful thought was devoted in the preparation of research plans for the next three years. It matches with the overall objective of AID research plan.

In preparing the budget, I have taken into consideration all the discussions we had at San Antonio pertaining to this subject. The proposed budget, if approved, will avoid the need for any supplementary budget in the future.

Let me take this opportunity to extend my warm best wishes to you and Dorothy for the holiday season and a happy New Year.

Aloha and Mahalo.

Wasim A. Siddiqui, Ph.D.
Professor
Acting Chairman

ENCLS.

AN EQUAL OPPORTUNITY EMPLOYER

University of Hawaii at Manoa

Office of Research Administration
Spalding Hall 557 • 2540 Maile Way
Honolulu, Hawaii 96822 • Cable Address: UNIHAW

December 20, 1983

Dr. James Erickson
Agency for International Development
Office of Health
Room 720, Rosslyn Plaza Building C
1601 North Kent Street
Rosslyn, Virginia 22209

SUBJECT: Continuation of Contract No. DPE-0453-C-00-1026

Dear Mr. Erickson:

Enclosed is an application for a research proposal entitled, "Malaria Immunity and Vaccination", under the direction of Dr. Wasim A. Siddiqui, Department of Tropical Medicine and Medical Microbiology, principal investigator.

Enclosed are seven copies, one of which carries the appropriate administrative signature. Thank you for your consideration of this proposal.

Sincerely yours,

M. Ghali

Moheb A. Ghali
Associate Dean
Research and Training

jn

Enclosures

cc: Dr. Wasim A. Siddiqui
School of Medicine Fiscal Officer
Contracts and Grants Office

AN EQUAL OPPORTUNITY EMPLOYER

8

RESEARCH CONTRACT APPLICATION

December 15, 1983

1. Title of Research Project: Malaria Immunity and Vaccination

1. Production, identification, characterization and purification of asexual blood stage *Plasmodium falciparum* protective antigen(s).

2. Vaccination studies with purified *P. falciparum* antigens in *Aotus* monkeys.

2. Sponsoring Agency: Agency for International Development, U.S. State Department, Washington, D.C. 20523.

3. Current Contract No.: DPE-0453-C-00-1026-00

4. Starting Date & Duration of the Proposed Contract: July 1, 1984 - June 30, 1987.

5. A. Principal Investigator:

Wasim A. Siddiqui, Ph.D
Professor, Dept. Tropical Medicine & Medical Microbiology,
John A. Burns School of Medicine,
University of Hawaii,
Leahi Hospital, 3675 Kilauea Ave,
Honolulu, Hawaii 96816

Social Security No.: 109-38-2139
Telephone No.: (808) 948-6418
(808) 734-0221

B. Organisation submitting Research Contract Applicaction:

University of Hawaii
Office of Research Administration
2540 Maile Way, Honolulu, HI 96822

C. Person to be Contacted on Contractual Matters:

Dr. Moheb A. Ghali
Associate Dean, Research & Training
Office of Research Administration
University of Hawaii
2540 Maile Way, Honolulu, HI 96822

Telephone: (808) 948-8658

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SIGNATURES:

Name: (Wasim A. Siddiqui, Ph.D.)

Title: Principal Investigator

Name: (Moheb A. Ghali, Ph.D.)

Title: Associate Dean
Office of Research Administration

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C. Curriculum Vitae

6. Progress report (F/Y 1981, '82, '83)

6.1 List of published papers:

1. Siddiqui, W.A. 1980. Vaccination against malaria. Is it for tomorrow? Afr. J. Clin. Exp. Immunol. 1(1)1980): 13-22.

2. Siddiqui, W.A. 1980 Immunization against asexual blood-inhabiting stages of plasmodia. Malaria, Vol. 3, Academic Press, Inc.

3. Taylor, D.W. and W.A. Siddiqui. 1980. Differentiating owl monkey T and B lymphocytes by rosette formation. J. Med. Primatol. 9:88-94.

4. Taylor, D.W., S. Richmond-Crum, K.J. Kramer and W.A. Siddiqui. 1980. Alterations in the distribution and proliferation responses of rhesus monkey peripheral blood and spleen cells during malaria (Plasmodium knowlesi) infection. Infec. Immun. 28(2):502-507.

5. Siddiqui, W.A., S.C. Kan, K. Kramer, S. Case, K. Palmer & J.F. Niblack. 1981. Use of a synthetic adjuvant in an effective vaccination of monkeys against malaria. Nature 289(5793):64-66.

6. Siddiqui, W.A. and K.L. Palmer. 1981. Propagation of malaria parasites in-vitro. In: Advances in Cell Culture, Vol.1, Academic Press.

7. Siddiqui, W.A. 1981. Continuous in-vitro cultivation of Plasmodium falciparum: replacement of human serum by calf serum and proteose peptone. Indian J. Med. Res. 73(Suppl), 19-22.

8. Peltola, H.O., S.E. Case, S.F. Perri and W.A. Siddiqui. 1982. Plasmodium falciparum merozoite vaccination in Aotus monkeys recovered spontaneously from P. falciparum infection: A Clinical Study. Scand. J. Infect. Dis. 14:217-224.

9. Kramer, K.J., S.C. Kan, S.W. Siddiqui. 1982. Concentration of Plasmodium falciparum-infected erythrocytes by density gradient centrifugation in Percoll. J. Parasitol. 68(2):336-337.

10. Palmer, K.L., G. Hui, W.A. Siddiqui. 1982. A large-scale in-vitro production system for Plasmodium falciparum. J. Parasitol. 68(6):1180-1183.

11. Taylor, D.W., W.A. Siddiqui. 1982. Recent advances in malarial immunity. Ann. Rev. Med. 33:69-96.

12. Peltola, H.O., S.E. Case, S.F. Perri and W.A. Siddiqui. 1983. Reactogenicity of a malaria merozoite antigen in Aotus monkeys compared with the effects caused by two new adjuvants, CP-20,961 and [D30]-MDP. Scand. J. Infecc. Dis. (In press).

13. Hui, G.S.N., K.L. Palmer and W.A. Siddiqui. 1983. Synchronization of Plasmodium falciparum in continuous in-vitro culture: use of Colchicine. Am. J. Trop. Med. Hyg. 32(6):1451-1453.

14. Kan, S.C., K.M. Yamaga, K.J. Kramer, S.E. Case and W.A. Siddiqui. 1984. Plasmodium falciparum: Protein antigens identified by sera from vaccinated Aotus monkeys. Infec. Immun. 43:539-545.

Reviews & Chapters in Books

1. Siddiqui, W.A. "Immunization against Asexual Blood Inhabi- ting Stages of Plasmodia": 'Research in Malaria', Vol. 3. - Kreier, J.P. Pub. Academic Press, New York. 1980.

2. Siddiqui, W.A., Palmer, K. "Propagation of Malaria Para- sites in-vitro" : 'Advance in Cell Culture'. - Maramorosch, K. Pub. Academic Press, New York. 1980.

3. Taylor, D.W., Siddiqui, W.A. "Recent Advances in Malarial Immunity": 'Annual Review of Medicine: Selected Topics in the Clinical Sciences', Vol. 33 - Creger, W.P., Coggins, C.H., Hancock, E.W., Pub. Annual Reviews Inc. California, 1982.

4. Siddiqui, W.A. "The Use of Immunoadjuvant in Vaccination Studies against Human Malaria." Int. J. Immunopharmacol 1982: 4(4):309.

Abstracts:

1. Siddiqui, W.A. 1980. Vaccination of experimental monkeys against Plasmodium falciparum: A critical review. 10th International Congress on Trop. Med. & Malaria.

2. Siddiqu, W.A. 1980. A strain of P. falciparum cultivation and drug susceptibility studies. 10th Int. Congress on Trop. Med. & Malaria.

3. Siddiqui, W.A. 1980. Recent work on immunology and malaria parasites. 2nd Congress Fed. Asian & Oceanian Biochemists, Bangalore, India.

4. Case, S.E., T. Ono, K.M. Yamaga, S.C. Kan, K.J. Kramer, L. Tam, W.A. Siddiqui. Characterization of P. falciparum schizont antigens using monoclonal antibodies. Am. Soc. Trop. Med. Hyg. 1982.

5. Palmer, K., G. Hui, W.A. Siddiqui, E. Palmer. A large scale semi-automated culture system for production of Plasmodium falciparum antigen. 2nd Int. Conf. on Malaria & Babesiosis, Annecy, France. p.19, 1983.

6. Kan, S.C., K.M. Yamaga, K.J. Kramer, S.E. Case, W.A. Siddiqui. Development of Plasmodium falciparum asexual blood stage vaccine. Identification of antigens using immune sera from vaccinated monkeys and monoclonal antibodies. 2nd Int. Conf. on Malaria & Babesiosis, Annecy, France, p.103, 1983.

7. Siddiqui, W.A., S.C. Kan, K.M. Yamaga, K.J. Kramer, L.Q. Tam, K.L. Palmer, S.E. Case. Development of Plasmodium falciparum asexual blood stage vaccine: Studies on the immunogenicity of purified antigens. 2nd Int. Conf. on Malaria & Babesiosis, Annecy, France, p.123, 1983.

8. Yamaga, K.M., S.C. Kan, K.J. Kramer, G. Hui, W.A. Siddiqui. Development of Plasmodium falciparum asexual blood stage vaccine: Characterization of antigens recognized by malaria antibody. 2nd Int. Conf. on Malaria & Babesiosis, Annecy, France, p.154, 1983.

6.2 Progress report:

6.2.1 Large-scale in vitro production of schizont stage Plasmodium falciparum antigen:

Our laboratory has been engaged in the *in vitro* cultivation of *P. falciparum* for the past 7 years utilizing a static flask culture system. This unique qualities of the system make it adaptable so that we are able to grow parasites just as readily in 1 ml tissue culture wells as in large-scale, 500 ml, culture vessels. The method is similar to the basic Trager-Jensen system except that we use flasks rather than petri dishes, and we pass a special gas mixture (90% N_2, 2% O_2, and 8% CO_2) constantly through each flask. Our cultures contain a 5% concentration of erythrocytes, with RPMI medium supplemented with 10% human serum. The cultures are initiated with parasitemias of 1 to 2% and we obtain peak parasitemias of 18 to 20% after 6 days. Fresh erythrocytes are added weekly and the medium is changed daily.

Our stock strains of *P. falciparum* are maintained as 10 ml cultures in standard 125 ml Erlenmyer flasks. The flasks are fitted with a hard rubber stopper which has two cotton plugged gas ports. The flasks are connected together using latex tubing and the special gas mixture constantly flows through the system at the rate of approximately 10 ml per minute.

Parasites grown in the system maintain good morphology even at parasitemias above 20%. The system is more fully described by Siddiqui & Palmer (Advances in Cell Culture v.1, K. Maramorosch (ed.) pp. 183-212, 1981).

6.2.1.1 In vitro cultivation of isolates of P. falciparum from different geographic regions of the world and their growth characteristics:

We currently have a collection consisting of 15 strains of P. falciparum including isolates from Asia, Africa, and Latin America. A majority of the strains were isolated in our laboratory primarily from travelers or military personnel; the others have come from laboratories in India and from CDC. Strains in our collection are listed in Appendix I.

Our two principle strains are FUP (Falciparum-Uganda-Palo Alto) and FVO (Falciparum-Vietnam-Oaknoll) which have been in culture for 8 and 6 years respectively. Before being maintained in culture, both strains were serially passaged in Aotus monkeys. We still maintain cryopreserved stocks of Aotus passaged stabilites of both strains. We periodically retrieve these parasites from liquid nitrogen storage and passage them in Aotus monkeys. In this way we maintain stocks of both the so called K^+ and K^- lines of FUP and FVO.

Recently, we added the CDC Honduras strain to our list of stock cultures because of the AID network's interest in this strain. Other strains are kept in liquid nitrogen and retrieved for specific experiments. Most have been characterized as to their drug sensitivities and in vitro growth patterns.

6.2.1.2 Gradual increase in culture size from 10 ml to 300 ml:

Table 1-1 shows the various size cultures that are used in our system. Starting with the standard 125 ml flask we have scaled up into 200 ml flasks, and 2.8 l Fernbach flasks. The 200 ml flask holds 20 ml and the 2.8 l flask 100 ml of culture. To further expand, we designed a dome type culture vessel which has a culture volume of 300 ml. as

diagrammed in Figure 1-1. These are constructed from 10 liter boiling flasks which are cut in half. The bottom edges are ground flat and the domes mounted on glass plates. The domes are attached to the plates with a silicone sealant which is inert and withstands repeated autoclaving.

Table 1-1 OUTLINE OF THE IN VITRO SYSTEM

Culture Vessel	Volume of Culture (ml)	Volume of Erythrocytes (ml)*	RPMI Medium (ml)	Serum (ml)
Microtiter Plates				
96 wells	0.2	0.02	0.16	0.02
24 wells	1.0	0.10	0.8	0.10
Standard Flasks				
25 ml	2.5	0.125	2.0	0.125
125 ml	10.0	1.0	8.0	1.0
500 ml	20.0	2.0	16.0	10.0
2.8 l	100.0	10.0	80.0	10.0
Large-Scale Vessels				
12 l dome	300.0	30.0	240.0	30.0
Plexi-box	500.0	50.0	400.0	50.0

*Washed erythrocytes that have been reconstituted 1:1 with RPMI Medium.

A unique feature of these domes are the two serum capped access ports (shown in Figure 1-1). They allow a technician using long needles to draw off spent medium and add fresh medium and serum without removing the dome from an incubator. This feature is especially important where contamination is a problem.

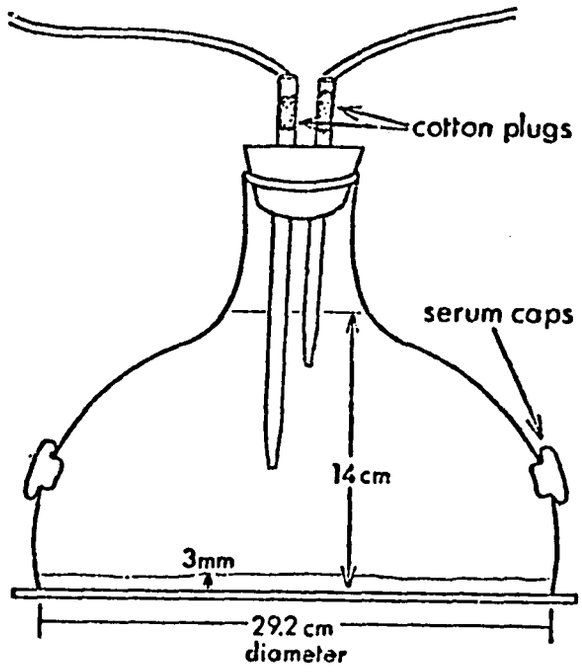


Figure 1-1 Diagram of dome culture vessel

6.2.1.3 Development of large-scale plexiglas culture vessels:

The dome type culture vessel represented one step toward the eventual development of even larger culture vessels. When it came time to design a larger size vessel we made the decision to fabricate it from plastic rather than glass because of the difficulty in obtaining larger glass containers and because plastic was easy to fabricate and therefore made possible numerous innovations in design and function which we did not feel were possible with glass.

The attached publication (Palmer et al., J. Parasitol. 68(6):1180-1183, 1982) describes our first version of the large-scale plexiglas culture vessel. The vessel is shown in Figure 1-2. Briefly, the vessel was designed to function as part of the static flask system. It was equipped with eight gas ports to provide an even flow of gas

through the vessel. Access ports located on the front of the vessel were used to add erythrocytes and medium and to take routine samples.

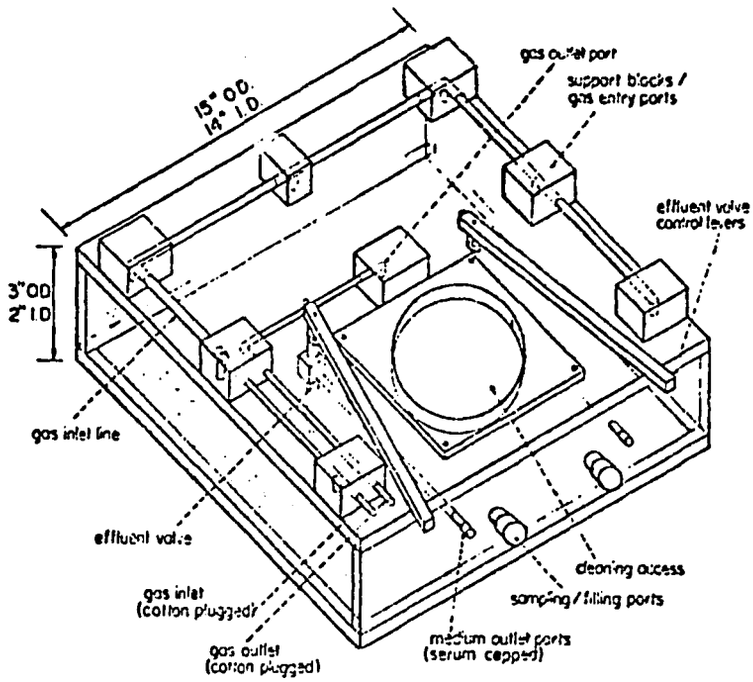


Figure 1-2 Design of the original large-scale plexiglas culture vessel

The key features of this culture vessel were the two effluent valves. A single valve is shown in Figure 1-3. The valve was specially designed to withdraw spent medium from the culture vessel without taking up any of the settled layer of erythrocytes. Each valve consisted of two sections labeled A and B. The principle of operation was that A rotated inside B. A had a vertical slit which matched with a spiral slit in unit B. Suction was applied through the center of A so that liquid is sucked through the vertical slit at the point where it matched with the spiral slit of element B. By rotating A, the point of suction could be moved up and down along the spiral slit. The idea was to suck off medium from the upper level of the medium layer thereby minimizing the disruption of the lower levels of medium and the layer of erythrocytes. As the medium was drawn off element A was rotated and the point of suction moved

down with the surface of the medium which thereby allowed us to withdraw the maximum amount of spent medium without taking up any erythrocytes.

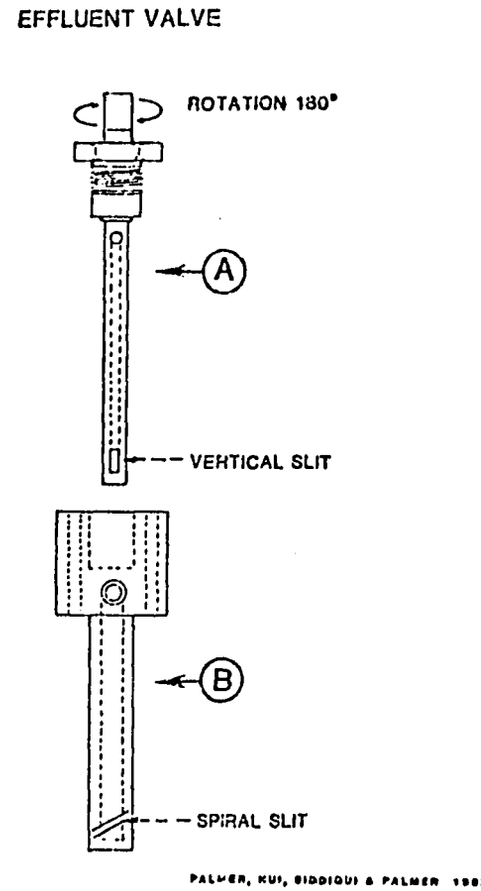


Figure 1-3 Diagram of the surface tracking effluent valve

Medium was drawn off by inserting long needles attached to a suction apparatus through the serum capped ports located on the front of each vessel. The valves were rotated by hand.

One major drawback of our original design was that it could not be autoclaved. The plexiglas used to fabricate the vessel had a low melting temperature so that the vessels could only be sterilized with ethylene oxide or gamma radiation. After repeated ethylene oxide sterilization a film apparently built up on the plastic which was toxic to the parasites. Repeated gamma radiation treatments caused a break down in the

molecular structure of the plexiglas so that after repeated treatments the vessels began to leak and fall apart.

Subsequent designs included numerous minor changes but the major development was the switch from plexiglas to polysulfone plastic. This change allowed us to sterilize the vessels in a standard steam autoclave. Using this redesigned large scale vessel we were able to harvest 10^{10} parasites per vessel per week. These results demonstrated that parasites could be grown in the large-scale vessels and that peak parasitemias were equivalent to those obtained in our standard 10 ml cultures.

6.2.1.4 Development of a semi-automated large-scale culture system:

Development of a micro-computer control system was the next step in the development of what we envisioned to eventually be a completely automated large-scale culture system. Using funds from the University of Hawaii Foundation we worked with a local electronics firm to design a control unit which would allow us to assemble a system consisting of 12 of our large-scale plastic culture vessels.

The control unit was designed to take over the tedious tasks of changing medium in the 12 large-scale culture vessels. In doing so, the unit had to have the capability to withdraw spent medium from each vessel on the system, and then replace it with fresh medium. The two critical aspects of these tasks were first to remove a maximum amount of spent medium without taking up erythrocytes using the effluent valve, and second the unit had to deliver the correct volume of fresh medium to each vessel. Addition of too much or too little medium would be detrimental to the parasites. While medium was being replaced the erythrocyte layer had to be resuspended so that fresh medium was in contact with a maximum number of erythrocytes.

Integration of the original large-scale culture vessels into the automated system required a number of modifications. The modified design is shown in Figures 1-4 and 1-5. Each vessel had a single effluent valve which was linked by gears to a stepper motor assembly. A sight glass and sight glass scanner were added to the front of each vessel. The number of access ports on the front were reduced to one with single gas inlet and outlet ports. Instead of cotton plugs, gas ports were fitted with sterile gas filters.

In addition to the control unit and vessels there was a third component consisting of the various pumps, valves, and a flow meter which handle the movement of medium in and out of the vessels.

The control unit operated the system on a preprogrammed medium replenishment cycle. Specific parameters of the cycle were set by the basic program but others were determined by the operator. The operator set parameters included: (1) interval between change cycles; (2) speed at which the effluent valve was rotated; and (3) number of steps that the valve rotated. The ability to control the effluent valve allowed the operator to set the volume of medium withdrawn during each cycle.

To understand how an individual vessel operated as part of the system it is necessary to follow a vessel through a complete change cycle. At the beginning of a cycle the control unit simultaneously activated the drainage pump and the stepper motors. The speed at which the motor turned the effluent valve was coordinated with the speed of the drainage pump. The speed of the motor and the arc through which the motor turned the valve was determined by the system's operator.

When the motor had turned the set number of steps, the motor and drainage pumps were turned off and the system entered a five minute

waiting period so that the system operator could sample individual culture vessels, add erythrocytes or carry out any other maintenance function.

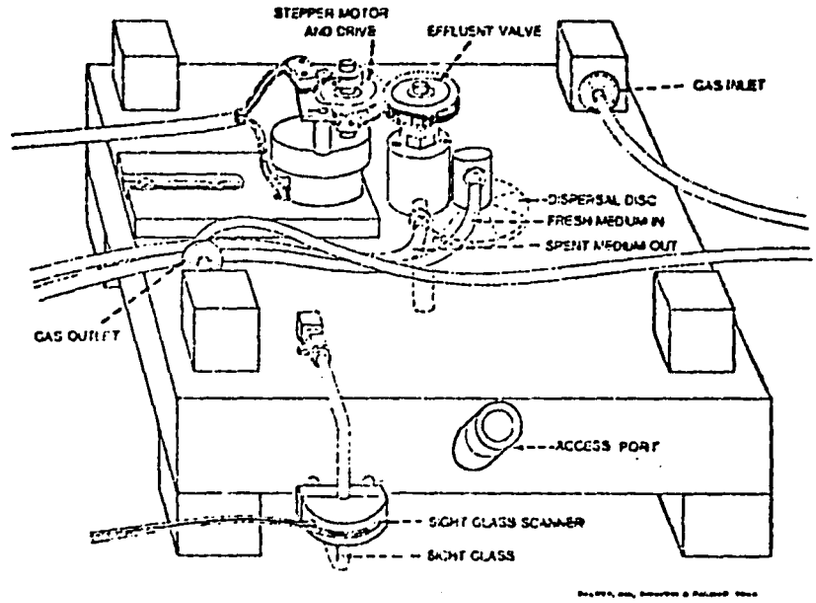


Figure 1-4 Full view of modified lare-scale culture vessel showing modifications that allow the vessel to function as part of the semi-automated system.

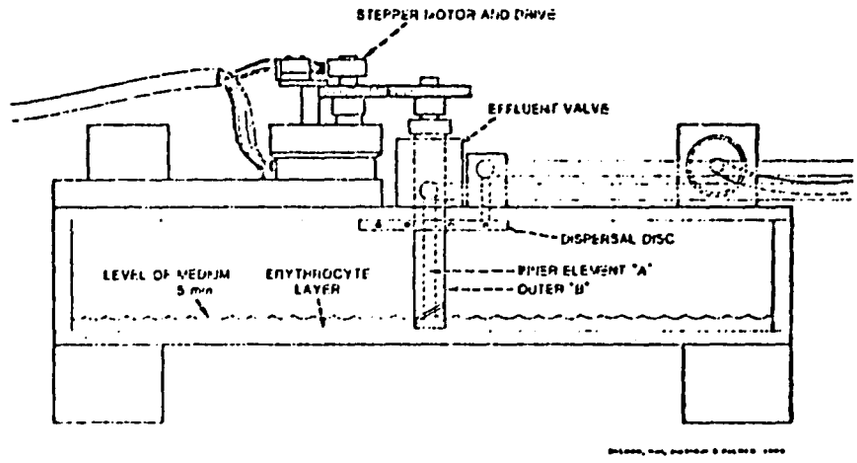


Figure 1-5 Cut-away view of the modified large-scale culture vessel showing the surface tracking effluent valve, stepper motor and drive, and dispersal disc.

Once the waiting period was over the control unit was able to fill each vessel starting with vessel one. The input pump was switched on and the solenoid valve to vessel one was opened.

As medium was pumped into each vessel it passed through a dispersal disc (shown in Figure 1-5). This broke the flow of medium into 8 separate streams and created a mixing action which resuspended the erythrocyte layer. This ensured that fresh medium was in contact with a majority of cells in the culture.

The pump continued to deliver medium to vessel one until either 480 ml were pumped in or the level in the vessel reached a predetermined point. The volume of medium was measured by a flow meter located in the delivery line. The 480 ml figure represented a fail-safe mechanism in case the sight glass system did not function.

The sight glass and scanner was a simple system. As the level of medium changed in the vessel it also changed in the sight glass. When each vesssel was filled with 500 ml of culture the scanner was set so that it "saw" the miniscus of the liquid in the tube. As medium was drawn out, the level of liquid in the sight glass tube dropped below the level of the scanner. On the fill cycle the liquid level slowly rose until it broke the light beam in the scanner. The scanner then signaled the control unit to stop filling vessel one and go on to vessel two, etc. Once the last vessel was filled the control unit printed out the volume of medium delivered to each vessel. By checking the volume delivered, the operator could determine if the system was operating within acceptable limits.

Once the control unit became operational it was used together with two culture vessels, to determine whether the entire system was functioning as intended. Growth curves for our three stock strains (FUP, FVO, Honduras) grown in the semi-automated system are shown in Figures 1-6 and 1-7. Figure 1-6 demonstrates that growth of the FUP strain is the

same in our standard 125 ml flasks as it is in the large-scale vessels. Figure 1-7 shows similar patterns of growth for the Honduras and FVO strains in the large-scale system. Parasite yields per culture vessel from six experiments are shown in Table 1-2. These results together with a description of the system were presented. (Palmer et al., 2nd International Congress on Malaria and Babesiosis in Annecy, France, p.19, 1983).

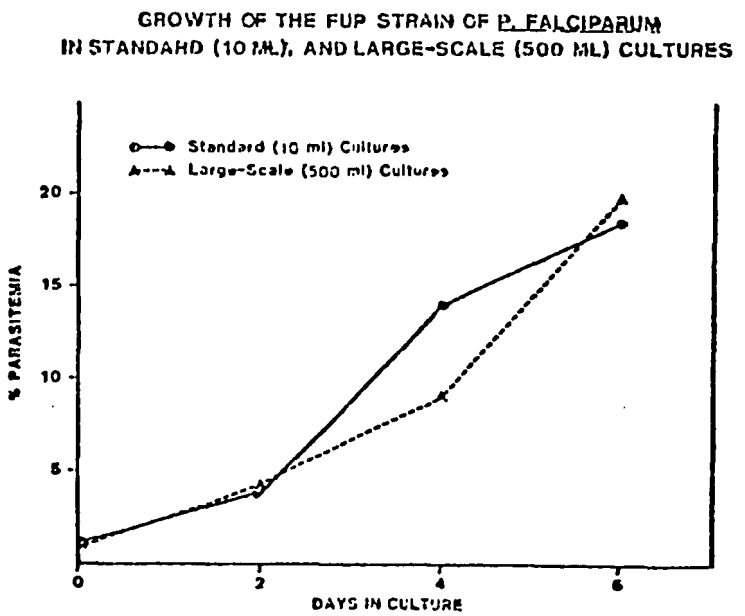


Figure 1-6 Growth curves for the FUP strain of P. falciparum grown in either standard 125 ml culture flasks or in large-scale vessels. In both cases cultures contained RPMI medium supplemented with 10% human serum.

Table 1-2 Large Scale Culture System Parasite Yields and Medium Consumed

Experiment Number	Parasitemia Starting (%)	Parasitemia Harvest (%)	Total Parasites (X 10^{10})	Medium Consumed* (liters)
1	1.2	22.2	2.7	2.1
2	1.2	21.2	3.0	2.3
3	1.2	18.4	1.9	2.5
4	1.6	19.0	2.1	2.7
5	1.8	18.8	2.9	2.3
6	1.2	19.7	2.1	2.1

* Contains 10% plasma.

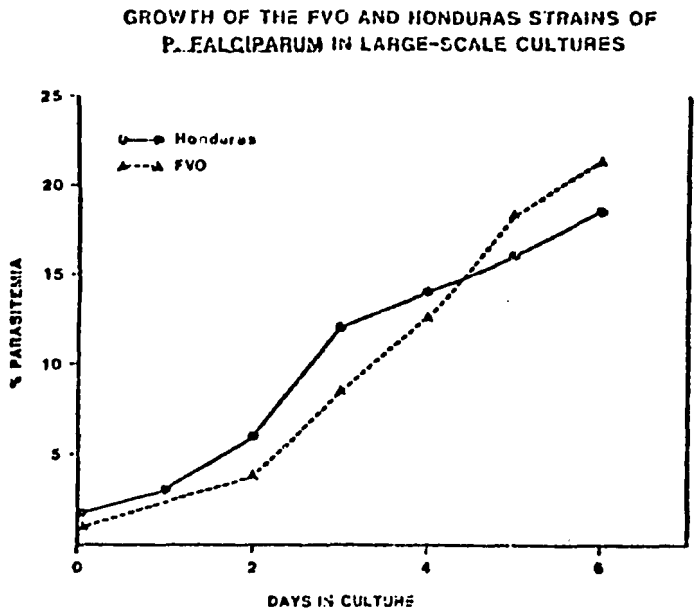


Figure 1-7 Growth curves for the Honduras and FVO strains of P. falciparum grown in large-scale culture vessels. Cultures contained RPMI medium supplemented with 10% human serum.

At the present time the system is still in an evaluation phase. Two vessels are in operation but it is hoped that the system will soon be expanded to include 12 large-scale vessels.

6.2.1.5 Use of colchicine to synchronize cultures:

Since our goal was to expand our ability to produce schizont-stage antigen, we sought to increase our harvest of schizonts by incorporating a method for synchronizing the cultures in our large-scale system. We were not satisified with the results obtained in our smaller cultures using the sorbital method because it produced good synchrony but at the expense of high parasite yields.

Using work done on P. knowlesi as a clue, (McColm & Trigg, Ann. Trop. Med. Parasitol. 74:479 483, 1980) we began to experiment with colchicine as a possible method of synchronization. In P. knowlesi cultures,

treatment with colchicine suspended parasites in the trophozoite stage of development. This led us to investigate whether we could use colchicine to stop development of P. falciparum at the trophozoite stage and then remove it and allow the parasites to continue growing synchronously. By merely stopping the development of the parasites in a specific stage we saw the possiblity of overcoming the major problem inherent in the sorbital method. Sorbitol works by eliminating all parasites except young rings. In doing so a majority of parasites in the culture were lost. By starting with fewer parasites the cultures treated with sorbitol reach peak parasitemias of only about 10% after six days. Colchicine on the other hand does not destroy any parasites thereby allowing the treated cultures to reach high parasitemias of 16 to 18%.

We experimented with various concentrations of colchicine together with numerous treatment times and methods of washing out colchicine from our cultures. A concentration of 4 mM in contact with the cultures for 24 hours followed by two changes of medium at 5 hours intervals was found to give us optimal results. Figures 1-8 and 1-9 show the results obtained using this colchicine methodology. Figure 1-8 clearly shows that we get good synchrony which lasts for up to 2 cycles. The growth curves shown in Figure 1-9 show that cultures treated with colchicine reach peak parasitemias that are as high or higher than peaks seen in unsynchronized cultures of the same strain. A detailed description of the methodology is presented in the attached manuscript (Hui et al., Am. J. Trop. Med. Hyg., 32(6):1451-1453, 1983).

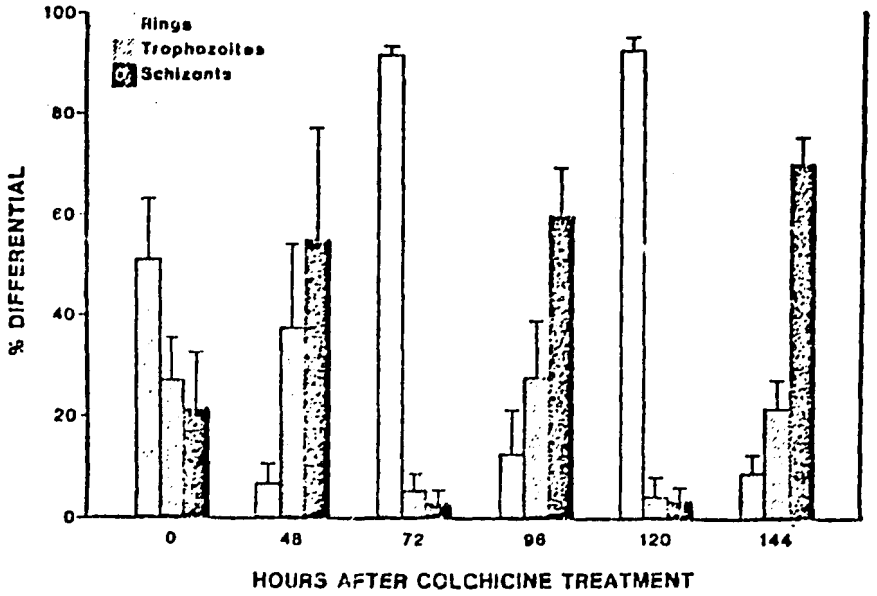


Figure 1-8 Distribution of three stages of P. falciparum (rings, trophozoites, and schizonts) in cultures treated with 4 mM colchicine (n=9).

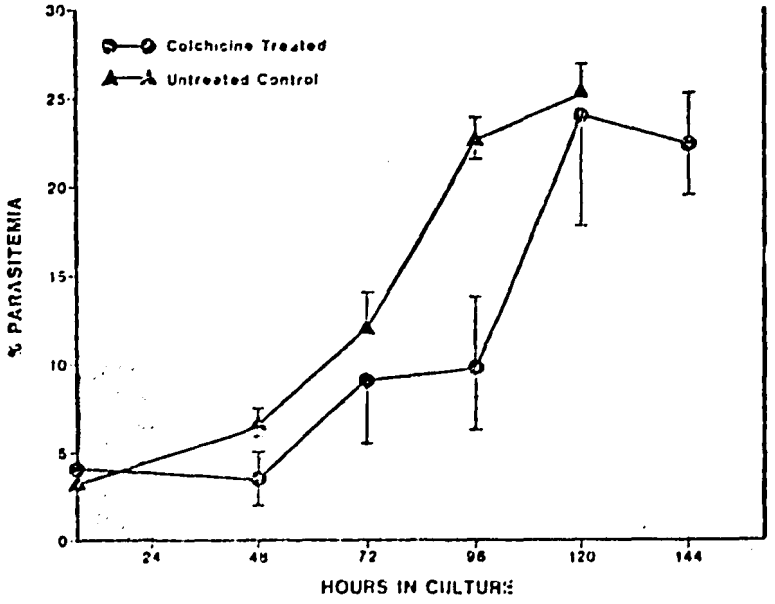


Figure 1-9 Growth curves for P. falciparum cultures treated with 4 mM colchicine (n=9) and normal untreated controls (n=4). Shaded area denotes time when cultures were exposed to colchicine.

6.2.2 Identification of *Plasmodium falciparum* antigens by sera from vaccinated *Aotus* monkeys:

The availability of sera from experimentally vaccinated *Aotus* monkeys has given us a unique opportunity to identify the possible protective antigens in *P. falciparum* malaria (Kan *et al.*, Inf. & Immun. 43: 539, 1984). Because of the consistent and permanent immunity observed in protected *Aotus* monkeys, sera from such monkeys are likely to contain antibodies against the relevant protective antigens. Moreover, the *Aotus* sera used are well-defined, since pre-immune, primary, secondary, and post-challenge sera from protected and unprotected monkeys are available, under conditions in which the amount and timing of antigenic stimulation are known. Metabolically labeled *P. falciparum* proteins were immunoprecipitated with sera from unprotected (A300, A299, and A305) and protected (A297, A320, A310, A321, and A301) *Aotus* monkeys. In Figure 2-1, lane A contains proteins immunoprecipitated with pre-immune sera, B with primary sera, C with secondary sera, and D with post-challenge sera. Lane S shows molecular weight standards. Lane L is the total parasite lysate and DF is the dye front. A total of 21 protein bands, having molecular weights from 180,000 to 30,000 and designated *a* to *u* were recognized. The major protein bands identified were *a* (Mr = 180,000), *d* (Mr = 132,000), *f* (Mr = 107,000), and *l* (Mr = 75,000).

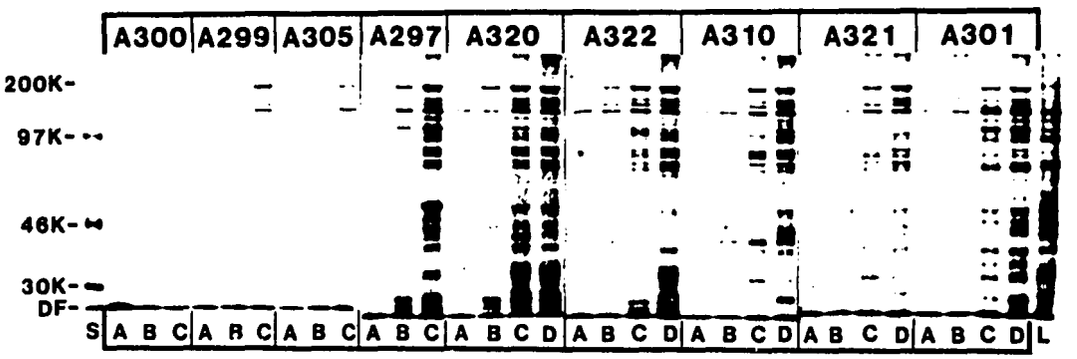


Figure 2-1

Representative lanes of fluorograms were scanned and the densitometer tracings are shown in Figure 2-2.

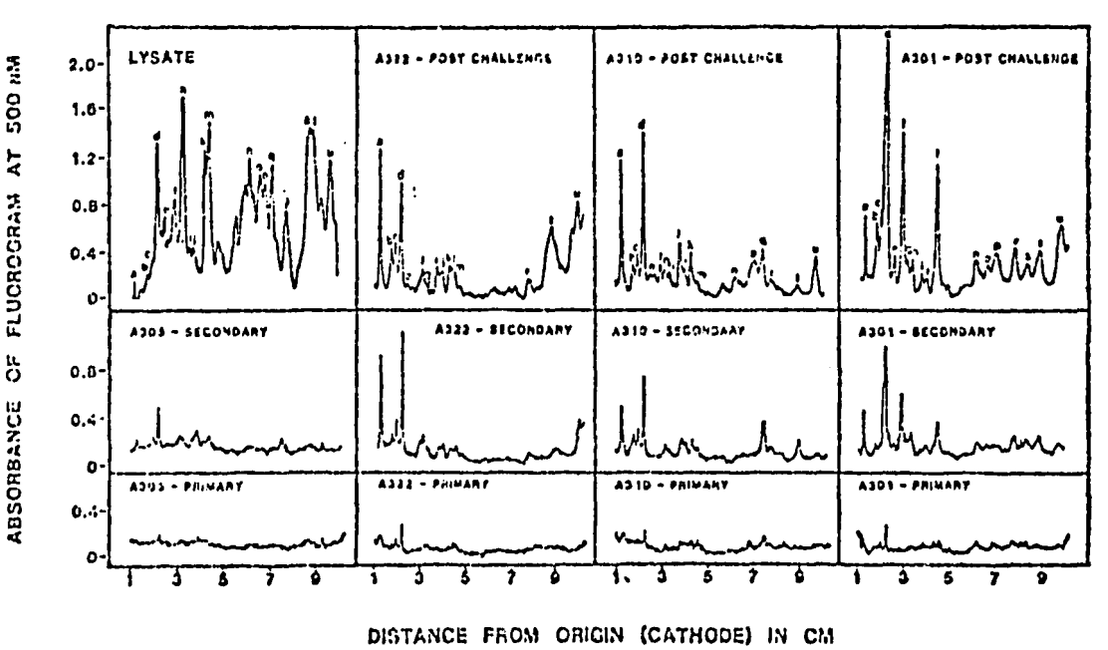


Figure 2-2

Each band visualized on SDS-PAGE may consist of more than one protein species. Thus we have analyzed labeled lysates and immunoprecipitates by

two-dimensional gel electrophoresis as shown in Figure 2-3. Our two dimensional patterns have not been consistent, and we are in the process of improving our techniques.

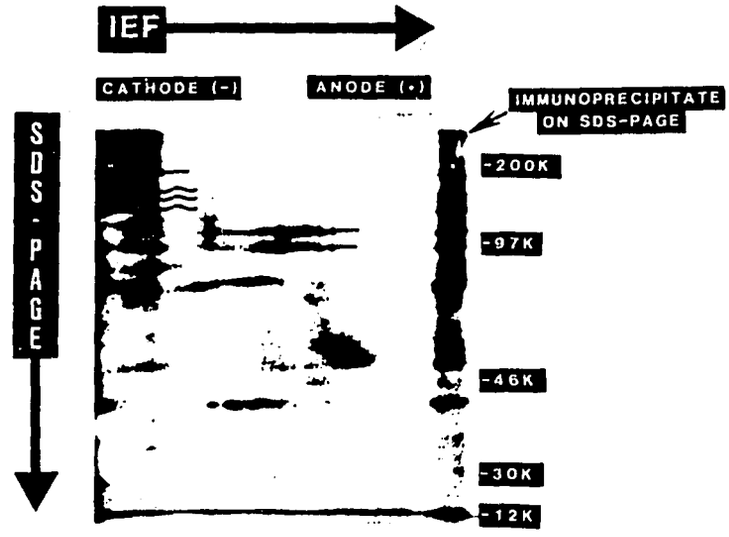


Figure 2-3

The pitfalls in using human sera for the identification of relevant P. falciparum protective antigens are plentiful. Often the clinical history of a patient is obscure; thus, documentation of the number of clinical infections and the infecting strain is unavailable to the investigator. Drug treatment may alter the immune response. Moreover, the immunity in humans is variable and transient. In general, we have noted diverse gel patterns when sera from endemic areas of Thailand or India were used to immunoprecipitate P. falciparum antigens. In addition, Aotus sera identify more antigens than human sera as shown in Figure 2-4. Lane A shows the antigens immunoprecipitated by a representative hyperimmune Aotus sera; lane N, normal human serum; lane A, lysate, lanes I1 - I3, sera from India; T11 - T13, sera from Thailand. For the latter, serum samples wre obtained from 0 to 4 weeks during drug treatment.

Figure 2-4

A clinically useful vaccine is one that protects against P. falciparum in different parts of the world. The protective antigens should be found among diverse isolates. Using Aotus antisera, we have analyzed a limited number of isolates. Our preliminary results, shown in Figure 2-5, indicate that the major antigens, are found among the isolates analyzed. Lanes A, B, C, D, and E refer to precipitates of metabolically labeled Honduras, FUP (Africa), FCH-1 (Philippines), FCH-13 (India), and FVO (Vietnam), respectively. The antigens were immunoprecipitated by sera from Aotus 301, 310 and 320.

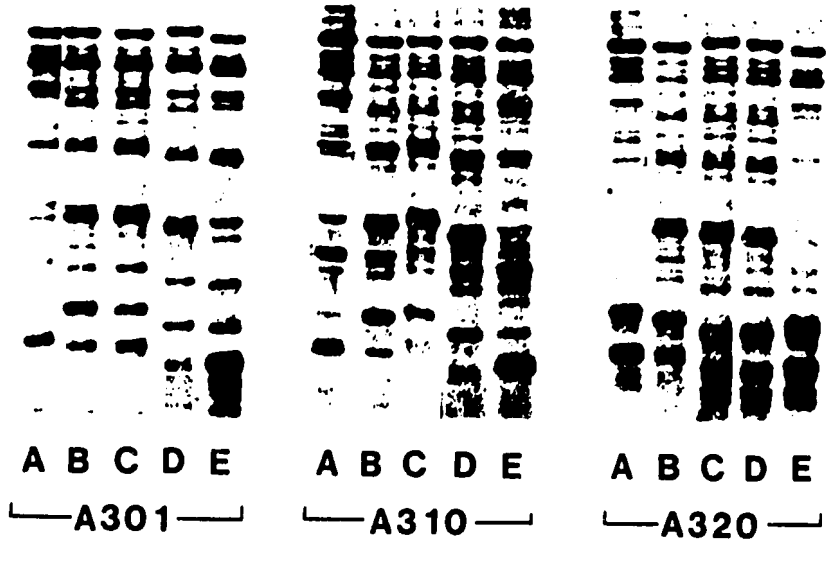


Figure 2-5

Possible antigenic differences between long term *in vitro* and *in vivo* derived parasites must be assessed. We have intiated such studies by comparing the antigens from synchronized *in vitro* and *in vivo* derived parasites. Long-term, *in vitro* derived *P. falciparum* cultures were synchronized with colchicine using a method recently developed in our laboratory (Hui *et al.*, Am. J. Trop. Med. Hyg. *32*: 1451, 1983) and metabolically labeled with ^{3}H-isoleucine. Cultures obtained at three different stages were solubilized, and metabolically labeled antigens were immunoprecipitated with pre-immune (lane P) or immune (lane I) *Aotus* sera in Figure 2-6. Lane L shows the lysate. Antigens having Mr greater than 95,000 were found only in schizonts; whereas, antigens having Mr less than 95,000 were found in all stages.

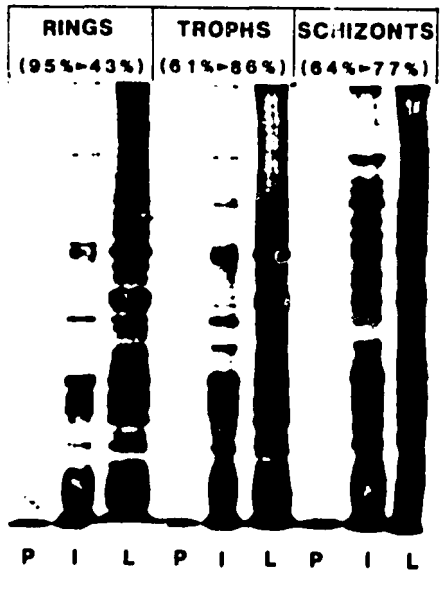


Figure 2-6

Similar high Mr antigens were found in schizont stage *P. falciparum* parasites derived from an ongoing infections of *Aotus* monkeys as shown in Figure 2-7.

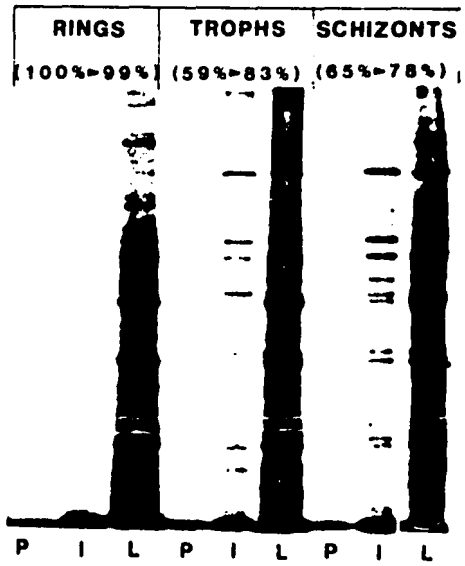


Figure 2-7

A direct comparison of the long term in vitro and short term in vivo derived schizont stage antigens was made. In the long term in vitro derived cultures (left) antigen bands b, c, i, m and t were more prominent than in the in vivo derived culture (right) (Figure 2-8). Additional experiments are required to explain these differences.

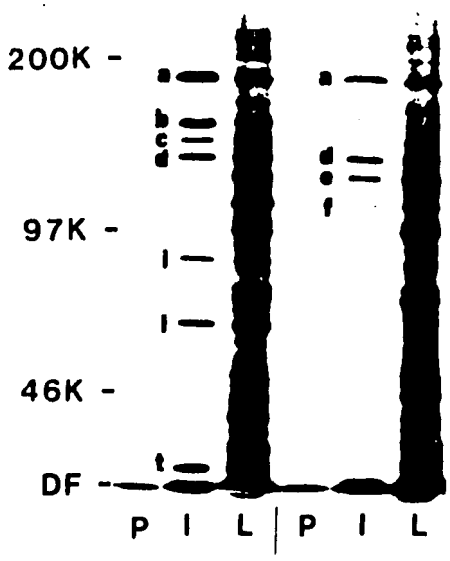


Figure 2-8

6.2.3 Characterization of P. falciparum antigens:

It is likely that relevant protective antigens are associated with membrane material (either erythrocytic or parasitic), since antibodies or immune cells must react with accessible determinants. To determine which antigens are membrane associated, metabolically labeled P. falciparum infected erythrocytes were separated into soluble and particulate fractions by passage through a Stansted hydraulic cell disruptor. Parasite membranes were purified from the particulate fraction by density gradient centrifugation. Labeled soluble and solubilized membrane proteins were immunoprecipitated as shown in Figure 3-1. Lane C contains the antigens solubilized from the membrane fraction, while lane D contains those antigens in this fraction which were immunoprecipitated by Aotus sera. The major protein bands a (Mr = 180,000), f (Mr = 107,000), l (Mr = 75,000) are membrane associated. Lane A contains the soluble antigens, and lane B contains the soluble antigens immunoprecipitated by Aotus sera. Band d (Mr = 132,000) is mainly in the soluble fraction.

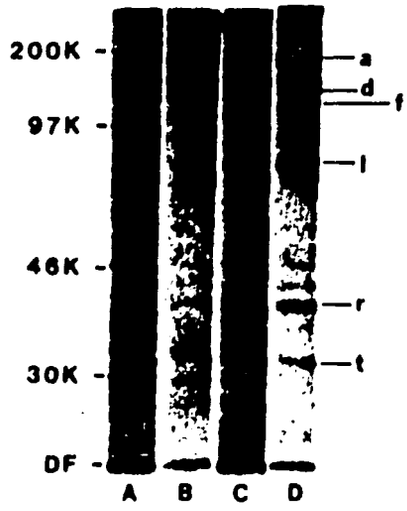


Figure 3-1

External membrane proteins are often glycosylated. ^{14}C glucosamine labeled parasite proteins were immunoprecipitated and SDS-PAGE patterns

are shown in Figure 3-2. Lane L shows the ^{3}H-glucosamine labeled lysate, while Lane IP shows the glycosylated proteins immunoprecipitated with Aotus sera. Major bands a, d, and 1 are all glycosylated and a is strongly labeled. DF is the dye front.

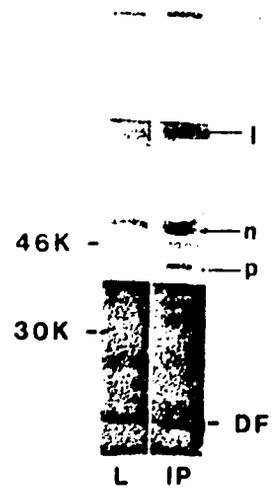


Figure 3-2

To determine if parasite antigens are composed of multiple polypeptide chains, two-dimensional gel electrophoresis was performed by running the immunoprecipitates under non-reducing conditions in the first dimension and under reducing conditions in the second dimension. Figure 3-3 indicates that the 21 P. falciparum bands identified by Aotus sera contain intrachain, but no interchain disulfide bonds. The immunoprecipitates were electrophoresed under non-reducing conditions in the first dimension (horizontal lane) and under reducing conditions in the second dimension. No antigens fell below the 45° diagonal, indicating that no inter-polypeptide chain disulfide bonds were present.

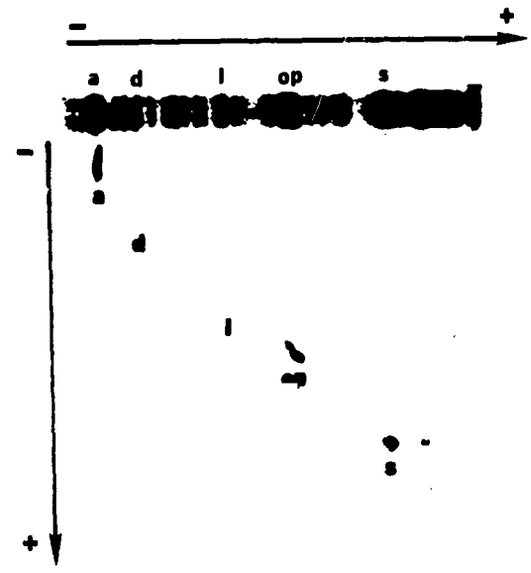


Figure 3-3

6.2.4 Purification of P. falciparum antigens:

We have used affinity chromatography to isolate P. falicaprum antigens. Two preliminary studies were required before a purification protocol could be established. The first involved accumulating substantial amounts of hyperimmune sera from Aotus monkeys. IgG from each was isolated and immobilized on Sepharose beads. SDS-PAGE of immunoprecipitates, using all such preparations, showed that identical patterns were obtained whether whole serum, purified IgG or immobilized IgG was used. No variation in antigens identified by the different antibody sources could be detected.

The second study involved analysis of various eluting agents to determine one that was most efficient in removing the parasite antigens from the columns without loss of antigenicity. Figure 4-1 gives preliminary results establishing that lithium diiodosalicylate is an effective eluting agent. Antigens bound to affinity columns were eluted with various eluting agents and equal cpm were added to the gels. Lithium diiodosalicylate in 0.5% NP-40 (buffer E) eluted 100 percent of bound

radioactivity. Other eluting buffers were: 1% SDS in phosphate, A; pH 10.5 carbonate-0.5% NP-40, B; pH 3.5 citrate-0.5% NP-40, C; pH 4.0 acetate-0.5% NP-40, D; pH 3.5 citrate-HCl, F; 8M urea, G; and 2M potassium isothiocyanate, H.

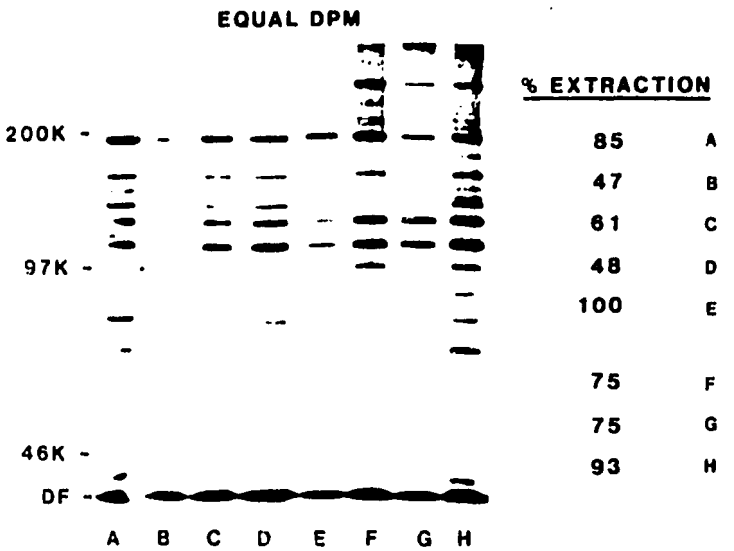


Figure 4-1

Figure 4-2 shows a scheme used for affinity purification of P. falciparum antigens. To remove red blood cell contaminants that were present in NP-40 lysates of saponin lysed parasites preparations, the crude extract was passed through an affinity bead column consisting of purified anti-red blood cell antibody conjugated to Sepharose (Step 1). Theoretically, the eluant should contain only parasite derived antigens. The P. falciparum antigens were specifically purified by passage through a second affinity column consisting of purified Aotus antibody covalently bonded to CNBr activated Sepharose (Step 2). The non-antigenic parasite material passed through the column. The column was thoroughly washed and all antigens were eluted from the column (Step 3, blue circles) with lithium diiodosalicylate buffer.

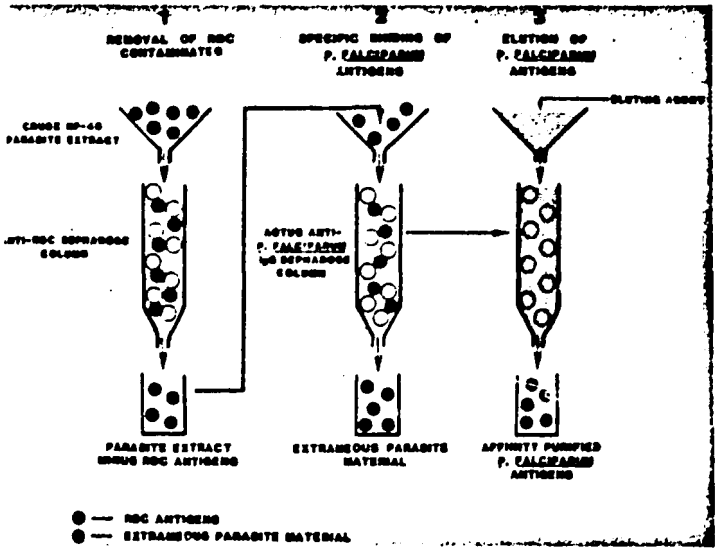


Figure 4-2

6.2.4.1 Retention of antigenicity:

To determine whether the affinity-purified antigen preparation had retained antigenicity, the following experiment was performed as shown in Figure 4-3. The crude, unpurified antigens were immunoprecipitated by *Aotus* immune sera (lane A), protein A-purified *Aotus* Ig (lane B), and the purified Ig conjugated to Sepharose beads (lane C). Lane D shows the eluted, undialyzed affinity-purified proteins, while lane E shows the eluted, dialyzed material. Finally, lane F shows the eluted, dialyzed, affinity-purified proteins re-immunoprecipitated with *Aotus* hyperimmune sera. In the latter, there was no loss of bands, which indicated that the eluted antigens had retained antigenicity.

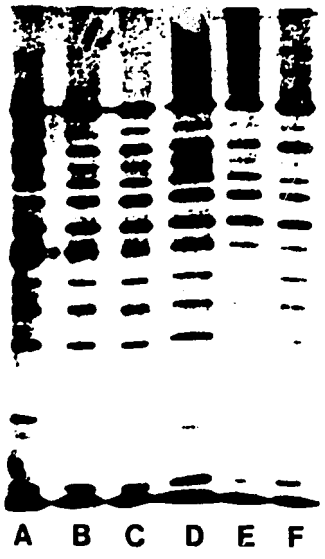


Figure 4-3

6.2.4.2 Retention of immunogenicity:

To study the immunogenicity of the pool of affinity-purified *P. falciparum* antigens, preliminary studies were performed in mice. Three mice received 5 ug of purified antigen emulsified in Freund's complete adjuvant (FCA) intraperitoneally. The mice were bled 13 days after the primary and secondary immunizations and 27 days after the third immunization. The antibody titers to whole *P. falciparum* antigens were evaluated by solid-phase radioimmunoassay. Binding of ^{125}I labeled Protein A was determined with five serum dilutions up to 1/31,250. Only data for the 1/250 serum dilution is shown in Figure 4-4. As can be seen, all three mice had increased antibody titers following the secondary and tertiary immunizations. This preliminary experiment, showed that the pool of affinity-purified antigens was immunogenic in a non-primate laboratory animal.

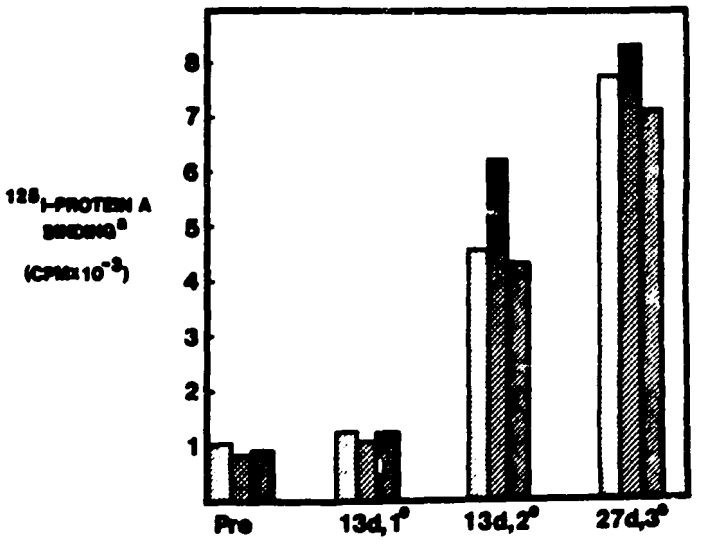


Figure 4-4

Immunogenicity was evaluated in a single Aotus monkey. Aotus 363 was immunized on day 0, and boosted on days 21 (secondary) and 42 (tertiary) with 200 ug of the affinity-purified P. falciparum antigen·pool emulsified in FCA. The animal was bled 13 days after each immunization, and the antibody titers were determined by solid-phase RIA. The antibody titers of the preimmune, primary, secondary and tertiary sera were also evaluated by indirect immunofluorescence. As shown in Figure 4-5, there was increased antibody activity following the secondary and tertiary immunizations.

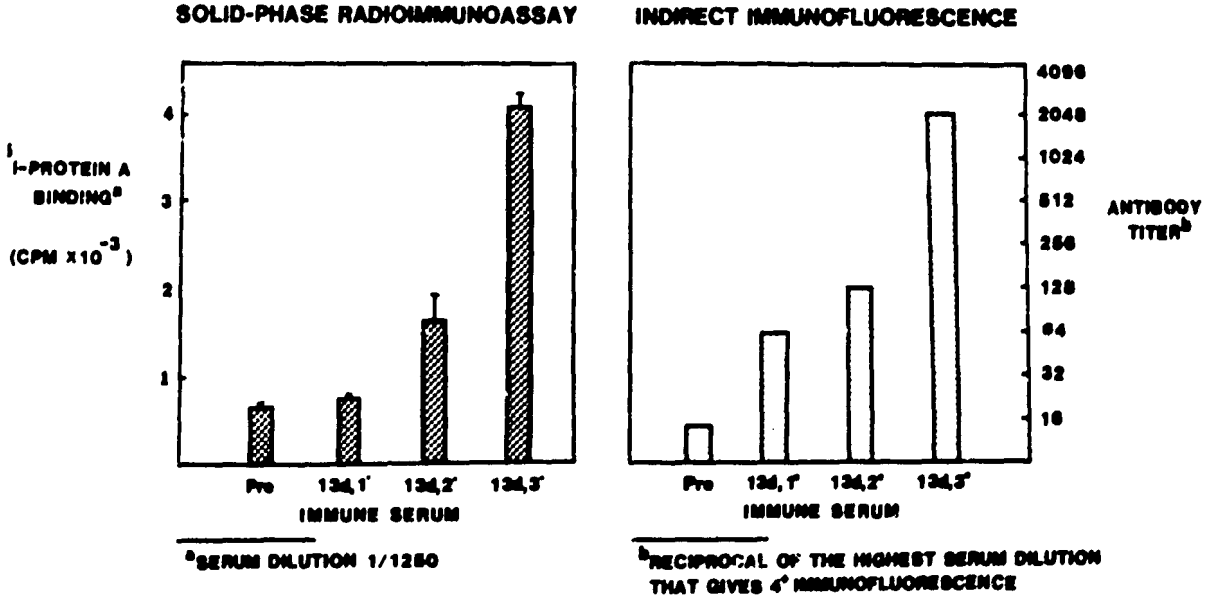


Figure 4-5

The ability of these serum samples to immunoprecipitate ^{3}H-isoleucine labeled P. falciparum antigens is shown in Figure 4-6. The preimmune, primary, and secondary sera from Aotus 363 did not precipitate significant amounts of parasite proteins; however, the tertiary serum immunoprecipitated proteins having Mr 180,000 (band a), 132,000 (band d) and 107,000 (band f). Many other proteins were not immunoprecipitated. Whether this reflects a loss of immunogenicity or the need for further immunization is currently under investigation.

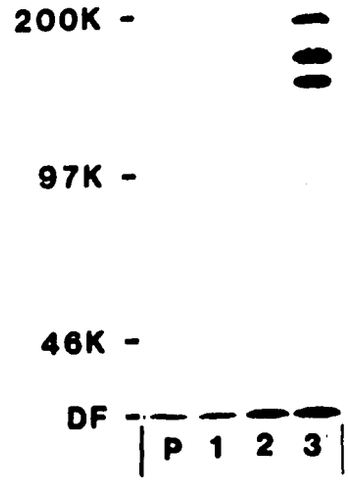


Figure 4-6

6.2.5 Production of Monoclonal Antibodies to P. falciparum:

The production of monoclonal antibodies (Mab) was not formally proposed during the past funding period. However, this aspect of research was deemed critical, and preliminary attempts were initiated. The results of these studies are described below.

6.2.5.1 Development of a rapid method for screening hybridoma supernatants:

A radioimmunoassay was developed to facilitate screening of hybridoma supernatants. The most notable difference among the published RIA methods is the preparation and fixation of parasite antigens. We evaluated different fixatives with the purpose of minimizing any changes to the epitopes of schizont-stage parasites. Fixation with formaldehyde-methanol gave high specific counts for three monoclonal antibody (Mab) supernatants. Early in the screening of hybidomas, we encountered a problem reported in the literature (Taylor et al., Exp. Parasitol. 53: 362, 1982) Some IFA assay positive hybridomas were negative by RIA. As shown in Table 5-1, this was resolved by air-drying instead of formaldehyde-methanol fixation. Two Mab's 21D79.4 and 21E42.4 became RIA positive.

Table 5-1

Antigen Treatment	^{125}I-Protein A (cpm) bound to monoclonal antibodies[a]					
	21D79.4		21E42.4		P3	
	I[b]	N[c]	I	N	I	N
Formaldehyde-Methanol fix	580	720	360	330	270	273
Air Dried	960	320	1900	270	393	220

[a]IFA pattern 21D79.4: Trophs to Schizonts, linear
21E42.4: Trophs to Schizonts, linear
P3: Negative control

[b]I = Schizont enriched whole infected erythrocytes
[c]N = Normal erythrocytes

Titrations of these Mab's have shown that this RIA will detect about 1-3 ng of purified Mab per 50 ul. Hybridoma supernatants generally contain about 500 to 5000 ng per 50 ul.

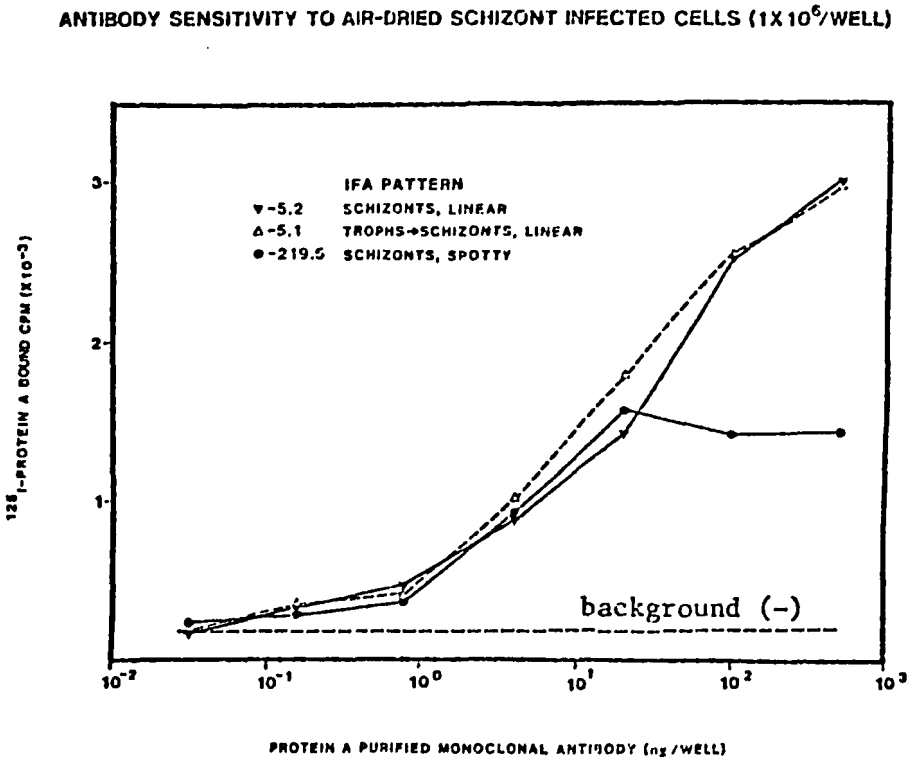
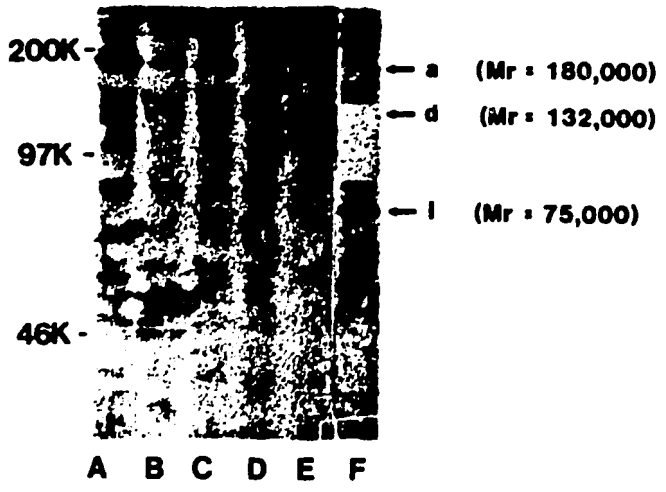


Figure 5-1

6.2.5.2 Use of monclonal antibodies to characterize P. falciparum antigens:

6.2.5.2.1 By comparing with Aotus serum-recognized SDS-PAGE bands:

Using the non-secretor myeloma cell line P3 X 63 Ag 8.653, hybrids were produced by the method of Kohler (EMBO, Cold Spring Harbor, 1980). Six hybridomas were found to immunoprecipitate P. falciparum proteins and the SDS-PAGE patterns are shown in Figure 5-2. Lanes A, B and C show a Mr = 180,000 P. falciparum antigen recognized by monoclonal antibodies 5.2, 23E2.1 and 24A1.5, respectively; lanes D and E show a Mr = 132,000 protein immunoprecipitated by monoclonals 219.5 and 8.311, respectively; lane F shows a Mr = 75,000 protein identified by monoclonal antibody 5.1.



LANE	MAB
A	5.2
B	23E2
C	24A1
D	219.5
E	8.311
F	5.1

Figure 5-2

The antibody repertoire of Balb/c mice may differ from the Aotus monkey. Therefore, a monoclonal antibody must formally co-identify an antigen recogized by Aotus sera. As shown in Figure 5-3, the proof was established for Mab 5.2, by two methods. In the first method, Mab 5.2 was Protein A-purified from ascites and conjugated to Sepharose 4B. A metabolically labeled P. falciparum lysate was then passed through the immunoabsorbent column. The unabsorbed (column passed) antigens were then immunoprecipitated with Aotus sera, and band a (Mr = 180,000) can be seen to be diminished (left). In the second method, the material bound to the Mab immunoabsorbent column was eluted with lithium diiodosalicylate. After dialysis, it was re-precipitated with hyperimmune Aotus sera and found to be band a (Mr = 180,000) (right).

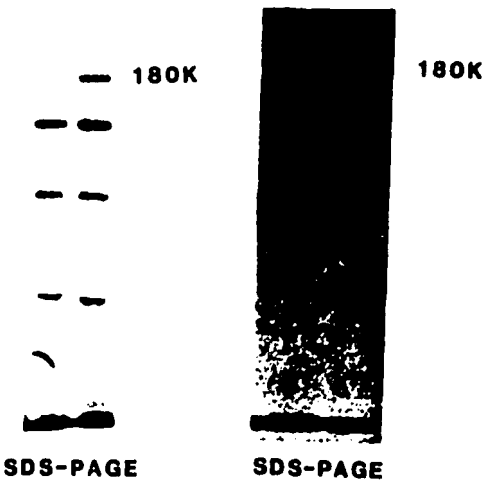


Figure 5-3

6.2.5.2.2 Characterization by immunofluorescence patterns:

By indirect immunofluorescence, the monoclonal antibodies gave different qualitative staining patterns. All Mab's to the 180,000 antigen stained schizonts; the pattern was even, linear staining over the merozoite surface. Mab's 219.5 and 8.311 immunoprecipitated a Mr = 132,000 protein and stain schizonts. However, the staining pattern was spotty. Possibly the apical end of the parsite was identified. Mab 5.1 gave a linear staining pattern. Schizonts and trophozoites were stained,and a Mr = 75,000 protein was immunoprecipitated. Mab's 8.183 and 30B8.3 defined a parasite protein, which is inserted into the erythrocyte membrane. The surface of infected erythrocytes was stained, while non-infected erythrocytes were not stained. Mab's 3E51.4 and 21E22.1 stained parasite and both infected and non-infected erythrocytes. They appear to detect a crossreacting antigen. For the latter four Mab's no metabolically labeled protein has been identified.

6.2.5.3 Use of monoclonal antibodies to characterize antigens in different strains of P. falciparum:

Honduras, FUP (Africa), FCH-1 (Philippines), FCH-13 (India), and FVO (Vietnam) were metabolically labeled (lanes A, B, C, D, and E, respectively) with ^{3}H-isolucine and antigens were immunoprecipitated with Mab's 5.2 and 219.5 as indicated in Figure 5-4. The antigens were present in all strains tested.

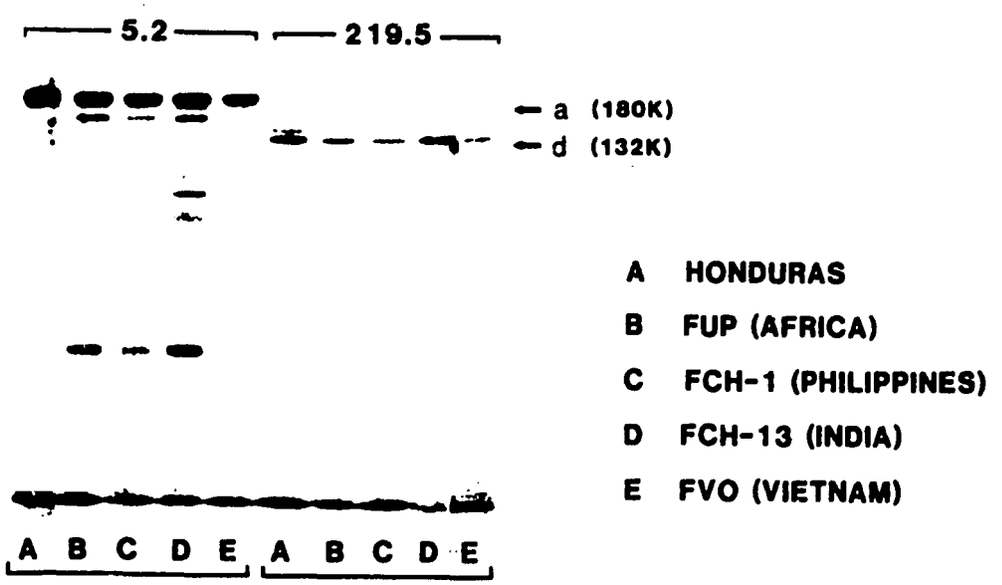


Figure 5-4

6.2.6 Vaccination of Aotus monkeys using P. falciparum antigens:

As directed by AID, no vaccination experiments were performed during this period, since a defined antigen preparation was not available. However, a solid-phase radioimmunoassay was developed to evaluate the anti-P. falciparum antibody titers in pre-immune, primary, secondary, and post-challenge sera from previous vaccination experiments. In the vaccination protocol, two immunizations were given prior to challenge; however, this strategy was decided without information about the antibody titers. We have now analyzed these sera and representative data is shown

in the Figure 6. The antibody titers in sera from animals which recieveda single injection of antigen in FCA approximately equalled the antibody titers in sera from animals receiving two doses of antigen in saline as shown in Figure 6. The latter group of animals were not protected. Only animals having antibody titers greater than about 1/500 by RIA were protected, and two immunizations in FCA were required to attain this antibody level. The information gives some justification for the two vaccination protocol, in retrospect.

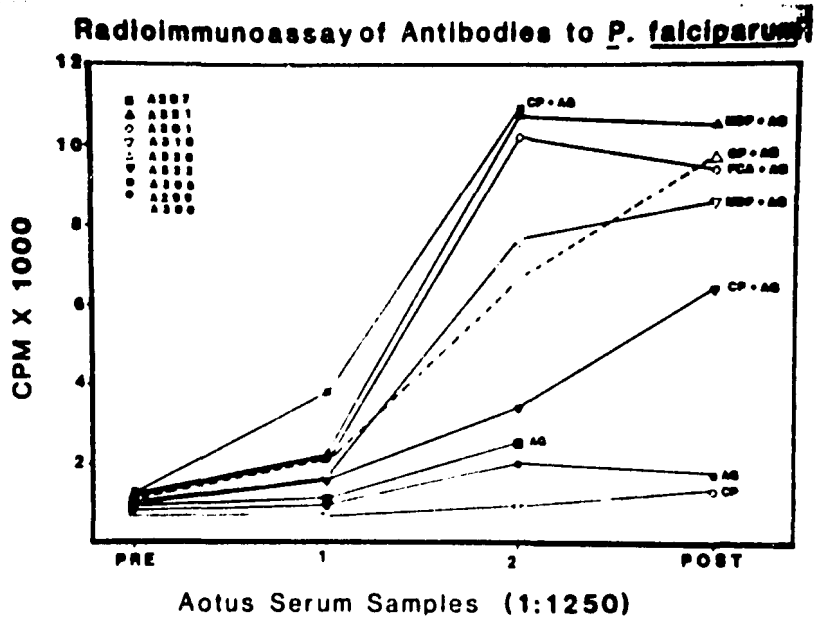


Figure 6

7. Research Plan

7.1 Objectives:

1. To improve technology related to *in vitro P. falciparum* cultivation by optimizing the culture system to obtain maximum parasite yield with minimum cost.
2. To identify, characterize and purify possible protective antigens of blood stages of *P. falciparum*.
3. To carry out vaccination experiments in *Aotus trivirgatus griseimembra* using purified and biochemically characterized defined blood stage

   P. falciparum antigens and a appropriate adjuvant.
4. To continue production of monoclonal antibodies, and to initiate feasibility studies on the use of recombinant DNA technology for the production of relevant antigens

7.2 Background and Specific Aims:

With the improvement of *in vitro* culture techniques (Section 6.2.1) we are able to produce sufficient schizont-stage *P. falciparum* for immunological studies and for vaccination experiments. We propose to work on the optimization of *in vitro* culture conditions so as to increase yield and reduce cost in parasite production. By using sera from vaccinated *Aotus* monkeys, we have identified multiple protein antigens which are likely to be involved in protection (Section 6.2.2). We have carried out preliminary experiments on the characterization of these antigens and have raised monoclonal antibodies to several. We propose that further work on the identification and characterization of these blood-stage *P. falciparum* antigens be done. Such work helps to provide adequate information for identifying the antigen(s) to be selected in vaccine experiments. Such information will also help in devising the most efficient method of

preliminary experiments on the characterization of these antigens and have raised monoclonal antibodies to several. We propose that further work on the identification and characterization of these blood-stage P. falciparum antigens be done. Such work helps to provide adequate information for identifying the antigen(s) to be selected in vaccine experiments. Such information will also help in devising the most efficient method of antigen purification. When sufficient quantities of molecularly and biologically defined antigens have been isolated, we propose to do vaccination experiments in Aotus monkeys. If the chosen antigens are found to be protective, we propose to carry out molecular biology experiments to study the feasibility of producing the antigen(s) by recombinant DNA technology.

7.2.1 In vitro cultivation: large-scale production of schizont-stage Plasmodium falciparum antigen

We will continue to produce large amounts of parasite antigen (with the existing in vitro technology referred to in Section 6.2.1) to be used in experiments designed to identify and characterize potential protective parasite antigens. The identification of protective antigens constitutes the primary thrust of our proposed work. However, we propose the following studies to further improve our large-scale in vitro production capabilities to more fully meet our present and future needs for parasite antigen. In doing so our goal will be to accomplish maximum yields of synchronized parasites within a shorter period of time and at minimum cost for labor and supplies. This work will fall into four main categories: (1) to replace serum with plasma in the in vitro culture medium; (2) to improve parasite yield by optimizing in vitro culture conditions; (3) to obtain schizont-enriched cultures by colchicine treatment; and (4) to further develop the large-scale in vitro culture system progressing toward a eventually fully automated system.

7.2.1.1 To replace serum with plasma in culture medium:

Since we add 10% serum to the culture medium for *in vitro* growth of *P. falciparum*, large-scale production of the parasite will lead to the consumption of a large amount of human serum. Serum represents the most expensive component in the culture medium and it is not readily available. Other sera, particularly calf and rabbit, have been successfully used in *P. falciparum* cultures, but even these sera are not readily available. Our preliminary experiments have indicated that unprocessed human plasma can serve as a replacement for human serum. Termed "salvage plasma", this is readily available from most blood banks at a cost which is approximately one sixth that of serum. Experiments will be conducted to systematically characterize plasma as a replacement for serum in *P. falciparum* cultures. Growth curves, morphology and yields of parasites will be compared in cultures with human plasma versus human serem.

7.2.1.2 To obtain schizont-enriched cultures by treatment with colchincine

We have been using Percoll gradient centrifugation to obtain schizont-enriched parasites from asynchronous *P. falciparum* cultures. This is time-consuming, labor-intensive, and costly. Recently, we reported that synchronous cultures could be obtained by treatment with colchicine (Hui, *et al*. Am. J. Trop. Med. & Hyg. 32, 1451, 1983). Presently, we are able to maintain synchrony for one harvest after a single colchicine treatment and obtain cultures with approximately 70% of parasites at schizont-segmenter stages. Thus, Percoll gradient centrifugation is not routinely used to harvest schizont-stage parasites. Parasite yield is greatly improved since substantial loss of parasites after Percoll gradient centrifugation is occurs

We will initiate studies to improve the method of colchicine treatment, such as additional short duration treatment, using lower

concentrations applied at specific times with the hope of extending the period of synchrony. Maintenance of synchrony for longer periods will additional harvests of schizont-stage parasites from a single colchicine treatment.

7.2.1.3 To improve yields of parasites by optimizing in vitro culture conditions

With our static culture system and our largest culture vessel, the plexiglas box which holds 500 ml medium and 50 ml blood, we are able to obtain 2-4 x 10^{10} parasites per culture vessel per week. We will attempt to maximize the yield of parasites by modifying the medium, by increasing the frequency of medium changes, by increasing the ratio of blood to culture medium, and by controlling pH changes in the medium. Presently, in our semi-automated culture system medium is changed once daily. Theoretically, as the parasitemia becomes higher, nutrients in medium are quickly depleted, pH changes faster and concentrations of harmful metabolic products become higher. Thus, it is logical to change the medium more frequently as parasitemia become higher. We hope that after these improvements, we will be able to harvest the same amount of parasites per culture vessel twice per week.

7.2.1.4 To further develop the large-scale culture system:

Our experience with the semi-automated system has shown that parasites will grow in the system and that the control unit and large-scale vessels function as designed. In the process of evaluation however we have identified some specific changes that can be made to improve operation of the system and eventually make the system fully automated. These include the incorporation of pH and temperature sensors, more sophisticated data acquisition and display capabilities, control and monitoring of gas flow, and a special colchicine synchronization cycle.

Having the pH sensors will allow for two modes of operation: (1) Either the system can change medium in individual culture vessels when the pH reaches a specified level, or (2) changes can be made according to predetermined time intervals. The importance of pH control for optimum parasite yields was recently discussed by Zolg et al. (J. Parasitol, 68(6): 1072, 1982).

The second generation system will incorporate data acquisition and display capabilities that will allow the operator to precisely define and monitor all factors which could affect the growth and multiplication of the parasites. This will include periodic temperature readings, continuous monitoring of gas flow in the sytem, as well as determination of the length of intervals between medium changes, volume of medium delivered to each vessel after each change cycle, and periodic pH readings on each vessel.

The incorporation of a special colchicine synchronization cycle will provide the means for automatically inducing synchrony in the system. To carry this out the control unit will have to be able to first add medium containing colchicine, then after 24 hours make two changes with regular medium at 5 hour intervals. This will necessitate having two medium reservoirs, one with and one without, colchicine.

The final innovation that will make the system fully automated will be the incorporation of an automated harvest cycle. Individual vessels will be harvested either at a preset time or by the operator when parasitemias reach a specified level. The culture will be drawn off into a separate collection system that can be connected directly to a continuous flow centrifuge rotor where the material can undergo the initial steps of the purification process.

The second generation control unit capable of carrying out these functions will consist of an IBM Personal Computer or similar micro-computer system connected to the present control unit that will be modified and upgraded. Use of the micro-computer will allow more operator interaction with the control unit so that parameters can be easily changed. With the present control unit the number of user-defined parameters is limited making it difficult to precisely adjust operation of the system.

7.2.2 Identification, characterization and purification of potential protective antigens of blood-stage *P. falciparum* to be tested in vaccination experiments.

7.2.2.1 Rationale:

Our proposed work on the identification, characterization and purification of *P. falciparum* antigens is based on the hypothesis that hyperimmune *Aotus* sera (i.e. sera from *Aotus* monkeys which have survived repeated challenge with live parasites after vaccination) will immunoprecipitate a pool of antigens inclusive of those that are intimately involved in protection. This hypothesis will be tested in our first proposed vaccination experiment (Section 7.2.3.2.1). For this experiment, we will vaccinate *Aotus* monkeys with a pool of antigens isolated by affinity chromatography, using purified IgG from hyperimmune *Aotus* sera covalently bonded to Sepharose beads. If the hypothesis is correct and the vaccinated *Aotus* monkeys are protected the next step will involve the identification of the critical protective antigens amongst this pool. Detailed physical characterization and indirect immunogenicity testing of these antigens will shed light on the identity of critical protective antigens. The selected antigens will ultimately be tested in vaccination

experiments. Thus the logical sequence of our work is (1) to determine the maximum number of antigens recognized by malaria antibody and to document their presence in different P. falciparum isolates, (2) to categorize the antigens into different groups based on their physical and biochemical characteristics, (3) to devise several schemes of purification so that different groups of antigens can be obtained in sufficiently pure preparations, and (4) to perform vaccination experiments with these preparations and establish protection with these antigens. Further sub-grouping and in vivo testing will continue until the end of the third year of this proposal. By then, we should be able to identify a minimal number of protective antigens with known physical, biochemical and biological characteristics.

We have immunoprecipitated ^{3}H-isoleucine labeled P. falciparum proteins with Aotus hyperimmune sera, separated them on SDS-PAGE and visualized them by fluorography. Twenty-one parasite protein bands with Mr between 30,000 and 180,000 were identified (Section 6.2.2). The detection of antigens in these bands depends on their being labeled by the ^{3}H-isoleucine. If the protective antigens are proteins in nature and yet not labeled by radioactive amino acids because of an extremely low intracellular turnover rate, they will have to be labeled with ^{14}C-acetaldehyde or Na^{125}I for detection. If they are glycolipids or polysaccharides, then they must be labeled metabolically by radioactive sugars or non-metabolically by ^{3}H-borohydride for detection. Alternative to radio-labeling, group specific biochemical stains may be used for detection of proteins, glycoproteins, lipids and sugars. Furthermore, each of the 21 parasite bands detected on one-dimensional SDS-PAGE may contain more than one protein species. Two-dimensional electrophoresis will be used to determine how many polypeptides are actually found in each band.

Significant differences exist between Aotus monkeys and humans in the degree and extent of immunity to P. falciparum. The acquired immunity is long lasting in Aotus monkeys, whereas, it is transient in humans. For this reason, sera from Aotus monkeys, protected through vaccination, are more likely to contain antibodies that react with protective antigens than hyperimmune sera from humans with transient or variable immunity. Nevertheless, it is important to determine experimentally if human sera might recognize antigens missed by Aotus sera.

A detailed characterization of antigen serves two pruposes. First, it will allow us to define potentially protective antigens on the basis of their physical characteristics. We have reasons to postulate that the relevant protective antigens are associated with membrane (either erythrocytic or parasitic), since antibodies or immune cells must interact with physically accessible determinants. Membrane bound antigens are oriented extracellularly and by analogy with most mammalian intrinsic membrane proteins are likely to be glycosylated. Thus, the identification of those P. falciparum antigens which are membrane-bound and glycosylated are important physical criteria of relevance. Such P. falciparum antigens, whether associated with parasite or erythrocyte membrane, are likely to be confronted with an immunological attack.

Second, it enables the design of a rational purification scheme. For example, we have shown (Section 6.2.4) that many P. falciparum antigens recognized by Aotus sera are present in the particulate fraction. This implies that isolation of membranes will represent a

significant purification step. If the membrane protein represents 1% of the total cellular protein (as it does in mammalian cells), the isolation of membrane alone will give a 100-fold purification of cell surface antigens. Thus we will perfect methods of isolation of P. falciparum surface membrane. Furthermore, since integral membrane proteins all possess hydrophobic tails enabling anchorage, an examination of the hydrophobicity of antigens will reveal the likelihood of their membrane association. Since many integral membrane proteins are glycosyated, we have labeled P. falciparum metabolically with sugars and shown that several proteins do contain polysaccharides. Our preliminary studies have indicated that the commonly used lentil lectin does not bind P. falciparum glycoproteins. A wide variety of commercially available lectins that react with different glycoproteins, glycolipids, or polysaccharides should be tested.

One means of identifying and localizing P. falciparum antigens is by the use of monoclonal antibodies (Mab). For example, by correlating immunofluorescent patterns of Mab's, and SDS-PAGE of Mab precipitated labeled antigens with results of other antigen characterization experiments we are able to ascertain that Mab's 5.2 and 24A1.5 recognize a membrane bound and heavily glycosylated protein antigen in band a (Mr = 180,000)(Section VI,B,5b). The likelihood that an antigen is relevant in protection can be assessed by using Mab's to that antigen in the in vitro growth inhibition of P. falciparum cultures. One or a combination of several Mab's to one or several antigens can be tested directly to see if in vitro growth of the parasite is inhibited by its (their) presence in culture medium. Alternatively, Mab's can be

conjugated to Sepharose beads and used to purify individual antigens. These can be used to raise antisera in rabbits or mice and the polyclonal antibodies tested for growth inhibition ability. Besides being useful in antigen characterization, Mab's can be used for the purification of individual antigens for physio-chemical, biochemical and biological studies. Thus in the near future we will hasten our Mab production work with a hope to obtaining more Mab's which will immunoprecipitate other SDS-PAGE bands.

The purification schemes will be designed largely on the results of studies on the physical, biochemical and biological properties of the antigens. However, two indispensible techniques will be included in any scheme that we employ. The first is affinity chromatography, using either immunoglobins from hyperimmune Aotus sera, monoclonal antibodies or lectins. Optimizing conditions of chromatography, especially assessing the most appropriate eluting agents that enable the retention of both antigenicity and immunogenicity of the antigens is critical. The second indispensible technique is high pressure liquid chromatography (HPLC), which in recent years has become a valuable tool for analytical and preparative separation of macromolecules. Purified antigens can be separated on the basis of molecular size using molecular sieving in the presence of chaotropic agents or ionic detergents, on charge properties using ion-exchange columns, and on hydrophobicity using reverse phase columns. Additional techniques that may be exploited include chromatofocusing (which will separate proteins into basic, neutral, and acidic fractions), Triton X114 (which will provide a rapid method of separating hydrophophilic and hydrophobic proteins), and preparative SDS-PAGE for size separation.

Groups of purified antigens obtained by using the combination of above techniques can be used as antigens either for Mab production or in vaccination experiments.

7.2.2.2 Experimental:

7.2.2.2.1 Identification of possible protective *P. falciparum* antigens:

7.2.2.2.1.1 To visualize antigens separated on SDS-PAGE by staining:

Unlabeled *P. falciparum* protein extracts will be isolated by affinity chromatography, electrophoresed on SDS-polyacrylamide gels and stained with silver nitrate (Sammons *et al.*, Electrophoresis 2:135, 1981) and "stains-all" (Green *et al.*, Anal. Biochem. 50:43, 1973). The Protein A purified *Aotus* anti-*P. falciparum* antibody used to coat Sepharose beads will be extensively adsorbed with normal erythrocytes, and the *P. falciparum* extract will be exhaustively adsorbed with insolubilized anti-erythrocyte antibody to eliminate staining of host erythrocyte antigens.

7.2.2.2.1.2 To further resolve identified antigens by 2-dimensional electrophoresis:

Two-dimensional electrophoresis to better resolve immunoprecipitated *P. falciparum* proteins will be carried out by the method of O'Farrell (J. Biol. Chem. 246: 6328, 1975).

7.2.2.2.1.3 To compare antigens recognized by *Aotus* sera with those recognized by human sera:

Aotus sera from past vaccination experiments and human hyperimmune sera from Indochina, India, Africa and South America will be compared by autoradiography, staining, and 2-dimensional electrophoresis to determine whether identical or different antigens are identified.

7.2.2.2.1.4 To compare *P. falciparum* antigens in isolates from different geographical regions:

Different isolates of *P. falciparum* from Central America, Africa,

India, Indochina, and the Philippines will be labeled with ^{3}H-isoleucine. Immunoprecipitated parasite proteins will be separated by SDS-PAGE and visualized by fluorography. Only antigens common to all isolates will be considered as potential candidates for a vaccine.

7.2.2.2.1.5 To compare parasite antigens in the course of *in vitro* cultivation:

An *in vitro* culture with *P. falciparum* from an infected *Aotus* monkey will be initiated and aliquots will be cryopreserved in liquid nitrogen bi-weekly for 6 months. Aliquots will be thawed and metabolically labeled with ^{3}H-isoleucine simultaneously. Using vaccinated *Aotus* sera for immunoprecipitation and using conventional SDS-PAGE and fluorography, the change in pattern of antigens during the course of *in vitro* cultivation will be followed.

7.2.2.2.2 Characterization of possible protective *P. falciparum* antigens:

7.2.2.2.2.1 To distinguish membrane bound antigens from soluble antigens:

Surface membrane material will be isolated to confirm the membrane-bound nature of some antigens. Parasites will be metabolically labeled with ^{3}H-isoleucine, released by saponin and treated with a non-ionic detergent, Tween 40. After breaking up the detergent treated parasites by passage through a Stansted cell disruptor, high speed centrifugation at 100,000 X g for 1 hr. will separate the parasite preparation into a supernatant soluble fraction and a pellet, the particulate fraction. By density gradient centrifugation of the resuspended particulate fraction, parasite surface membrane will be obtained as a light fraction, since the bouyant density of membrane is lowered by the Tween 40 bound to its lipids. Both the membrane fraction solubilized by NP-40 and the soluble fraction will be immunoprecipitatied with hyperimmune sera and the antigens recognized will be separated on

SDS-PAGE and visualized by fluorography.

7.2.2.2.2.2 To define glycosylated vs. non-glycosylated antigens:

A panel of purified lectins will be assayed for their ability to bind parasite antigens. A solid phase assay will be developed to screen the lectins. The assay will involve plating the lectin to poly-L-lysine coated wells of microtiter plates. Metabolically labeled parasites solubilized by NP-40 will be added, and after washing, the amount of label associated with the well will be determined. A partial list of purified lectins that are commercially available (and their specificities) include peanut agglutinin (galactose), mistletoe lectin (D-galactose), Concanavalin A (alpha-methyl-D-mannoside), soybean lectin (N-acetyl galactosamine), limulus hemagglutinin (sialic acid, glucuronic acid, and phosphoryl choline analogs), and asparagus pea lectin (L-fucose).

7.2.2.2.2.3 To classify antigens by high pressure liquid chromatography:

A pool of P. falciparum antigens obtained from affinity chromatography using immobilized IgG from hyperimmune Aotus sera will be fractionated by HPLC. A column filled with 300 angstrom or 500 angstrom pore size glass matrix coated with glycerylpropyl-silane will be used for separation according to size. The chromatography will be done in 6M guanidine-HCl. A column of large pore silica with the ligand octadecyl will be used for separation according to hydrophobicity. The chromatography will be done by decreasing the polarity of the mobile phase with increasing amounts of acetonitrile or n-propanol. A column filled with a glycophase-coated, silica-based matrix will also be tried for separation according to charge. These experiments will help determine the most appropriate type of column and the optimum elution conditions to be used for the purification of antigens by preparative HPLC.

7.2.2.2.2.4 To separate antigens according to size:

The use of the ionic detergent SDS enables the separation of parasite proteins according to size. Non-ionic detergents such as NP-40 result in micelle formation and prevent such separation. In future work, P. falciparum antigens will be isolated preparatively according to size. However, we must determine whether or not SDS has adverse effects on antigenicity and immunogenicity. To study antigenicity we will do the following experiment. An aliquot of ^{3}H-isoleucine labeled P. falciparum culture will be divided into two portions. One will be solubilized with NP-40 and the other with SDS. SDS from the latter will then be removed and replaced with NP-40. Hyperimmune sera immunoprecipitation patterns of the two lysates will be compared following SDS-PAGE and fluorography. To study immunogenicity, we will immunize mice separately with NP-40-prepared and SDS-prepared (SDS eventually will be removed and replaced by NP-40) P. falciparum antigens and then compare antisera raised by the two different preparations. If there is no adverse effect, then SDS can be used in antigen purification by gel chromatography. This will facilitate purification greatly since SDS is a powerful solubilizer and is very easily removed.

7.2.2.2.2.5 To localize antigens using monoclonal antibodies:

Production of monoclonal antibodies which co-identify antigens defined by Aotus sera will continue. As new monoclonals become available, the defined antigens will be localized to trophozoite, schizont, merozoite or erythrocyte by indirect immunofluorescence.

7.2.2.2.2.6 To evaluate antigens that inhibit merozoite reinvasion in vitro:

As monoclonal antibodies are produced to P. falciparum antigens, the antigens will be isolated by affinity-purification. Purified antigens

will be used to immunize rabbits and the Ig will be purified on Protein A columns. The purified Ig will be used to evaluate *in vitro* growth inhibition. We believe that inhibition of merozoite reinvasion is more reliably assessed using polyvalent antisera rather than monoclonal antibodies, since multiple epitopes on the antigen will be recognized and greatly increasing their functional affinity.

7.2.2.2.3 Purification of possible protective *P. falciparum* antigens:

7.2.2.2.3.1 To purify a pool of antigen by using *Aotus* hyperimmune sera:

As mentioned in Section 6.2.4, a pool of *P. falciparum* antigens isolated by affinity chromatography using IgG from hyperimmune *Aotus* sera has retained their antigenicity and immunogenicity. The eluting agent, Lithium diiodosalicylate, though efficient is to expensive, and is easily precipitated upon slight change in pH. Other methods of elution, such as elution by electrophoresis with an ISCO electroporetic concentrator will be tested. Once the method of elution is perfected, we will use this preparation as a vaccine in our proposed first vaccination experiment.

7.2.2.2.3.2 To separate antigens into soluble and membrane-bound fractions:

The analytical method described in Section 7.2.2.2.2.1, will be extended for the preparation of *P. falciparum* surface membrane. Since the starting parasite materials will not be labeled, the purity of Tween 40 incorporated *P. falciparum* surface membrane will be assessed by visualizing SDS-PAGE bands with $AgNO_3$ staining.

7.2.2.2.3.3 To separate antigens into glycosylated and non-glycosylated fractions:

Immobilized lectins on Sepharose beads will be used to separate glycosylated from non-glycosylated proteins. Preliminary data on antigen characterization (Section 6.2.3) have shown that quite a number of

antigens recognized by Aotus sera are glycosylated. We have found that the commonly used lentil lectin does not bind P. falciparum glycoproteins. Thus, we envision using a lectin having stronger binding affinities, such as Con A. Alternatively, lectins having other sugar specificities may be used (cf. Section 7.2.2.2.2.2).

7.2.2.2.3.4 To isolate antigens by high pressure liquid chromatography:

The information used to classify antigens by analytical HPLC will be used for preparative HPLC (Section 7.2.2.2.2.3).

7.2.2.2.3.5 To purify antigens according to isoelectric points:

A pool of affinity-purified parasite proteins, obtained by using immoblilized IgG from hyperimmune Aotus sera, will be separated into basic, neutral and acidic proteins by "chromatofocusing". Both column medium and buffers are available commercially from Pharmacia Fine Chemicals. The methodology will be that of Sluyterman & Elgersma (J. Chromatogr. 150: 31, 1978) and Sluyterman & Wijdenes (J. Chromatogr. 150: 31, 1978).

7.2.2.2.3.6 To separate antigens according to hydrophobicity by Triton X-114:

Triton X-114 , a non-ionic detergent of the Triton X series having a low cloud point, was found to be most suitable for separating hydrophobic from hydrophilic proteins (Bordier, J. Biol. Chem. 256:1604, 1981). Parasite proteins can be extracted by Triton X-114 at 37C (above the cloud point). After centrifugation at the same temperature to remove insoluble materials, the detergent solution with parasite proteins will be placed at room temperature (below the cloud point) to allow phase separation to occur. Hydrophilic proteins will remain in the upper, clear aqueous phase; whereas, hydrophobic proteins will be in the lower, turbid detergent phase.

7.2.2.2.3.7 To separate antigens according to molecular size:

The pool of affinity purified parasite proteins will be fractionated into three size groups by preparative SDS-PAGE: Mr above 100,000, Mr between 60,000-100,000, and Mr below 60,000. Both SDS-PAGE and Sephacryl gel chromatography with SDS will be used for separation. The latter will employ 3 mm slab gels. After electrophoresis, slabs will be cut into the three sections according to the arbitrary Mr ranges above, and parasite proteins will be eluted electrophoretically by a ISCO protein concentrator according to Allington et al. (Anal. Biochem. 85, 1978). SDS will be removed during concentration and replaced by NP-40 present in the buffer used in the concentrator. In gel chromatography a maximum of 3 mM SDS will be used in the buffer so that Sephacryl properties will not be altered. Buffer of ionic strength 0.5M will be used so that the critical micellar concentration of SDS will fall far below 3 mM. Under such conditions, SDS will not be lost from the SDS-parasite protein complexes and the parasite antigens will remain solubilized. Groups of proteins obtained from the Sephacryl gel chromatography will also be concentrated and SDS removed likewise. Immunogenicity of these preparations will be evaluated by vaccination of mice and antigenicity will be checked by re-immunoprecipitation with Aotus sera. It is likely that SDS will cause some protein denaturation. However, sequential antigenic determinants should remain unaltered and some conformational determinants should remain intact.

7.2.2.2.3.8 To purify antigens using monoclonal antibodies:

If a set of antigens is shown to protect Aotus monkeys in vaccination experiments, the individual antigens will be purified by using appropriate monoclonal antibodies covalently bonded to Sepharose beads. Details of

this will appear in Section 7.2.4, which specifically deals with production of monoclonal antibodies to P. falciparum antigens.

7.2.3 Vaccination studies in Aotus trivirgatus using purified blood stage P. falciparum antigens in conjunction with an appropriate adjuvant.

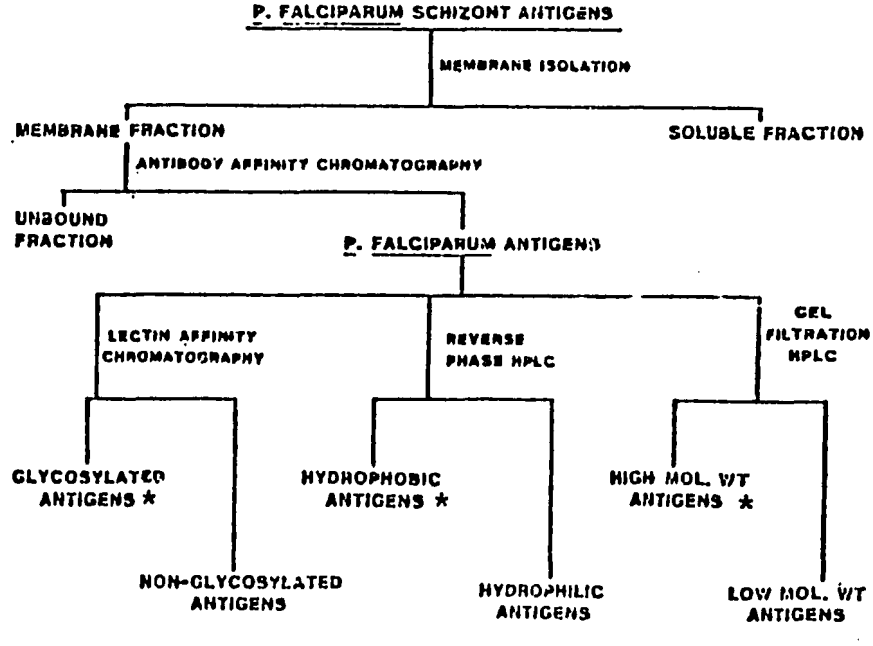
7.2.3.1 Rationale:

To date, the most effective vaccines in Aotus monkeys have been composed of partially purified whole cell schizont or merozoite antigens derived from the short-term culture of infected cells obtained from an infected monkey or human (Mitchell, C.H., et al., Lancet 1: 1335, 1977. Siddiqui, W.A., et al., Nature 289: 64, 1981). Attempts to repeat these experiments with whole cell antigens derived from long-term in vitro cultures have not been as effective (Reese RT., et al., Proc. Natl. Acad. Sci. USA 75: 5665, 1978. Trager, W. et al., Parasite Immunol. 5: 225, 1983). The reasons for this discrepancy are not fully understood at this time. Perhaps the putative protective antigens: 1) are diminished either in number or in quantity after long periods of in vitro cultivation or 2) are masked by immunodominant antigens which appear during long-term in vitro cultivation.

To date, no vaccination studies have reported using purified antigens to protect Aotus monkeys against P. falciparum. We propose to purify a pool of P. falciparum antigens from in vitro cultures using hyperimmune Aotus sera as described in Section 6.2.4, and as detailed in Section 7.2.2.2.3.1. This pool of purified antigens will be used in a series of vaccination studies. If these vaccination experiments are successful, then we will reduce the number of antigens in the purified preparation to a much smaller pool and carry out further vaccination experiments. Our proposed studies on antigen identification, characterization and

purification of antigens will aid us in choosing the most appropriate combination of antigens for further vaccination experiments.

During the next three years we propose to conduct at least four full-scale vaccination experiments using Aotus type II and III monkeys. The first series of experiments will test the effectiveness of a purified pool of antigens isolated by antibody affinity chromatography from both long term in vitro and in vivo derived cultures. Depending on which antigen preparation is most potent in conferring protection, we will further dissect the antigens into groups and conduct additional vaccination experiments. An example of a purification scheme that may be followed is given below. The asterisks indicate high priority groups that might be tested. The rationale for priority has been discussed earlier (Section 7.2.2.1). Purification schemes may change and priorities will shift as more information becomes available.



EXAMPLE OF A PROPOSED PURIFICATION SCHEME

7.2.3.2 Experimental:

7.2.3.2.1 First vaccination experiments:

7.2.3.2.1.1 To prepare a pool of antigens from *in vitro* cultures:

The parasites will be grown in large culture vessels as described in Section 7.2.1. Long-term cultured parasites will be synchronized using colchicine. This treatment will not be needed for short-term cultures, since these remain synchronized for approximately two months. Short-term cultures will be established from a frozen stabilite of a monkey passaged FUP strain and harvested over a two month period only. Mature parasites from both long and short-term cultures will be released by saponin and stored at -70° C unitl purification. After sufficient amounts of parasites have been accumulated, they will be solubilized by NP-40. Unsolubilized parasite material will be removed by centrifugation at 100,000 x g for 1 hour. The solubilized *P. falciparum* antigens will be purified by affinity chromatography as described in Section 7.2.2.2.3. Protein A-purified antibody from *Aotus* hyperimmune sera will be coupled to Sepharose. Lithium diiodosalicylate will be the eluting buffer. This antigen preparation will be concentrated and lithium diiodosalicylate will be removed and replaced by NP-40 in an ISCO electrophoretic concentrator with NP-40 present in the buffer. All protein determinations will be carried out by spectrofluorometry using fluorescamine (Sims & Carnegie, Anal. Biochem. 63: 578, 1975).

7.2.3.2.1.2 To test the antigenicity of affinity purified antigens prior to vaccination:

The purified antigens will be analyzed for retention of antigenic determinants by re-immunoprecipitation with hyperimmune *Aotus* sera following column elution. A sample of eluted antigen and a sample of re-immunoprecipitated eluted antigen will be co-electrophoresed on a

SDS-acrylamide slab gel. Antigens will be visualized by silver staining. If similar bands appear in both lanes, antigenicity was not lost in the course of purification.

7.2.3.2.1.3 To test the immunogenicity of the affinity purified antigens in non-primates:

This antigen preparation, with FCA, will be used to immunize rabbits and mice. Sera will be obtained before immunization and at several intervals after each immunization. Total antibodies to *P. falciparum* will be assayed by RIA and IFA. Reactivity to individual antigens will be analyzed by immunoprecipitation of ^{3}H-labeled parasite proteins, SDS-PAGE and fluorography. Growth inhibition studies will be performed with the Ig fraction from immunized rabbit sera to evaluate development of antibodies which neutralize merozoite reinvasion. Additional tests to evaluate the ability of these purified proteins to induce delayed type hypersensitivity will
be performed in mice using antigen proliferation assays of splenic lymphocytes under conditions previously described (Taylor D.W., W.A. Siddigui, Bull. WHO., 57(suppl. 1): 247, 1979) and foot pad swelling.

7.2.3.2.1.4 To vaccinate Aotus monkeys with a pool of affinity purified antigen:

Two vaccination experiments will be carried out if the antigen preparations are shown to retain antigenicity and immunogenicity. The first vaccination will test antigens derived from short-term *in vitro* derived cultures and another experiment will evaluate the antigens from long-term *in vitro* cultures. The vaccination protocol is as follows:

7.2.3.2.1.4.1 Composition of the vaccine:

Monkeys will be vaccinated with purified antigens in FCA. Monkeys

receiving either antigen only or FCA only will serve as negative controls and monkeys receiving whole parasite antigens with FCA will be positive controls. If more Aotus monkeys are available another group of monkeys will be given purified antigens with a synthetic adjuvant, either CP20,961 (Pfizer) or B-30 MDP. The number of monkeys used per group will depend on the number of monkeys available. Karyotype II and III Aotus monkeys from Columbia will be used because these monkeys have given, in our laboratory, reliable responses to P. falciparum vaccination and challenge.

7.2.3.2.1.4.2 Collection of blood samples from Aotus monkeys:

Blood from all experimental monkeys will be collected before and two weeks after each vacccination. After challenge with live parasites, blood will be collected from surviving monkeys after the parasitemia has cleared and the hematocrit has returned to normal.

7.2.3.2.1.4.3 Immunological assessment:

Sera will be analyzed prior to challenge for total malaria antibodies by RIA and IFA and for reactivity with specific P. falciparum antigens by immunoprecipitation and SDS-PAGE. Aotus antibody will be evaluated for inhibition of merozoite re-invasion in vitro. The presence of bound anti-erythrocyte antibody will be tested by the Coombs anti-globulin test

7.2.3.2.1.4.4 Challenge with live parasites:

When the immune responses are maximal (two to four vaccinations may be needed), we will challenge the vaccinated monkeys with live parasites obtained from an infected donor Aotus monkey. The challenge inoculum will be 7.5×10^5 infected erythrocytes per monkey.

7.2.3.2.2 Other vaccination experiments to determine the minimum number of affinity purified antigens required for protection:

7.2.3.2.2.1 To vaccinate with an antigen sub-group:

If the first vaccination experiment is successful, the pool of

antigens will be fractionated into sub-groups by the purification scheme discussed in 7.2.3.1. The methodology specified for evaluation of antigenicity, immunogenicity in non-primates, and vaccination of Aotus monkeys described above, will be followed using a selected antigen sub-group.

7.2.3.2.2.1 To vaccinate with monoclonal antibody purified antigens:

If an antigen sub-group is protective, Balb/c mice will be immunized with the fractionated antigens and monoclonal antibodies will be produced. The antibody will be protein-A purified from Ascitic fluid and conjugated to Sepharose 4B. The specific antigens will be affinity purified in a single step from crude, saponin-lysed, NP-40 antigen extracts. Again, either single antigens or combinations of antigens will be evaluated for protection, adhering to the basic vaccination protocol described above. If a single antigen or a restricted set of antigens is defined, the feasibility of production by recombinant DNA technology will be evaluated.

7.2.4 To continue monoclonal antibody production, and to initiate feasibility studies on the use of recombinant DNA technology for the production of relevant antigens:

7.2.4.1 Monoclonal Antibodies:

7.2.4.1.1 Rationale:

Improvements in monoclonal antibody production can be made in two areas: immunization of Balb/c mice and screening of hyridomas. In the past, we have immunized mice with crude parasite preparations. Future immunizations will employ charaterized antigens obtained through preparative purification schemes such as those described in Section 7.2.2.2.3. This will increase the probability that selected hybridomas co-immunoprecipitate antigens identified by Aotus sera.

Once a hybridoma is identified by solid-phase RIA, much time and labor

is invested in identifying the P. falciparum antigen. The identification involves indirect immunofluorescence and SDS-PAGE. As we produce more and more hybridomas, we will encounter the problem of redundancy. That is, monoclonal antibodies will be preferentially detected against the most immunogenic P. falciparum antigens. The 180,000 (band a) and 132,000 (band d) antigens are good examples. We have produced several Mab's to both antigens in separate fusions. To minimize redundancy, Balb/c mice will be immunized with restricted sets of antigens. For example, the 21 antigens isolated by Aotus serum will be reduced to 18 by adsorption with affinity columns containing Mab 5.2 (band a), Mab 219.5 (band d), and Mab 5.1 (band 1). As more and more monoclonal antibodies are produced, fewer antigens will be used for immuumization.

Secondly, the screening assay can be improved. The majority of labor in monoclonal antibody production lies in screening. Thousands of hybridoma supernatants need be surveyed and the effort is laborious. Identified parental cultures must be cloned twice, adding to the number of supernatants that must be screened. About half of the identified clones are unstable and must be continually monitored for retention of activity. Isotype must be determined, ascites fluids must be titered, and the different fractions from Protein A-Sepharose chromatography must be monitored for antibody activity. Thawed aliquots of purified Mab must be examined for loss of activity upon storage, and the various fractions during conjugation to Sepharose 4B must be titered. The RIA involves greater labor than an enzyme linked screening assay (ELISA). Protein A must be iodinated monthly, and the mechanics of counting individual wells in a gamma counter is time-consuming. The major advantages of ELISA are the elimination of radioactive iodine, which is a biological hazard, and the time saved using an ELISA reader. The latter is about 100 times more

rapid than the counting of individual wells by RIA. Switching over should pose little difficulty. Coating of plates with P. falciparum antigens, the major problem in immunoassays, has been solved and the same plates used for RIA will be used in the ELISA system.

7.2.4.1.2 Experimental:

7.2.4.1.2.1 To immunize Balb/c mice with defined antigens:

As purification schemes are devised for the isolation of potentially protective antigens, whether by preparative HPLC, lectin affinity columns, or preparative isoelectric focusing, the purified antigens will be used to immunize Balb/c mice for monoclonal antibody production. Balb/c mice will recieve a primary immunization 50 ug of purified antigen in FCA intraperitoneally. Secondary and tertiary immunizations will be at two week intervals with 50 ug of antigen in Freund's incomplete adjuvant (FIA). (Alternatively, 5×10^7 unprimed Balb/c spleen cells will be sensitized in vitro by culture in 20 ml Balb/c thymocyte conditioned media containing 50 ug characterized P. falciparum antigen.) Four days prior to fusion, mice will receive 5 ug intravenously through the tail vein. Mice will be exsanguinated by cervical dislocation and washed splenocytes fused with the nonsecretor P3 X 63 Ag 8.653 myeloma cell line according to the method of Kohler (EMBO, Cold Spring Harbor, 1980) using 45% polyethylene glycol 1540 and 7.5% DMSO in serum free media. The fused cells will be resuspended in HAT selective media and planted in 96 well microculture trays (1000 cultures per fusion) containing syngeneic peritoneal macrophage feeder cells. Ten days following fusion, wells will be screened by ELISA (see below) on assay plates containing air-dried, P. falciparum sonicated material. Positive wells will be double cloned by limiting dilution.

7.2.4.1.2.2 To adsorb antigens with monoclonal antibodies prior to immunization:

Ascitic fluid containing monoclonal antibodies to dominant immunogens within a group will be produced. The ascites will be delipidated with heparin (Lindqvist, K. and D. C. Bauer, Immunochem. 3: 377, 1966). The ammonium sulfate fraction will be passed through Protein A-Sepharose (Pharmacia) and the Ig eluted with pH 3.5 citrate buffer. One hundred mg of purified monoclonal antibody will be conjugated to 10 ml (about 3 grams) of cyanogen bromide activated CH Sepharose 4B (Pharmacia), washed by three cycles of pH 4.0 acetate buffer followed by pH 8.0 Tris buffer, and stored in borate buffer until required. The set of characterized antigens will be passed through the monoclonal antibody affinity column prior to immunization of Balb/c mice. This step appears unnecessary when the theory of monoclonal antibody production is considered; however, we believe that it will hasten the detection of monoclonal antibodies to weakly immunogenic P. falciparum antigens by eliminating redundancy.

7.2.4.1.2.3 To convert the solid-phase RIA to an enzyme-linked immunoabsorbent assay:

Assay plates coated with the equivalent of 1 X 10^6 sonicated, air-dried P. falciparum will be used. These are the plates developed for RIA. For screening, 50 ul of culture supernatant will be added to a microtiter well and allowed to react 60 min. at room temperature. The plates will then be washed with 0.05% Triton X-100 in water followed by pH 7.2 phosphate buffered saline (PBS) containing 0.02% Tween 20. The wells will then be filled with 50 ul of goat anti-mouse antibody coupled with alkaline phosphatase, diluted pH 7.0 PBS-Tween 20, and incubated at room temperature for 60 min. The plates will be washed and 50 ul Sigma 104 substrate in pH 10.2 buffer will be added. The plates will be scanned at 405 nm colorimetrically after 60 min. on an ELISA reader. Supernatants

giving greater than three standard deviations above negative controls will be considered positive.

7.2.4.2 Molecular biology related to vaccine production by recombinant DNA technology:

It is conceivable that the protective antigens will be polypeptides which can be synthesized by recombinant DNA technology. This is desirable from a production standpoint, and emphasis will be given to those protective antigens which are polypeptides. Currently, the most common method for producing plasmodial proteins by recombinant DNA techniques is by construction of cDNA banks (Ellis, J. et al., Nature 302: 536, 1983; Kemp J. et al., PNAS 80: 3787, 1983). A cDNA bank is simpler to screen than a genomic bank. In eucaryotic cells, for example, a genomic bank will contain between 100,000 to 1,000,000 DNA fragments of a size suitable for cloning; whereas, a cDNA bank will contain between only 10,000 to 30,000 different mRNA sequences. Nevertheless, the screening of a cDNA bank still represents a heroic task for a number of reasons: First, the frequency of a particular clone in a cDNA bank is proportional to its abundance. The problem of detecting a low frequency gene is difficult for plasmodial proteins, since mRNA encoding for antigens that react with immune sera can be found in both poly (A)+ and poly (A)- mRNA fractions (Riveros-Moreno, B. and K. P. Hastings; 2nd Int. Conf. Malaria and Babesiosis, p.27, 1983). Second, the optimal conditions for obtaining full length mRNA and full length transcripts for large proteins are difficult to attain, because there is preferential selection for cDNA that encodes for low molecular weight proteins.

Several techniques may be used to simplifly the problem of screening cDNA banks. First, mRNA can be fractionated by size on density gradients. Second, the particular polyribosomes can be purified by reacting the

nascent polypeptide chains with antibodies. A modification by Shapiro and Young (J. Biol. Chem. 256: 1495, 1981) has simplified the technique considerably. In pilot feasibility studies, we propose to isolate antigen-specific mRNA by reacting polysomes with polyvalent Aotus serum using standard techniques (Maniatis et al.,"Molecular Cloning. A Laboratory Manual," Cold Spring Harbor Laboratory, 1982). The complexes will be absorbed on protein A-Sepharose columns, and the eluted mRNA will be used to prepare a double stranded cDNA clone. Eventually, when protective antigens are identified, monoclonal antibodies will be used to purify specific messages. This technique has been applied successfully to the human HLA-DR antigen, which represents only 0.01-0.05% of the total mRNA (Korman, A. J. et al., PNAS 79: 1844, 1982). Although the initial expenditure of time and resources is great, the task of screening for a specific sequence is obviated when monoclonal antibodies are used.

Work on the above will not be initiated until protective antigens have been formally documented by vaccination experiments. At the University of Hawaii, there are a number of experts working on various aspects of recombinant DNA technology. Two individuals who have expressed enthusiam in serving as consultants are Dr. John Hunt (Department of Genetics) and Dr. Morton Mandell (Department of Biochemistry and Biophysics). Dr. Hunt is an established investigator in the area of alpha- and beta-globin mRNA isolation, synthesis and characterization. Currently, he is constructing cDNA from globin mRNA. He has already provided help in formulating this proposal and in planning of preliminary experiments to isolate P. falciparum mRNA. Dr. Mandel is a pioneer in the field of transferring E. Coli with naked DNA and currently is involved in cloning sea urchin genes at different stages of the developmental cycle. Their experience in molecular biology is documented in their enclosed curriculum vitae.

7.3 Significance & Rationale

An appraisal of malaria as a medical and public health problem today reveals that malaria still persists in many areas of the world. This is especially turc in less developed countries (LDCs), despite widespread use of prophylactic drugs and residual insecticides. The development of drug resistance in strains of malaria parasites has been reported in various areas of Southeast Asia and South America. Drug resistant strains of human malaria parasites have been found recently in the Indian and African subcontinents. The resurgence of malaria has created alarming concern among medical and public health officials in LDCs as well as among malariologists all over the world. The impact of malaria on the economy of the LDCs can hardly be over emphasized, especially with regard to the loss of manpower not only in terms of mortality, but, most importantly, in terms of morbidity.

Attempts to find a vaccine against malaria began in the 1930s, then gave way to searches for new drugs during World War II and was later followed by anti-mosquito programs. Resistance to drugs and insecticides, lack of money and political instability, however, have reduced the future prospects of malaria eradication and thoughts have again turned to the possibility of developing a successful vaccine.

There has been a marked change of view over recent years concerning the possibilities of developing effective vaccines against malaria. Former pessimism concerning such vaccines was based on the view that since natural infections did not induce complete immunity, vaccination was unlikely to succeed. Recent advances in knowledge of the immune response and, in particular, the demonstration of immunization against *P. falciparum* in *Aotus* monkeys have caused the views to be revised. It is

the belief of many malaria experts that vaccination against malaria is feasible and that the time is now appropriate for a major effort to be made to develop and test such vaccines.

To emphasize the above point, let me quote from a report of the WHO Scientific Working Group on the immunology of malaria (TDR/IM/ 76.3). "The effectiveness of such a vaccine has been demonstrated in a primate model. In vitro cultivation now opens the way to obtain sufficient material to prepare a vaccine. The major obstacle is the development of a suitable adjuvant for use in man. Once this has been achieved and all necessary investigations have been undertaken on safety and efficacy, including studies in the most suitable primate models, such a vaccine would be available for clinical trial in man."

Provided that the objectives are accomplished within a reasonable period of time, the proposed research will bridge the gap between the existing knowledge of the immune response in malaria and the possibility of developing an effective and safe malaria vaccine.

8. Facilities Available

The facilities to be utilized are in the Leahi Hospital which is the location of the Department of Tropical Medicine & Medical Microbiology. Space available to the Department consists of an area of approximately 11,000 sq. ft. which is divided into eighteen fully furnished and equipped laboratory rooms, three offices, a conference- library room, glassware preparation room and six animal rooms.

In addition to the usual glassware and small equipment, the Department is furnished with glassware preparation and decontamination equipment, power, gas, water, benches, cubicles, refrigerated centrifuges, ulta-low temperature freezers, a Beckman L2-65B preparative ultracentrifuge, a Packard PRIAS model PLD liquid scintillation counter, a Gilford spectrophotometer Model 2400S, an Aminco-Bowman spectrophotofluorometer, a Beckman Gamma counter Model 4000, LKB Multiphor flat bed electrophoretic system, several Bio-Rad vertical electrophoretic apparatus, a Shandon preparative electrophoretic apparatus, an ISCO UA-5 UV monitor, fraction collectors, peristaltic pumps, a walk-in cold room, and two walk-in warm rooms (37°C), one of which is used exclusively for in vitro cultivation, and 3 tissue-culture rooms equipped with Laminar Flow hoods.

There is also a modern animal facility covering an area of 4,200 sq. ft. available for housing normal and experimental animals. There is one animal room which is exclusively designed to maintain a temperature of 84°F and 50-70% humidity for housing Aotus monkeys. Food storage, cage washing facilities, animal and waste disposal, and trained primate caretakers are also avilable. Additional long-term holding facilities for primates are available in a trailer located at the basement level of Leahi Hospital and at the Pacific Biomedical Research Center on the Manoa Campus of the University of Hawaii.

9. PERSONNEL

Dr. S. C. Kan: A research biochemist who has been actively involved with this project for the last eight years. He will be involved in all phases of the proposed project, with special emphasis on biochemical aspects of the project.

Dr. K. M. Yamaga: An established immunologist and regular faculty member of the Department of Tropical Medicine & Medical Microbiology, is paid by the University. She has become very much interested in our ongoing research project and will be involved in all phases of immunological work of this project.

Dr. Kevin Palmer: He has been involved with this project for the last four years. His main area of expertise and responsibility will be for large-scale *in vitro* production of *Plasmodium falciparum* antigen.

Dr. Leslie Tam: He has expertise in the area of immunology and will be involved in the production of monoclonal antibodies and other immunological studies.

Mr. Kenton Kramer: He has been involved with this project for the last nine years. He has the major responsibility for conducting vaccination experiments and will assist Dr. Kan in all phases of immuno-biochemical work.

Mr. Steve Case: He has been involved with this project for the last four years and has played a key role in developing monoclonal antibodies in this laboratory. His area of responsibility includes vaccination studies, production of monoclonal antibodies and IFA tests.

Ms. Joann Olejarczyk: She has been involved with this project for the past two years and will be responsible for RIA tests as well as assisting with *in vitro* production of *P. falciparum* antigen.

10. COLLABORATIVE WORK WITH OTHER RESEARCH ORGANIZATIONS

Dr. S. Kotani of the Osaka Institute of Microbiology, Osaka, Japan, will supply us with a series of synthetic adjuvants and will be available for consultation with no cost involved.

Dr. John Miblack of Pfizer Central Research Center, Groton, Connecticut, will also supply us with a series of synthetic adjuvants and will be available for consultation with no cost involved.

11. OTHER RESEARCH PROJECTS CURRENTLY IN PROGRESS

NONE.

12. OTHER SPONSORS

NONE.

13. BUDGET

Summary AID Budget

(July 1, 1984 to June 30, 1987)

		Yr. I FY 1984-85	Yr. II FY 1985-86	Yr. III FY 1986-87
Personnel:	Salaries & Wages	171,660	190,606	200,129
	Fringe Benefits	55,455	61,958	65,053
Supplies		56,500	55,000	45,000
Animals		23,820	23,500	20,000
Travel		11,585	11,585	11,585
Repairs		9,500	9,500	9,000
Other Expenses		3,500	3,500	3,500
Sub-total		332,020	355,649	354,267
Overhead (41%)		136,129	145,816	145,250
		468,149	501,465	499,517
Major Equipment		152,200	30,500	--
TOTAL		620,349	531,965	499,517

Grand total for Yrs. I, II and III = $1,651,831

AID BUDGET

July 1, 1984 to June 30, 1985 (Yr. I)

1. PERSONNEL

Dr. Wasim A. Siddiqui, Principal Investigator	--
Dr. Karen Yamaga, Assistant Professor	--
Dr. S.C. Kan, Assistant Researcher	29,196
Dr. Leslie Tam, Assistant Researcher	25,716
Dr. Kevin Palmer, Assistant Researcher	21,588
Kenton Kramer, Research Assoc. III	19,752
Stephen Case, Research Assoc. II	17,364
Joann Olejarczyk, Research Assoc. I	15,336
Administrative Supervisor, 50%	9,876
Microbiologist (50%), for safety check of specimens	7,668
Student helps (glassware washers, lab. assistant, etc.)	25,164
Total Salaries	171,660
Fringe Benefits	55,455

2. MAJOR EQUIPMENT (Justification enclosed)

High pressure liquid chromatography system	25.000	
ELISA reader	10,000	
Fluorescent microscope with camera	15,000	
Preparative ultracentrifuge with	25,000	
Two rotors and	10,000	
Zonal rotor	10,000	
Microscope with dark field condenser	7,000	
Apparatus for automation for large-scale *in-vitro* cultivation	17,000	
Fraction collector	4,500	
Laminar flow hood	4,500	
Refractometer	3,500	
Conductance meter	1,000	
Immunofuge	500	
Gas mixer	4,500	
IBM personal computer with printer	5,700	
X-ray film processor	4,500	
Large 37°C upright incubator	4,500	
		152,200

3. SUPPLIES

Chemicals, media for in-vitro cultivation	9,000	
Blood, serum, etc.	8,000	
Supplies for monoclonal antibodies	5,000	
Isotope	9,500	
X-ray films and chemicals	4,500	
Affinity beads	3,000	
Protein-A-sepharose beads	4,000	
Chemicals for electrophoresis	3,000	
Disposable glassware, slides, coverslips, etc.	5,500	
Pipets, flasks, beakers, etc.	5,000	
		56,500

4.	ANIMALS		
	Mice (includes per diem)	3,000	
	Rabbits (includes per diem)	3,000	
	Per diem for 30 Aotus monkeys ($1.65/day x 30 days x 30 monkeys x 12 months)	17,820	
			23,820
5.	TRAVEL		
	East Coast conference for 5 professional people ($1,200 + per diem @ $65/day x 7 x 5)	8,275	
	P.I. to visit AID for annual meeting and to attend one scientific conference ($1,200 x $65/day x 7 x 2)	3,310	
			11,585
6.	REPAIRS		
	Old equipment, centrifuge, Scintillation counter, etc.	9,500	
			9,500
7.	OTHER EXPENSES		
	Telephone, postage, cables, drawings and charts	3,500	
			3,500
8.	TOTAL DIRECT COSTS		484,220
9.	INDIRECT COST (41% of MTDC)		136,129
10.	TOTAL AMOUNT REQUESTED		620,349

MAJOR EQUIPMENT

		Yr. I	Yr. II
1.	High pressure liquid chromatography system	25,000	14,000
2.	Small electrophoresis apparatus	--	5,000
3.	ELISA reader	10,000	
4.	Fluorescent microscope with camera	15,000	
5.	Preparative ultracentrifuge with two rotors and Zonal rotor	25,000 10,000 10,000	
6.	Microscope with dark field condenser	7,000	
7.	Apparatus for automation for large-scale in-vitro cultivation	17,000	
8.	Fraction collector	4,500	4,500
9.	Absorbance monitor		3,000
10.	Lyophilizer		4,000
11.	Laminar flow hood	4,500	
12.	Refractometer	3,500	
13.	Conductance meter	1,000	
14.	Immunofuge	500	
15.	Gas mixer	4,500	
16.	IBM personal computer with printer	5,700	
17.	X-ray film processor	4,500	
18.	Large 37°C upright incubator	4,500	
		152,200	30,500

JUSTIFICATION FOR MAJOR EQUIPMENT

1. High pressure liquid chromatography system (HPLC)--We have proposed to use preparative HPLC as one of our major methods for the purification of *Plasmodium falciparum* antigens. In the first year of the present contract, we will need $25,000 for an analytical HPLC system which includes 2 pumps, a solvent delivery programmer, a high pressure mixing chamber, 3 analytical columns (one each of reverse phase, gel permeation and anion exchange), a UV detector, sample injection valves and loops, and a recorder/data processor. In the second year, we will use data from analytical HPLC for preparative work in *P. falciparum* antigen purification. We will then need $14,000 for 2 preparative pumps, one or two preparative columns, a fraction collector and other accessories for adapting the analytical HPLC into a preparative one.

2. Small Electrophoresis apparatus

   a. A multiple slab gel apparatus--We will need it in the beginning of the second year for the separation of *P. falciparum* antigens according to molecular size groups. The cost inclusive of accessories is $1,800.

   b. An electrophoretic blotting cell--We will need it in the second year for analyzing DNA and RNA from *P. falciparum*. The cost inclusive of accessories is $500.

   c. A preparative nucleic acid electrophoresis apparatus--We will need it at the end of the second year for fractionating mRNA mixtures. The cost is $2,700.

3. ELISA reader--We will speed up our production of monoclonal antibodies to *P. falciparum*. Even though the RIA method we have developed for screening hybrid cells is much faster than indirect fluorescent antibody tests, it is still too slow. We will need a much quicker ELISA method, using a sensitive fluorometric enzyme assay and an automatic ELISA reader is $10,000.

4. Fluorescent microscope with camera--Presently, we share the use of a fluorescent microscope with other researchers in our department. In order to avoid our work on monoclonal antibody production being held up by irregular accessibility to the fluorescent microscope, we need one exclusively for malaria research in the beginning of the second year. The cost for a fluorescent microscope with camera of average quality is $15,000.

5. Preparative ultracentrifuge with rotors--Presently, we share the use of a preparative ultracentrifuge with other researchers in our department. Thus the ultracentrifuge is not always available when we need it. In the next 3 years, we anticipate more frequent use of the ultracentrifuge. It is essential for us to have one exclusively for malaria research. We will need to acquire a basically preparative ultracentrifuge with optics for *in situ* analysis of macromolecules during centrifugation. The cost inclusive of 2 rotors (large capacity, 1 angled, 1 swing-bucket) is $25,000. We would also like to have 2 more rotors (1 angled and 1 swing-bucket) of small capacity and the cost is $10,000. In addition, we need a zonal rotor to harvest schizont-enriched parasites by Percoll gradient centrifugation. The cost of a zonal rotor is $10,000.

6. Microscope with dark field condenser--We will need it in the second year to monitor the morphological changes (i.e. presence or absence of knobs) on the furface of infected RBC. The presence of absence of knobs is an indication of both the virulence of the parasite and the time it has been in culture. The cost of a microscope with dark field condenser is $7,000.

7. Additional components and accessories for semi-automated *in vitro* culture system--In the next three years we will work on optimizing conditions of *in vitro* production of parasites in our semi-automated system. These include monitoring pH changes and controlling pH changes with a pH stat, programming the duration of medium changes to coordinate with parasite growth, and monitoring the regulating gas flow. The total cost is $17,000.

8. Two fraction collectors--Presently, we have only one Gilson Mini-Escargot fraction collector. Since we will carry out a lot more liquid chromatography, we will need to acquire 1 fraction collector in the first year and one more in the second year. Each fraction collector inclusive of security valve and refrigeration unit costs $4,500.

9. Absorbance monitor--Presently, we only have 1 ISCO UA5 absorbance monitor. As work progresses, we will need an additional absorbance monitor in the beginning of the second year. The cost is $3,000.

10. Lyophilizer--We will need a lyophilizer in the beginning of the second year to freeze-dry samples in the course of antigen purification. The cost of a satisfactory lyophilizer is $4,000.

11. Laminar flow hood--Presently, we have 4 Laminar flow hoods placed in different rooms exclusively for use in malaria research. Two are for the handling of *in vitro P. falciparum* cultures, one for medium preparation and one for general use. As our work on the production of monoclonal antibodies is expending, we will assign one laboratory exclusively for this purpose in the second year. We will need to install a Laminar flow hood in that laboratory. The cost is $4,500.

12. Refractometer--We will need a refractometer to measure the refractive indices of fractions collected after density gradient centrifugation. The cost is $3,500.

13. Conductance meter--We will need a conductance meter to measure the conductance which is a function of the ionic strength, of fractions collected in gradient liquid chromatography in our work on antigen purification. The cost is $1,000.

14. Immunofuge--Presently, we have two table-top small centrifuges for quick processing of small samples for analytical SDS-PAGE. We will need an additional one to increase our capacity in sample handling. The cost of an Immunofuge is $500.

15. Gas mixer--Until now, we order gas mixtures from a local gas company for use in the *in vitro* culture of *P. falciparum*. The cost of one cylinder of gas mixture is more than 10 times that of O_2, and N_2 and about 7 times that of CO_2. If we purchase a gas mixer, we will be able to prepare gas mixture from pure gases to the right proportions for in vitro culture and save 90% of the gas money. The cost of a gas mixer is $4,500.

16. IBM personal computer with printer--Needed for acquisition, storage and analysis of data generated by work with monoclonal antibodies, *in vitro* cultivation, antigen characterization, and immunological studies in primate and nonprimate animals.

17. X-ray film processor--Towards the identification of *P. falciparum* antigen, the number of fluorographs generated through SDS-PAGE work has reached a level where hand processing of the x-ray films is a major task and it lacks the reproducibility and consistency. An automated system will handle the task in a shorter period of time with more consistency and reproducibility of the quality of fluorographs.

18. Large 37°C upright incubator--In order to accommodate the proposed expansion of our large scale semi-automated culture system, a large size incubabor is needed. We intend to expand the total capacity of our culture system to 12 large scale vessels, but our current incubator has a maximum capacity of only six vessels.

14. APPENDICES:

A. List of strains

B. Copies of published papers

C. Curriculum vitae

APPENDIX I

STRAINS OF *P. FALCIPARUM* AT
THE HUMAN MALARIA RESEARCH LABORATORY
UNIVERSITY OF HAWAII SCHOOL OF MEDICINE

FCH-1 Philippines

Source	U.S. Marine recently returned from Subic Bay, Philippines.
Cultures	Started June 27, 1980.
Stabilites	Frozen December 12, 1982.
Drug Sensitivity	Resistant to pyrimethamine, sensitive to amodiaquine, quinine, and chloroquine.

FCH-2 Papua-New Guinea

Source	American anthropologist recently returned from a study trip in Papua New Guinea.
Cultures	Started November 15, 1980.
Stabilites	Frozen January 7, 1983
Drug Sensitivity	Sensitive to chloroquine

FCH-3 Philippines

Source	U.S. Marine recently returned from Subic Bay, Philippines.
Cultures	Started November 21, 1980.
Stabilites	Frozen December 12, 1983.
Drug Sensitivity	Resistant to chloroquine.

FCH-4 Philippines

Source	U.S. Marine recently returned from Subic Bay, Philippines.
Cultures	Started December 2, 1980.
Stabilites	Frozen November 9, 1981.
Drug Sensitivity	Resistant to chloroquine.

FCH-5

Source	U.S. Marine recently returned from Subic Bay, Philippines.
Cultures	Started December 8, 1980.
Stabilites	Frozen January 19, 1981.
Drug Sensitivity	No tests yet carried out.

FCH-7

Source	3 year old child recently arrived in San Francisco from the Cameroons. Blood was drawn at Mt. Zion Hospital, San Francisco, California and sent to Hawaii. Sample was improperly frozen and an accompanying sample (FCH-6) was lost.
Cultures	Started August 15, 1981.
Stabilites	Frozen October 9, 1981.
Drug Sensitivity	Tests in progress.

FCH-11 Kenya

Source	28 year old American female.
Cultures	Started July 9, 1981
Stabilites	Frozen July 13, 1981.
Drug Sensitivity	No testing done.

FCH-13 India

Source	India (Utar Pradesh)
Cultures	Started August 29, 1981.
Stabilites	Frozen April 27, 1982.
Drug Sensitivity	Sensitive to chloroquine, amodiaquine, and pyrimethamine.

FCH-14 India

Source	20 year-old female Indian.
Cultures	Started August 29, 1981.
Stabilites	Frozen November 13, 1981.
Drug Sensitivity	No tests yet done.

FCH-17 Tanzania

Source	31 year-old female Tanzanian
Cultures	Started January 19, 1982.
Stabilites	Frozen January 7, 1983.
Drug Sensitivity	Resistant to chloroquine.

Honduras I/CDC Honduras

Two vials of stabilite received from CDC/Atlanta on March 2, 1983. Parasitemia at time of freezing was 3.2%.

Source	Choluteca, Honduras. Isolated from a patient on January 25, 1980. Stabilites frozen July 29, 1982.
Cultures	Cultures initiated at MMRL on March 2, 1983.
Stabilites	One original vial saved. Additional stabilite frozen after one passage, March 10, 1983.
Drug Sensitivity	Data from CDC indicate strain was initially sensitive to chloroquine, and resistant to pyrimethamine. Recent information indicates strain has lost some of its sensitivity to chloroquine.

Indochina I/CDC Viet Nam

Two vials of stabilites received from CDC on March 2, 1983. Parasitemia prior to freezing was 3.4%.

Source	Indochina. Isolated from a patient on May 22, 1980. Samples were frozen on August 13, 1982.
Cultures	Begun at HMRL on May 2, 1983. After two passages all culture material was frozen.
Stabilites	One original vial saved. Stabilite frozen after two passages on May 17, 1983.
Drug Sensitivity	Clinically resistna to chloroquine and pyrimethamine-sulphadiazine. In-vitro tests have confirmed the isolate's resistance to chloroquine and pyrimethamine. We have not confirmed these findings.

PAP 135/CDC Haiti

One vial of stabilite received from CDC on March 2, 1983. Parasitemia prior to freezing was 2.0%.

Source	Port-au-Prince, Haiti. Isolated from a patient on November 19, 1982. Samples frozen January 27, 1983.
Cultures	Cultures begun at HMRL on March 2, 1983 and maintained as stock cultures since.
Stabilites	Frozen ?
Drug Sensitivity	In-vivo the parasite were sensitive to Fansidar. In-vitro experiments indicate it to be sensitive to chloroquine and pyrimethamine.

In all cases adequate supplies of all strains are maintained in liquid nitrogen. At regular intervals strains are recovered and grown-up so that additional stabilite can be frozen and made available for specific experiments. An attempt is made to preserve at least one vial of the original isolate for future reference needs.