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P237a
1978/1979

PN-AAG-924

CONTRACT AID/ta-C-1440

AUGUST 15, 1978 to AUGUST 14, 1979

ANNUAL REPORT

MALARIA IMMUNITY AND VACCINATION

ANNUAL REPORT SUMMARY SHEET

Malaria Immunity and Vaccination, AID/ta-C-1440
Project Title and Contract Number

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August 15, 1977 - August 14, 1980
Contract Period (as amended) From - To

August 15, 1978 - August 14, 1979
Reporting Period From - To

Total Expenditures and Obligations through previous contract year	$228,536.00
Total Expenditures and Obligations for current contract year	$331,945.00

NARRATIVE SUMMARY OF ACCOMPLISHMENTS AND UTILIZATION

Plasmodium falciparum, African strain FCR-3, has been grown in flow flasks, candle jars and by stationary culture in gassed 125 ml Erlenmeyer flasks. Antigens from these sources have been used in attempts to isolate and characterize semi-purified and purified parasite antigens. Isolation has been accomplished by $(NH_4)_2 \cdot SO_4$ precipitation and polyacrylamide gel electrophoresis, and characterization has been by biochemical and serological methods. Candidate antigens have been injected into rabbits and the resultant antibodies have been studied by indirect fluoresent antibody techniques as well as in the *in vitro* parasite inhibition test. Serum from immune and non-immune *Aotus* monkeys has been evaluated in the I.F.A.T. tests in conjunction with the *in vitro* parasite inhibition test. These are being used as known positive immune sera in the parasite inhibition blocking test. Purified antigens were evaluated for their ability to block the action of immune antibody in this test. Seven primary clones of *P. falciparum*, FCR-3 strain, have been isolated by limiting dilution techniques *in vitro*, and three of these are being re-cloned through secondary and tertiary cloning steps to provide greater assurance of single-cell origin.

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ANNUAL RESEARCH REPORT

I. General Background

Malaria continues to be the most serious threat to life in the world community of nations, causing more than a million deaths and untold suffering and economic loss annually. Present control and treatment measures are proving to be inadequate against the present day widespread resurgence of malaria; an effective vaccine is sorely needed for use in conjunction with established procedures. The Trager-Jensen(1) system of *in vitro* cultivation of *Plasmodium falciparum* continues to provide materials for serology and vaccination studies in many research laboratories concerned with malaria vaccine development. The usefulness of these cultures in providing parasite materials for these areas of study has been documented (2, 3, 4). When free merozoites are harvested from culture by differential centrifugation red cell contaminants are present and are easily demonstrated serologically. These red cell antigens make the use of such whole merozoite preparations adjuvanted with Freund's Complete Adjuvant undesirable for human vaccination. Thus, there is an obvious need for the development of purified and well-characterized immunogens derived from the cultured parasites. This laboratory continues to isolate and characterize plasmodial antigens derived from culture. The parasite inhibition test described in our previous report, and improved in this report,

may prove capable of measuring one parameter of protective immunity. Development of this test continues, together with a parasite inhibition blocking test to be used for purified antigen evaluation.

The question of antigenic variation occurring in Plasmodium falciparum and its impact upon vaccine development is an important one, and should be addressed relatively early in any such developmental program. We present early results of cloning of P. falciparum by limiting dilution techniques in this report.

References

1. Trager, W. and J. B. Jensen, 1976. Human Malaria in Continuous Culture. Science 193: 673-675.

2. Hall, C., J. D. Haynes, J. D. Chulay and C. L. Diggs, 1978. Cultured Plasmodium falciparum used as Antigen in a Malaria Indirect Fluorescent Antibody Test. Am. J. Trop. Med. Hyg. 27 (5): 849-852.

3. Siddiqui, W. A. 1977. An Effective Immunization of Experimental Monkeys Against a Human Malaria Parasite, Plasmodium falciparum. Science 197: 388-389.

4. Mitchell, G. H., G. A. Butcher, W. H. G. Richards and S. Cohen, 1977. Merozoite Vaccination of Douroucouli Monkeys Against Falciparum Malaria. Lancet 1: 1335-1338.

II. Project Objective

The objective of the project is the development of a safe and effective merozoite vaccine against *Plasmodium falciparum*.

III. Continued Relevance of Objectives

No modifications in our project objective are indicated by the present status of our research, except to state that a possible purified subunit vaccine may be better described as "merozoite derived".

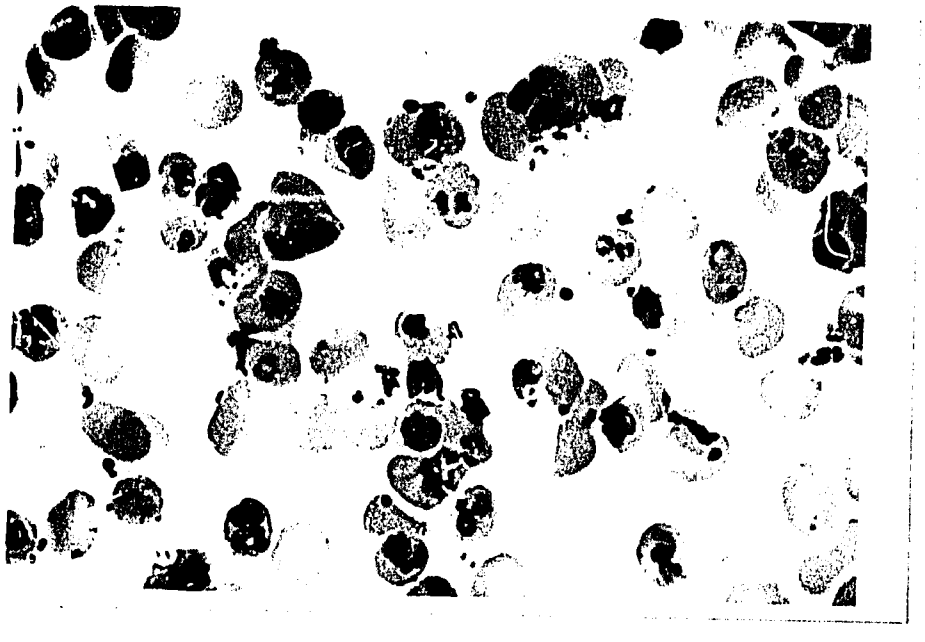
IV. Accomplishments: Aug. 15, 1978 - Aug. 14, 1979

This report details the work accomplished in this laboratory since Feb. 15, 1979. The first six months of this annual period were reported in detail in the Six Month Report for the period Aug. 15, 1978 to Feb. 14, 1979, and will not be reiterated in this report.

During the last six month period of the Project, we have investigated the following aspects of *Plasmodium falciparum* vaccine development:

- Antigen purification and characterization
- Production of anti-malarial serum antibodies in rabbits
- Evaluation of *Aotus* serum antibody levels against *P. falciparum* by indirect fluorescent antibody technique and the *in vitro* parasite inhibition test.
- Evaluation of rabbit serum antibody levels against *P. falciparum* by indirect fluorescent antibody technique

Figure 1



An *in vitro* culture of *P. falciparum* grown by the flow flask culture method with a parasitemia of 60%

and the *in vitro* parasite inhibition test.

- Antigen evaluation in the *in vitro* Parasite Inhibition Blocking Test.
- Cloning of the FCR-3 isolate of *Plasmodium falciparum*.

A. Antigen Purification and Characterization

Parasite cultures for all the procedures discussed below were obtained from flow flask cultures, or from cultures in gassed 125 ml Erlenmeyer flasks. Typical flow flask cultures approximate 8% parasitemia; however, parasitemias as high as 60% have been obtained (see Figure 1).

Procedures:

1. Particulate antigens from culture supernatants, and subfractionation.

   After 2-3 days in culture, the parasite culture supernatants were harvested by centrifugation at 400 g x 10 minutes. The supernatant was removed and centrifuged again at 400 g x 10 minutes. The resultant supernatant (containing free merozoites) was centrifuged at 30,000 g x 30 minutes. The pellet containing merozoites and particulate material was then solubilized at 22°C (in SDS [0.1%] buffer and centrifuged at 12,000 g x 45 minutes to remove hemazoin and other particulates. The supernatant was then precipitated with $(NH_4)_2 \cdot SO_4$ at 30% saturation. After precipitating for 60 minutes, the

suspension was centrifuged at 13,000 g x 45 minutes. The pellet was resuspended in Tris·glycine·SDS buffer and then examined by polyacrylamide gel electrophoresis (P.A.G.E.). All of the above centrifugations and precipitations were carried out at 4°C.

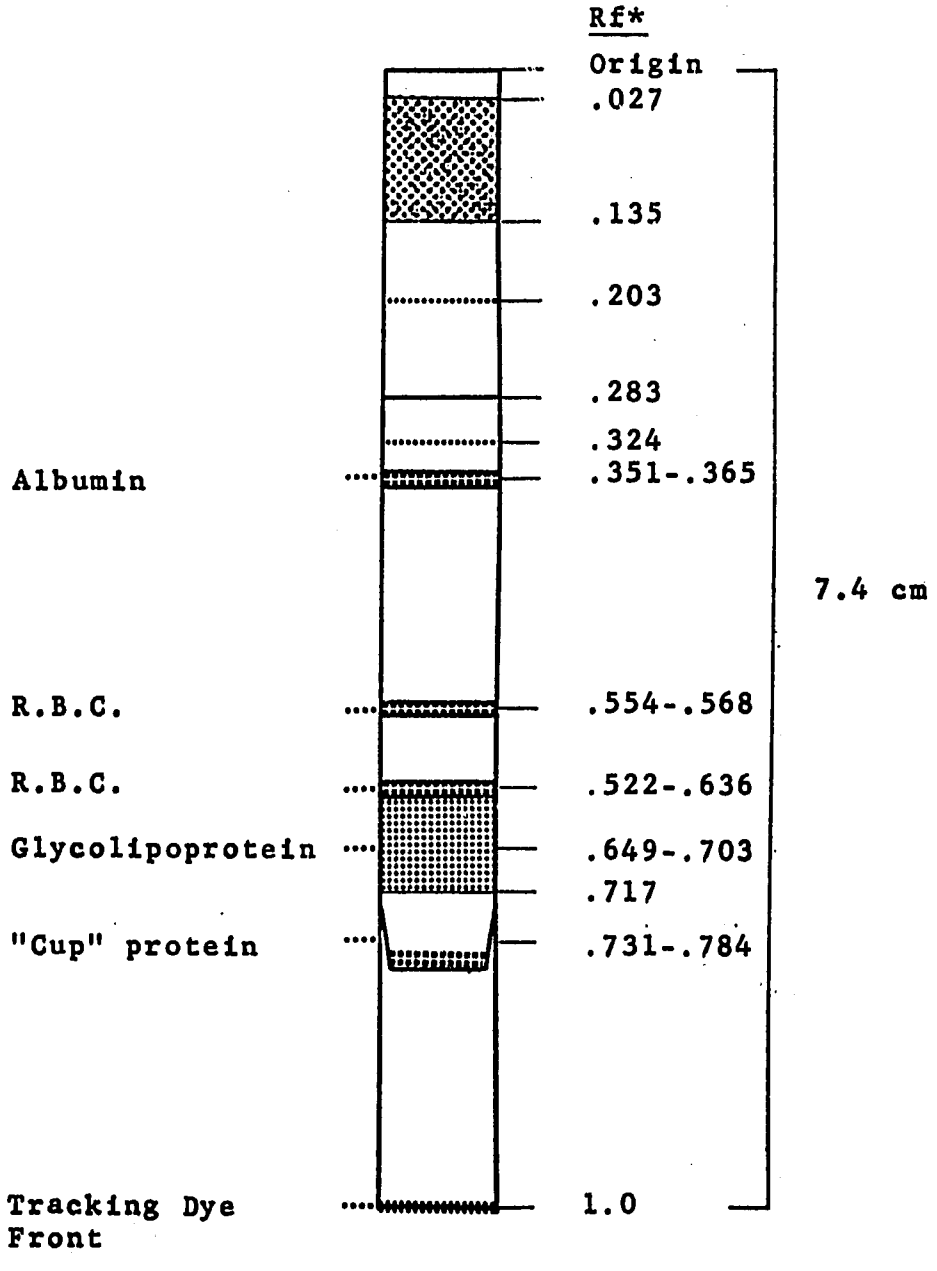
2. Antigens from cultures heated by sorbitol.
Sorbitol has the ability to selectively lyse erythrocytes containing mature stages of malarial parasites. Pellets (400 g x 10 minutes) of infected erythrocytes were mixed with several volumes of 5% sorbitol in PBS, pH 7.2 and then centrifuged (400 g x 10 minutes) for recovery of the supernatant. The supernatant was removed and precipitated with $(NH_4)_2 \cdot SO_4$ at 50% saturation as previously described. Uninfected erythrocyte and medium controls were treated similarly. The precipitated protein fractions were examined by P.A.G.E.

3. Analytical and Preparative Polyacrylamide Gel Electrophoresis (P.A.G.E.)
All antigen preparations were examined by analytical P.A.G.E. using a continuous Tris·glycine·SDS [0.025 M Tris · 0.14 M Glycine · 0.1% SDS] buffer system, pH 8.5. Triplicate gels were stained for protein, carbohydrate or lipid by the Coomassie Blue, Periodic acid Schiff (PAS) or Sudan Black methods respectively.

Figure 2

Schematic Illustration of a P.A.G.E./S.D.S.

Separation of P. falciparum Merozoite Antigens



$$*Rf = \frac{\text{Distance Migrated by protein}}{\text{Distance Migrated by front}}$$

Sufficient amounts of candidate antigens for characterization were obtained by preparative P.A.G.E. Each slab gel (7.4 cm x 7.4 cm) contained six samples of the antigen preparation, with a total of four slab gels used in each electrophoresis. One sample from each slab gel was sliced out intact (vertically), and stained with Coomassie Blue. The remainder of the gel was sliced into 2 mm slices (horizontally). After locating a prospective antigen on the Coomassie Blue stained portion of the gel, the distance from the origin was determined and the corresponding gel slices were identified. The protein was then eluted from the gel with PBS, pH 7.2. The eluted protein was dialyzed against distilled water at 4°C for 24-48 hours and lyophilized.

Results:

Figure 2 is a schematic composite of the P.A.G.E./SDS separation of major merozoite antigens.

Generally, specific antigens are chosen initially by comparison of parasite and control preparations. Several such candidate antigens have been chosen from the preparations described above. The first is in the material precipitated at 50% $(NH_4)_2 \cdot SO_4$ saturation from the supernatant phase of sorbitol-lysed cultures (see above) and also in the material precipitated at 30% $(NH_4)_2 \cdot SO_4$

saturation from the solubilized merozoite fraction obtained from the culture supernatant (see above). This antigen is characterized by a low molecular weight, fast migrating band with a characteristic cup-shaped migration pattern. It is usually located at Rf 0.731 - 0.784 after a two hour electrophoresis at 20 ma per slab gel. A partially purified preparation rich in the cup-shaped protein was used to immunize rabbits as described below. The immune serum was tested using the indirect fluorescent antibody technique (I.F.A.T.). The fluorescent staining was positive for both red blood cell membranes and parasites. The cup-shaped antigen was then purified by preparative P.A.G.E. The migration distance was similar to hemoglobin however, the benzidine peroxidase test, radioimmunoassay tests and amino acid analysis were all negative for hemoglobin. Rabbits were immunized intraperitoneally with 1-2 mg of the purified antigen which was adjuvanted by alum or Complete Freund's Adjuvant. The rabbits were boosted with an aqueous antigen preparation at two week intervals. The immune serum was adsorbed 2-3 times with normal human erythrocytes, type O^+ and then tested using the I.F.A.T. The fluorescent staining was located on the red blood cell membranes and did not appear to be parasite specific. The purified antigen was also used in an attempt to block the parasite inhibition test (details in section IV. E. on Parasite Inhibition Test). This attempt was not successful.

A second antigen preparation was obtained by preparative P.A.G.E. of the 30% $(NH_4)_2 \cdot SO_4$ precipitate from the merozoite fraction (see above). Proteins eluted from the gel slices included in Rf regions from 0.027 to 0.622 were used to immunize rabbits as described above. This preparation represented the above partially purified antigen minus the cup-shaped antigen and a lipid band found immediately above it (see below). The immune serum obtained showed staining of uninfected red cells in the I.F.A.T. test, as well as parasite-specific staining following adsorption with normal human red cells. This preparation is currently being examined more closely in order to locate the individual antigen(s) responsible for the specific parasite staining. A similar antigen preparation as just described was obtained by preparative P.A.G.E. of the 50% $(NH_4)_2 \cdot SO_4$ precipitate of the sorbitol-lysed cultures and is also being investigated.

A third candidate antigen was identified, following analytical P.A.G.E., by differential staining of the gels. The antigen was isolated from SDS solubilized pellets obtained from flow flasks. The antigen is rich in lipid and carbohydrate and has a detectable protein component as well. This antigen migrates with a relative mobility of Rf 0.649 - 0.703 in a 2 hour electrophoresis. The protein component was barely detectable with a Coomassie Blue stain. A protein control experiment using serial dilutions of BSA gave an estimate of 8 μg/ml of protein

in a total antigen concentration of 3 mg/ml (by weight). This glycolipoprotein (GLP) antigen was purified by extraction of the 30% $(NH_4)\cdot SO_4$ precipitate of the merozoite fraction with chloroform:methanol at the ratio 1:2:0.8 (chloroform:methanol:sample). The extracted GLP was moderately soluble in PBS, pH 7.2, and was checked for purity by analytical P.A.G.E. It was found to be relatively pure, except for a minor protein band at approximately Rf 0.324 - 0.336. The possiblity that this may be protein dissociated from the GLP antigen is being investigated. A red blood cell membrane control was extracted in a similar manner and the resulting lipid extract was found to be insoluble in PBS; the red cell lipid migrated similarly to parasite-derived GLP. The GLP antigen which was isolated from merozoites was sometimes found in the preparations prepared by 50% $(NH_4)\cdot SO_4$ precipitation of sorbitol lysed cultures.

The solvent-extracted purified parasite GLP was used to immunize rabbits as described above. Immune serum was collected after two weeks and tested by I.F.A.T. This serum was not adsorbed with red blood cells before the test. The fluorescent staining was positive for parasites with no significant red blood cell staining occurring. This antigen will be tested for blocking activity in the

parasite inhibition blocking test, and for its ability to produce antibodies in rabbits capable of inhibiting parasite reinvasion in the parasite inhibition test. These antisera are currently being raised. Further characterization of the GLF antigen is underway.

B. Production of Anti-Malarial Serum Antibodies in Rabbits

Test antigens were evaluated by examination of the antibodies stimulated in rabbits. Young New Zealand White rabbits were immunized intravenously or intraperitoneally with specific parasite antigens at doses of 1-2 mg, adjuvanted with either alum or Freund's Complete or Incomplete Adjuvant. Antigen boosts were given either with the appropriate adjuvant or in aqueous solution. The animals were bled 7-10 days after antigen injections; bleedings were designated A, B, C, etc. with A being the preimmunization sample, and B, etc. being taken following successive antigen injections. The specific antigens, adjuvants and routes of injection for individual rabbits can be found in Appendix 1. Indirect fluorescent antibody titers of anti-parasite and anti-red cell specificities are also given in Appendix 1. Additional rabbits, not listed in Appendix 1 have been immunized for the production of specific anti-globulins and for specific anti-red cell antibodies for use in the fluorescent antibody procedures.

Table 2
Serological Evaluation of Immune *Aotus* Serum
(Heterologous System)

Immune Monkey #	Date Infected with Strain	Date Tested	I.F.A.T. Titer of 50% Serum/Medium		Parasite Inhibition Test % Parasitemia/1000 Cells (I.S.* per 50 fields) 50% Serum Day 0		50% Serum Day 2	10% Serum Day 0		10% Serum Day 2
B14	FVO 10/28/77 (1/28/78)**	5/7/79	160	C_1	0.7	->	2.2 (0)	C_1 0.9	->	3.8 (0)
				C_2	0.7	->	2.4 (0)	C_2 0.6	->	4.1 (0)
			320	I_1	0.8	->	0.7 (8)	I_1 0.7	->	2.5 (1)
				I_2	0.5	->	0.3 (6)	I_2 0.8	->	2.6 (0)
B18	FVO 11/16/77 (3/1/78)	5/21/79	10	C_1	0.7	->	1.8 (1)	N.D.		
				C_2	0.7	->	1.9 (1)			
			1280	I_1	0.5	->	1.2 (42)			
				I_2	1.0	->	1.8 (15)			

*I.S. = "Inhibited Schizonts" - see description IV. C. page 11

**date of last known parasitemia given in parentheses

C. Evaluation of Aotus Serum Antibody Levels Against P. falciparum by Indirect Fluorescent Antibody Technique and the in vitro Parasite Inhibition Test

The details of the I.F.A.T. test procedure are presented in Appendix 2, and the procedure for the *in vitro* P.I.T. is presented in Appendix 3, with results in Tables 1 & 2.

An additional feature of the *in vitro* parasite inhibition test which was not reported in the previous 6 month report is the observation that numerous schizonts from which the merozoites have failed to disperse are present in the test cultures containing immune serum. These forms always have pigment associated with them, and the presence of a red cell membrane (typically fragmented) can be readily detected with interference microscopy and I.F.A.T. There is a strong correlation between the presence of these forms which we have termed "inhibited schizonts", and the presence and concentration of immune serum. We believe that, at the moment of schizont rupture, antibody enters the cell and agglutinates the enclosed merozoites, thereby preventing their dispersal if the specific antibody is present in sufficient titer to accomplish this before dispersal can occur. The gross membrane damage probably is an artifact of the blood film preparation, since pigment granules are invariably present in these forms. The assessment of the

Table 1
Serological Evaluation of Immune *Aotus* Serum
(Homologous System)

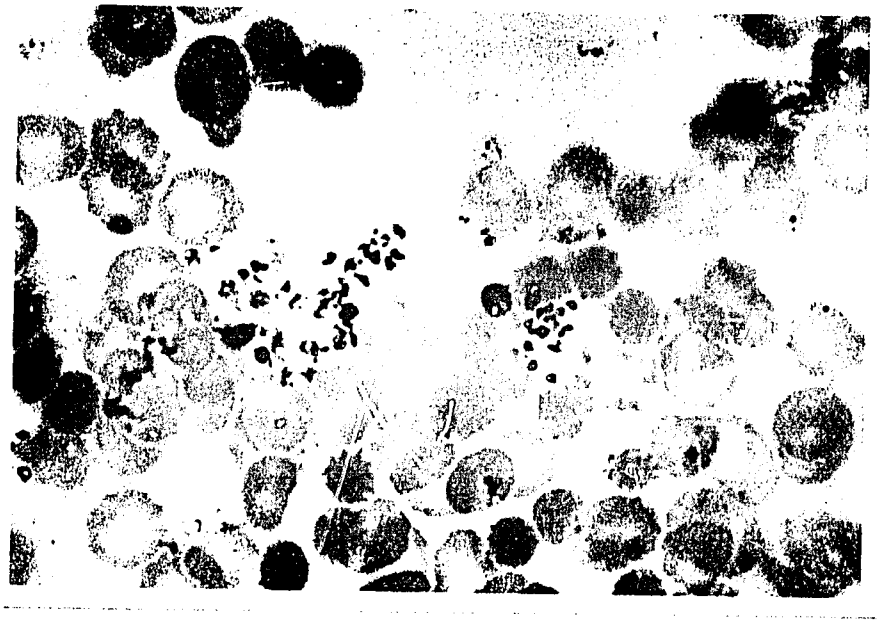
Immune Monkey #	Date Infected with Strain	Date Tested	I.F.A.T. Titer of 50% Serum/Medium		Parasite Inhibition Test: % Parasitemia/1000 Cells (I.S.* per 50 fields) 50% Serum Day 0		50% Serum Day 2	10% Serum Day 0		10% Serum Day 2
B3	FCR-3	4/27/79	80	C_1	0.7	->	1.5 (2)	C_1 0.6	->	2.7 (0)
	12/30/77			C_2	0.6	->	2.5 (5)	C_2 0.7	->	2.7 (0)
	(4/6/78)**		320	I_1	0.8	->	0.8 (24)	I_1 0.5	->	1.8 (4)
				I_2	0.8	->	0.5 (14)	I_2 0.7	->	1.9 (4)
B9***	FCR-3	7/10/79	10	C_1	1.0	->	4.8 (1)	N.D.		
	11/3/77			C_2	1.0	->	3.6 (1)			
	(7/3/78)		640	I_1	1.4	->	1.1 (41)			
				I_2	0.7	->	1.4 (22)			
B20	FCR-3	4/5/79	80	C_1	0.8	->	2.3 (1)	C_1 0.7	->	4.3 (0)
	2/2/79			C_2	0.9	->	3.0 (6)	C_2 0.5	->	2.9 (0)
	(4/5/79)		1280	I_1	0.6	->	1.7 (24)	I_1 0.7	->	3.2 (0)
				I_2	0.6	->	1.9 (30)	I_2 0.6	->	3.1 (1)
B21	FCR-3 a)	3/23/79	160	C_1	0.6	->	2.1 (7)	C_1 1.3	->	6.0 (1)
	4/7/78			C_2	0.6	->	1.7 (2)	C_2 1.4	->	7.6 (0)
			2560	I_1	0.4	->	0.4 (45)	I_1 0.9	->	4.2 (6)
	(4/27/78)			I_2	0.5	->	0.2 (33)	I_2 0.9	->	4.4 (6)
	b)	4/16/79	40	C_1	0.7	->	2.8 (2)	C_1 0.5	->	2.9 (0)
				C_2	0.8	->	1.3 (6)	C_2 0.4	->	3.1 (0)
			2560	I_1	0.6	->	0.4 (34)	I_1 0.6	->	1.2 (6)
				I_2	0.9	->	0.4 (41)	I_2 1.0	->	1.4 (6)

*IS ="Inhibited Schizonts" - see description IV. C. page 11

**date of last known parasitemia given in parentheses

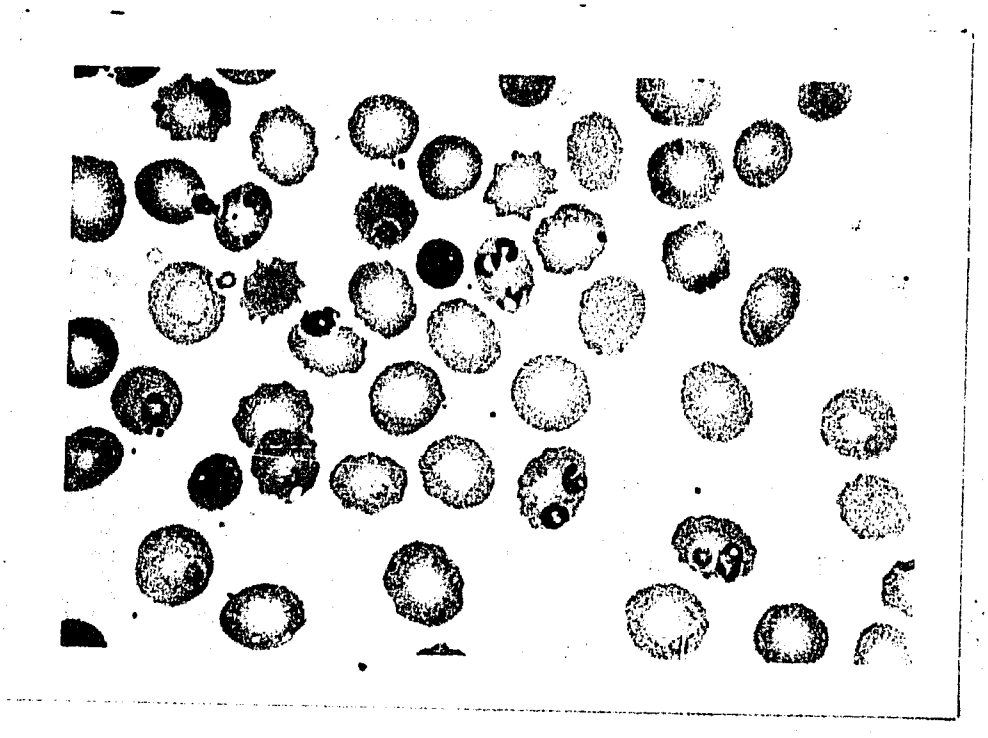
***splenectomized

Figure 3



A photomicrograph of a typical Day 2 culture containing "inhibited schizonts" of *P. falciparum* found in an *in vitro* parasite inhibition test of immune *Aotus* serum.

Figure 4



A photomicrograph of a typical Day 2 culture containing normal rings of *P. falciparum* found in an *in vitro* parasite inhibition test of normal *Aotus* serum.

relative numbers of these forms has been made by counting the number of "inhibited schizonts" per 10,000 cells (approximately 50 fields). Figure 3 is a photomicrograph of a typical field containing "inhibited schizonts" found in an *in vitro* parasite inhibition test of immune *Aotus* serum. Figure 4 is a photomicrograph of a typical field found in the normal serum control in the same test. The parasitemia antibody concentration is important to the mechanism acting in the parasite inhibition test to reduce reinvasion. Also, the presence and numbers of "inhibited schizonts" were a dramatic indicator of the ability of immune sera to inhibit reinvasion by merozoites in culture. Even in the heterologous FVO/FCR-3 test system these forms were found when no clear cut effect on parasitemia was apparent (e.g. B18).

D. Evaluation of Rabbit Serum Antibody Levels Against P. falciparum Antigens by Indirect Fluorescent Antibody Techniques and the in vitro Parasite Inhibition Tests

The results of the I.F.A.T. tests performed on rabbit serum raised against a variety of parasite-derived antigens are presented in Appendix 1. These results are summarized briefly below, together with comments on the parasite inhibition test when appropriate. The antigens are identified by the method of their preparation as well as their characteristic relative mobilities (Rf) in P.A.G.E. (Figure 2). The antigens of primary interest are:

1. Intact merozoites. Whole merozoites as antigens stimulate the production of rabbit antibodies against both red cells and parasites.

2. $(NH_4)_2 \cdot SO_4$ precipitation of sorbitol lysate of schizont-infected blood. These semi-purified antigens stimulated rabbit antibodies against both red cells and parasites. The parasite stain showed peculiar surface-type staining. This preparation included the main cup-shaped antigen (Rf 0.731 - 0.784) as well as variable amounts of the glycolipoprotein (GLP) found at Rf 0.649 - 0.703. Additional minor bands are found in the upper portion of the P.A.G.E. separation.

3. Subfraction of sorbitol lysate antigen (2) above further purified by elution of antigens found in P.A.G.E. gel in region from Rf 0.0 to Rf 0.636. This portion of the sorbitol lysate excluded the G.L.P. and the cup-shaped antigen found lower in the separation. These antigens stimulated rabbit antibodies against red cells, but not parasites after one immunization. Further boosts and testing will be done and the possibility of SDS denaturation of proteins is being investigated.

4. $(NH_4) \cdot {}_2SO_4$ precipitation of SDS solubilized whole merozoites obtained by differential centrifugation. These antigens when injected into rabbits produced high titers of antibodies against red cells, and moderate to low

titers against parasites. This preparation includes both the GLP found at Rf 0.649 - 0.703 and the "cup shaped" protein found at Rf 0.731 - 0.784, as well as numerous minor bands found above these areas in P.A.G.E. separations.

5. "Cup-shaped protein" band antigen purified by elution from P.A.G.E. preparative gels (Rf 0.649 - 0.703). This antigen is a purified major component of antigens 1, 2 and 4 above. In rabbits it produced antibody titers against human red cells, but not against P. falciparum parasites. These antibodies agglutinate human red cells in vitro, but fail to lyse them in the presence of fresh guinea pig complement.

6. Glycolipoprotein (GLP) purified by extraction of merozoites with chloroform:methanol and shown to be pure by P.A.G.E. It is found in the region Rf 0.649 - 0.703. A minor protein band, apparently dissociated from the complex after extraction is also seen at Rf 0.324 - 0.336. This GLP antigen is present in the partially purified antigens 1, 2 and 4 above. When this purified material was injected into rabbits, it stimulated the production of antibodies against parasites of all stages, but not against red cells. The fluorescent staining in I.F.A.T. defined parasite membranes rather than internal structures.

This rabbit antiserum will be tested in the *in vitro* P.I.T. procedure when higher titers are achieved, and the GLP antigen will be evaluated for its ability to block the P.I.T. with known positive *Aotus* serum. See "E. Antigen Evaluation in the *in vitro* Parasite Inhibition Blocking Test" below.

7. $(NH_4)_2 \cdot SO_4$ precipitation-derived merozoite antigens semi-purified by elution of preparative P.A.G.E. gels from the region Rf 0.0 to Rf 0.636. This preparation is a subfraction of (4) above which excludes the GLP and "cup-shaped" protein found at Rf 0.649 through 0.784. This antigen preparation resembled (4) above in the stimulation of rabbit antibodies in moderate titer against human red blood cells and low titer against *P. falciparum* parasites.

E. Antigen Evaluation in the in vitro Parasite Inhibition Blocking Test

This test, which is based upon the *in vitro* Parasite Inhibition Test described in Appendix 3 is intended to provide a method to evaluate the protective potential of a specific antigen. This is done by measuring the test antigen's capacity for blocking the ability of immune serum to inhibit parasite reinvasion *in vitro*. The test is still under development in this laboratory. However, test data on the "cup-shaped" protein (Rf 0.731 - 0.784) described in D.5.

above are presented in Table 4. When this pure material was present at either 17 μg or 1000 μg per 1.5 ml of culture medium containing 50% immune Aotus serum, the ability of the immune serum to inhibit reinvasion was not hindered. Control cultures with non-immune serum were used as well as a hemoglobin antigen control. Testing of other parasite antigens will proceed as such pure antigens become available.

F. Cloning of the FCR-3 Isolate of Plasmodium falciparum

Seven clones of the FCR-3 strain of Plasmodium falciparum were obtained using limiting dilutions of parasitized human O^+ red blood cells (P.RBC). The P•RBC suspension was diluted to give one P•RBC every fifth culture sample. Fifty-four cultures were started on Day 0 and seven were found to be positive on Day 14 (the greatest number of positives expected was 10 or 11). From the seven primary clones, three have been chosen, based on initial growth rates, for secondary and tertiary cloning. Seven primary and one secondary clones have been successfully frozen in liquid nitrogen. Presently, we have one tertiary and another secondary clone ready for storage in liquid nitrogen. The clones have been named following the suggestions of Jensen and Trager.* Thus, the primary clones are FCR-3/PD-2 (Cl, 1°)

*J. B. Jensen and W. Trager. 1978. Plasmodium falciparum in culture: Establishment of additional strains. Am. J. Trop. Med. Hyg. 27 (4): 743-746.

through FCR-3/PD-2 (7, 1°); the secondary clones are FCR-3/PD-2 (C5, 2°) and FCR-3/PD-2 (C6, 2°); and the tertiary clone is FCR-3/PD-2 (C5, 3°).

The medium used throughout was RPMI-1640 containing 2 gm/liter $NaHCO_3$, 5.95 gm/liter HEPES buffer and human O^+ serum at a final concentration of 10%. For the first 14 days, cultures were maintained in antibiotic-free medium. After that time, gentamicin was added at a concentration of 50 mg/liter. Spent supernatant medium was removed daily and replaced with fresh medium. Fresh washed red cells were added every 3-6 days, effecting a red cell dilution of 1:2 or 1:4 by 1 of 2 methods: (1) transferring the entire contents of each culture well to progressively larger wells to which RBC's at the same hematocrit, have been added; or (2) increasing the hematocrit by adding an equal volume of RBC's to the same well. Three sizes of culture cluster plates (Linbro, division of Flow Labs) were used: 96 well, 0.5 cm^2; 24 well, 2 cm^2; and 6 well, 9.6 cm^2. Hematocrits used in the procedure were 3%, 6% and 12.5%.

V. Dissemination and Utilization of Research Results

Presentations of research results during the period included in this Annual Report were made at the NMRI, USAID, WHO Workshop held in Bethesda, MD in October, 1978 and at the International Conference on Malaria and Babesiosis held in Mexico City, Mexico, April 30 - May 3, 1979.

VI. Work Plan for the Coming Six Months

A. We will continue to expand our culture capability to meet our increased demand for antigens.

B. The *in vitro* parasite inhibition test will be employed extensively to evaluate immune sera, and the *in vitro* parasite inhibition blocking test will undergo further development and will be utilized in attempts to identify protective antigens.

C. Isolation of merozoite antigens will continue together with their biochemical and serological characterization. Promsing candidate antigens will be tested *in vitro* before being considered as primate immunogens.

VII. Involvement of Minority Personnel and Women

Six women including one minority were involved in project activities during the reporting period.

VIII. Financial Statement

The expenditures and obligations related to the budget plan from 8/15/78 through 8/14/79 and from 8/15/79 through 8/14/80 are presented as follows:

Expenditures	Period 8/15/78 to 8/14/79	Period 8/15/79 to 8/14/80 (est.)
Salaries and Wages including Fringe Benefits	$179,016	$182,500
Consultant Fees	93	3,000
Travel and Transportation	0	1,150
Other Direct Costs	0	500
Overhead	119,404	14,245
Research & General Administration	9,041	10,971
Supplies	24,391	35,738
TOTAL COST TO GOVERNMENT	$331,945	$348,104

Appendix 1

IFAT Summary: Rabbits Immunized with Malarial Antigens

Rabbit #	Immunogen	Route	Adjuvant	Serum	Titer as whole serum IFAT ps	Titer as whole serum IFAT rbc	Description and/or Comments
G'	Fresh merozoites harvested by differential centrifugation	IP	CFA				Staining against all parasite stages with late stages brighest and easiest to distinguish
	boosted 4x	IP	aqueous	1 week post last boost adsorbed 3x vs RBC	5120	10,000	Red cell membranes also fluoresce after 3x adsorbtion
G2		IP	CFA		2560	≥ 10,000	See 1
G3		IP	CFA		2560		See 1
G5		IV	None				See 1
	boosted 2x	IV	aqueous				
	boosted 2x	IP	aqueous		2560		
G6	Fresh merozoites harvested by differential centrifugation	IV	None				See 1
	(see 5)	IV	aqueous				
		IP	aqueous		2560		

Appendix 1

IFAT Summary: Rabbits Immunized with Malarial Antigens

Rabbit #	Immunogen	Route	Adjuvant	Serum	Titer as whole serum IFAT ps	IFAT rbc	Description and/or Comments
G7	Fresh merozoites harvested by differential centrifugation	IV	None				
	boosted 1x	IV.	aqueous		no apparent titer	> 320	
	Dead						
G11	Pool of 50% $(NH_4)_2SO_4$ (sorbitol) cuts	IP	Alum				C. A more discriminate antibody than that of the pool (G1-6D) at the same level of fluorescent intensity i.e., G-11 stains fewer parasites yet their morphology is easier to distinguish because of excellent edge (surface) staining
	boost 50% (sorbitol)	IP	Alum	C. adsorbed 3x	160		
	boost 50% (sorbitol)	IP	Alum	D. adsorbed 3x	640	2560	D. Strong red cell titer inteferes Some discriminate parasite surface staining found - not as frequent or as obvious as before (C)
G12	Pool of 60% $(NH_4)_2SO_4$ sorbitol cuts	IP	Alum				
	boost 50% (sorbitol)	IP	Alum	C. adsorbed 3x	80	≥ 1280	Not as vivid as G-11 - Good surface staining of late stage parasites
	Dead						

Appendix 1

IFAT Summary: Rabbits Immunized with Malarial Antigens

Rabbit #	Immunogen	Route	Adjuvant	Serum	Titer as whole serum IFAT ps	IFAT rbc	Description and/or Comments
G13	50% $(NH_4)_2SO_4$ merozoite harvest	IP	CFA				
	boost 50% M's	IP	aqueous	adsorbed not adsorbed	20	640	Strong red cell antibody makes it difficult to distinguish parasites
	boost 50% M's	IP	aqueous				
	boost 50% M's	IP	aqueous				
G14	50% $(NH_4)_2SO_4$ cut Merozoite Pool	IP	CFA				See 13
	50% cut M pool	IP	aqueous	C 3x adsorbed unadsorbed	20	1280	
G16	50% $(NH_4)_2SO_4$ cut Merozoite Pool	IP	Alum				See 13
	50% cut M pool	IP	Alum	C 3x adsorbed unadsorbed	40	640	
	30% cut	IP	aqueous				
	Dead						

Appendix 1

IFAT Summary: Rabbits Immunized with Malarial Antigens

Rabbit #	Immunogen	Route	Adjuvant	Serum	Titer as whole serum IFAT ps	IFAT rbc	Description and/or Comments
G18	50% $(NH_4)_2SO_4$ cut Merozoite pool	IP	Alum				See 13
	50% cut M pool	IP	Alum	C 3x	80		
				adsorbed		160	
				unadsorbed		320	
	30% cut M pool	IP	aqueous				
G19	Sorbitol lysate 50% $(NH_4)_2SO_4$ cut	IP	CFA	C			
				adsorbed	20	160	
				unadsorbed		320	
G23	"Cup" shaped band eluted from gel	IP	Alum	C 3x adsorbed	neg	intact > 160 dehem. > 160	Strong relief staining around edges of cells. Membrane staining is spotted with the outside not as well defined as the inside of the rbc - dehemoglobinized cells stain brighter than fixed ones
G28	Merozoite derived gel above lipid	IP	Alum		20	intact 40	All parasite stages stain ≡ red cell staining looks like it may define an internal membrane antigen
	boost	IP	Alum	C 3x adsorbed		dehem. 320	

Appendix 1

IFAT Summary: Rabbits Immunized with Malarial Antigens

Rabbit #	Immunogen	Route	Adjuvant	Serum	Titer as whole serum IFAT ps	IFAT rbc		Description and/or Comments
G29	Merozoite derived gel above lipid	IP	Alum	3x adsorbed	20	intact dehem.	40 320	See 28
	boost	IP	Alum					
G30	Merozoite derived gel above lipid	IP	CFA	C 3x adsorbed	40	intact dehem.	40 160	
	"	IP	IFA					
G31	Merozoite derived gel above lipid	IP	CFA	C 3x adsorbed	40	intact dehem.	40 160	
	"	IP	IFA					
G33	Sorbitol derived gel above lipid	IP	Alum	B 3x adsorbed	neg		> 320	Strong red cell membrane staining
G34	Sorbitol derived gel above lipid	IP	Alum	B 3x adsorbed	neg		> 320	
G35	Sorbitol derived gel above lipid	IP	CFA	B 3x adsorbed	neg		> 320	

Appendix 1

IFAT Summary: Rabbits Immunized with Malarial Antigens

Rabbit #	Immunogen	Route	Adjuvant	Serum	Titer as whole serum IFAT ps	IFAT rbc	Description and/or Comments
G38	Sorbitol derived gel above lipid	IP	CFA	3x adsorbed	10?	> 320	
G41	"Cup" shaped gel band	IP	Alum				N.D.
G43	lipoglycocomplex (GLP)	IP	Alum*	B not adsorbed	40	0	Good discriminate parasite staining with no red cell background - membranes of parasitized red cells do not fluoresce
G44	lipoglycocomplex (GLP)	IP	CFA	B not adsorbed	20	~10	Parasite staining not as discriminate as G43B. Red cells positive @ 1/10 and @ 1/20 - more nonspecific background as well

*Alum precipitate not washed. Precipitate and supernate mixed for injection.

Appendix 2

Indirect Fluorescent Antibody Test - Procedure

Reagents & Materials:

Fluorescein conjugate: F.I.T.C. - Goat anti-rabbit IgG* at 1:20 dilution in PBS, pH 7.2. Adsorbed 2 x with mouse liver powder (50 mg/cc).

Antigen slides: Smears (medium) made from flow flask packed cells (4000 x 10 min), preferably > 10% parasitemia, rich in late stages. Slides divided across middle with barrier of nail polish and silicone grease. Cells are dehemoglobinized with 0.1% HCl and rinsed in distilled water once and PBS twice.

Antibody: Serum complement was inactivated at 56°C for 30 minutes, after which the serum was adsorbed 1 x 4°C overnight, followed by 2 x 37°C for 1 hour. (Adsorption overnight at 37°C was found to be detrimental to antiparasite titer. Normal and immune sera were serially diluted 2-fold in PBS, pH 7.2.

Procedure:

Antigen slides were flooded with normal or immune serum dilutions and incubated at 37°C for 30 minutes. The slides were then rinsed 3 x 5 minutes in PBS pH 7.2 at 37°C. Following the rinses, the slides were flooded with goat anti-rabbit IgG fluorescein conjugate and incubated for 30 minutes at 37°C. The slides were again rinsed 3 x 5 minutes in PBS pH 7.2 at

*Microbiological Associates, Walkersville, MD.

37°C. The wet slides were then coverslipped with one drop of mounting medium (50% glycerol + 50% PBS) followed by a small glass coverslip.

Microscopic examination:

A Zeiss fluorescence microscope with a mercury vapor ultraviolet light source was used to examine the fluorescent preparations. The exciter filter was the UGI and the barrier filters were -65 and 47, and the oil immersion objective was 43 x. Slides were rated from 4+ to 1+, titer was taken as the last 1+ reading while +/- readings were noted.

Appendix 3

Procedure for the in vitro Parasite Inhibition Test

Inactivate sera to be tested at 56°C for 30 minutes. Adsorb both the immune and normal control serum with an equal volume of packed human erythrocytes overnight at 4°C, then twice more at 37°C for one hour. Separate the serum from the red cells by centrifugation at 640 g x 10 minutes.

Day 0

Prepare RPMI 1640 culture medium with 10% or 50% v/v adsorbed normal or immune serum supplement.

Treat 1 volume of packed infected red cells with 5 volumes of 5% sorbitol in pH 7.2 PBS for 1 minute at room temperature, then wash with RPMI 1640 and dilute packed cells 1:16 (6% cell suspension) with RPMI 1640 supplemented with the appropriate serum. Plate 1.5 ml volumes in triplicate in 35 mm Falcon plastic petri dishes (or 0.5 ml volumes in triplicate in 17 mm wells using Linbro Tissue Culture Multi-well plates) and culture in burned candle jars at 37°C.

Make Giemsa-stained blood cell films before sorbitol treatment and at the time of plating of the suspension.

Day 1

Make Giemsa-stained blood cell films from all plates at the hour corresponding to when the cultures were plated on Day 0.

Refeed all plates by removing the supernatant medium and replacing it with an appropriate volume of complete fresh medium.

Day 2

Make Giemsa-stained blood cell films from all plates at the hour corresponding to when the cultures were plated on Day 0.

On all of the blood films taken, count and plot the total number of parasites per 1000 red cells (expressed as percent parasitemia) and count the percent of parasites present in the ring form. Note the presence or absence of merozoites associated with red cells and any degenerate older forms. Then count 10,000 cells (50 fields averaging 200 cells per field) for the presence and number of "inhibited schizonts". These forms which we have termed "inhibited schizonts" have been regularly found in cultures containing immune serum. They appear to be segmenters from which the merozoites have failed to disperse. These forms always have pigment associated with them, and the presence of a red cell membrane (typically fragmented) can be readily detected by interference microscopy or I.F.A.T. See Figure 3 in report.

Interpretation of Results

Day 0 culture after sorbitol treatment should contain viable parasites almost entirely in the "ring" trophozoite form. Day 1 cultures should consist of parasites in the older

trophozoite stage and the schizont stage. These should be examined carefully for evidence of degeneration due to non-antibody factors in inhibitory serum. Day 2 cultures may show a reduction or absence of ring forms if the serum tested has inhibitory activity against parasite multiplicatio Any decrease in the percent parasitemia in those cultures containing immune sera could also reflect inhibition. The presence of numerous "inhibited schizonts" has thus far only been associated with immune sera, and is seemingly a direct result of protective antibody action. Since many factors could cause a fall in parasitemia, this "inhibited schizont" phenomenon may prove to be a much more meaningful parameter than percent parasitemia, or other growth-related methods such as radiolabeled amino acid incorporation by parasites.