9310453-52 new
PD-AAC-774
ISN 26735
9310453005302
attached 9310453005802

CONTRACT AID/ta-C-1440

AUGUST 15, 1979 TO FEBRUARY 14, 1980

SIX-MONTH REPORT

MALARIA IMMUNITY AND VACCINATION

ANNUAL REPORT SUMMARY SHEET

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

Robert G. Brackett, Ph.D. and Theodore J. Green, Ph.D., Warner-Lambert/Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company
Co-Principal Investigators and Contractor

P.O. Box 118, Detroit, Michigan 48232
Contractor's Address

August 15, 1977 - August 14, 1980 Contract Period (as amended) From - To	August 15, 1979 - February 14, 1980 Reporting Period, From - To
Total Expenditures and Obligations Through Previous Contract Year	$331,945.00
Total Expenditures and Obligations For Current Contract Year	$319,446.00

Narrative Summary of Accomplishments and Utilization

Plasmodium falciparum, African strain FCR-3/FMG has been grown in flow flasks, candle jars and by stationary culture in gassed plastic tissue culture flasks. Merozoites from these sources have been used for antigen isolation and purification. This has been accomplished by $(NH_4)_2 \cdot SO_4$ precipitation, polyacrylamide gel electrophoresis, column chromatography and solvent extraction techniques. Candidate antigens were subsequently used to immunize rabbits. The resultant antibodies have been studied by indirect fluorescent and ferritin-labeled antibody techniques. A purified antigen has been isolated and partially characterized which stimulated no antibodies against either uninfected or infected host erythrocytes, nor against immature *P. falciparum* parasites but did stimulate the production of antibodies which were specific for the merozoite surface coat.

Evaluation and refinement of the *in vitro* parasite inhibition test has continued, using *Aotus* and human malaria-immune sera.

Three single-cell clones of *P. falciparum* FCR-3/FMG have been isolated by limiting dilution techniques repeated serially three times. These three clones have been characterized by their rates of growth in culture, their behavior in gelatin enrichment techniques (thought to be associated with the presence of "knobs" on the host cell surface), by electronmicroscopic morphological examination, and by pyrimethamine sensitivity *in vitro*.

Twenty-four new *Aotus trivirgatus griseamembra* monkeys have been obtained and are currently being karyotyped. The complete karyotyping procedure, adapted to *Aotus* monkeys, is presented.

In summary, new and interesting findings are presented in this report in the areas of identification of merozoite surface coat antigen, evaluation of malaria immune sera and characterization of *P. falciparum* clones.

Table of Contents

Six Month Research Report

		Page
I.	General Background	1
II.	Project Objective	4
III.	Continued Relevance of Objectives	4
IV.	Accomplishments: Aug. 15, 1979 to Feb. 14, 1980	4
	A. Antigen Purification and Characterization	5
	B. Production of Rabbit-origin Anti-malarial Serum Antibodies and Their Evaluation by Indirect Antibody Techniques	6
	C. The *in vitro* Parasite Inhibition Test for the Evaluation of Malaria-immune Sera (manuscript)	9
	D. Characterization of Three *P. falciparum* Clones	11
	E. Procedure for Karyotyping of *Aotus* Monkeys	13
V.	Dissemination and Utilization of Research Results	13
VI.	Work Plan for the Coming Six Months	13
VII.	Development of Minority Personnel and Women	15
VIII.	Financial Statement	16

Six-Month Research Report

I. General Background

The need for additional means to effect malaria control worldwide is becoming more urgent as the resurgence of this disease continues in spite of the application of traditional control measures. The development of an effective malaria vaccine is viewed as a major goal in the search for additional malaria control methods.

The basic source for asexual parasite antigens for vaccine development is the recovery of free merozoites from *in vitro* cultures of *P. falciparum* cultivated by the Trager-Jensen (1) method. When harvested from culture supernatants by differential centrifugation techniques these merozoite antigens are contaminated with host red cell antigens, making these preparations unsuitable as vaccines without further purification. Thus, there is a need for the identification of purified, well characterized immunogens derived from merozoites recovered from parasite cultures.

As an adjunct to vaccine development, there exists a basic need for an *in vitro* method for the evaluation of the immune status of individual sera against malaria. Considerable progress has

been made in this laboratory on such a test. This test should prove of benefit in the evaluation of individual sera, candidate immunogens and immunization regimens.

The question of antigenic variation of plasmodial strains remains unresolved at this time. Toward this end we have isolated several P. falciparum clones in culture, and are currently characterizing them by various methods; specifically, the presence of "knobs" (2) on the infected host cells, the behavior of the parasitized cells in gelatin-based enrichment procedures (3), the average rates of growth in culture and the sensitivity of the parasites to pyrimethamine in vitro based upon the method of Desjardins et al. (4).

Karyotyping of Aotus monkeys is believed to have predictive value as to the relative susceptibility of the animals to P. falciparum infection (5). Interpretation of the karyotypes is generally based upon the work of Ma et al. (6), but the precise laboratory procedures as adapted to Aotus monkeys are not readily available; therefore, we have presented the technique currently in use in this laboratory in sufficient detail that other investigators may utilize it.

References

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

2. Langreth, S. G., J. B. Jensen, R. T. Reese and W. Trager. 1978. Fine Structure of Human Malaria *In Vitro*. J. Protozool. 25 (4): 443-452.

3. Jensen, J. B. 1978. Concentration from Continuous Culture of Erythrocytes Infected with Trophozoites and Schizonts of *P. falciparum*. Am. J. Trop. Med. Hyg. 27 (6): 1274-1276.

4. Desjardins, R. E., C. J. Canfield, J. D. Haynes and J. D. Chulay. 1979. Quantitative Assessment of Antimalarial Activity *In Vitro* by a Semiautomated Microdilution Technique. Antimicrobial Agents and Chemotherapy 16 (6): 710-718.

5. Taylor, D. W. and W. A. Siddiqui. 1979. Susceptibility of Owl Monkeys to *Plasmodium falciparum* Infection in Relation to Location of Origin, Phenotype, and Karyotype. J. Parasitol. 65 (2): 267-271.

6. Ma, N. S. F., T. C. Jones, A. C. Miller, L. M. Morgan and E. A. Adams. 1976. Chromosome Polymorphism and Banding Patterns in the Owl Monkey (Aotus). Laboratory Animal Science 26 (6): 1022-1036.

II. Project Objective

The objective of the project is the development of a 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 best described as "merozoite derived."

IV. Accomplishments: Aug. 15, 1979 - Feb. 14, 1980

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 rabbit-origin anti-malarial serum antibodies and their evaluation by indirect antibody techniques
- the *in vitro* Parasite Inhibition Test for the evaluation of malaria-immune sera
- characterization of three *P. falciparum* clones
- standardization of karyotyping procedure for *Aotus*

A. Antigen Purification and Characterization

Over the past six months we have focused our efforts on the glycolipoprotein antigen reported in the preceding Annual Report (Aug. 15, 1978 - Aug. 14, 1979). This antigen can be briefly characterized as follows. Under S.D.S. polyacrylamide gel electrophoresis the antigen migrates at an Rf of 0.649 to 0.703, and has a molecular weight of 15,000 to 20,000 daltons. The complex stains positive for lipid by Sudan Black, for carbohydrate by Periodic Acid Schiff stain, and for protein by Coomassie Blue. It should be noted that certain lipids may show a positive P.A.S. stain; therefore, the presence of carbohydrate in the complex is tentative at present. The material is currently being obtained by solvent extraction of a $(NH_4)_2 \cdot SO_4$ precipitate of solubilized merozoites at a ratio of 1:2:0.8 of chloroform:methanol:aqueous sample. The protein moiety is present at approximately 8 μg of protein per 3 mg of total antigen complex. Repeated extractions result in the loss of the protein component. The antigen is believed to occur as an aggregate of approximately 100,000 daltons M.W. and to be dissociated by S.D.S. into subunits since the material is

found to migrate at approximately 15,000 to 20,000 M.W. on S.D.S./PAGE in samples taken from the void volume and first fractions through a Sephadex G-100 chromatographic column. Similar PAGE separations without S.D.S. failed to demonstrate the antigen.

B. Production of Rabbit-origin Anti-malarial Serum Antibodies and Their Evaluation by Indirect Antibody Techniques

Two indirect labeled-antibody techniques are being employed to evaluate the previously mentioned glycolipoprotein antigen (GLP). These are fluorescein-labeled second antibody for use in the indirect fluorescent antibody test (I.F.A.T.) and ferritin-labeled second antibody for use in electronmicrographic evaluations.

A rabbit anti-GLP antiserum titering 1:160 against late-stage parasites was obtained which contained no host cell antibodies when tested by the indirect fluorescent antibody technique. This antiserum was then tested in the ferritin-labeled indirect antibody test. The test was conducted in three parts:

(1) the negative control utilizing normal rabbit serum;

(2) the positive control utilizing rabbit serum raised

against the whole merozoites recovered from culture supernatants and (3) the test serum which was raised against the GLP antigen. The merozoites used in this test were recovered from P. falciparum culture by differential centrifugation and were contaminated with infected and non-infected erythrocytes. The sera were complement-inactivated at 56°C for 30 minutes; no red cell adsorptions were performed. The individual sera at 1:10 dilutions in phosphate buffered saline (PBS), pH 7.2, were incubated with merozoite preparations for 10 minutes at 1°C. Unbound antibody was removed by washing the merozoites three times in P.B.S. The pellets were resuspended by trituration with a glass Pasteur pipet. The resuspended merozoites were then incubated for 10 minutes at 1°C with goat anti-rabbit Ig: ferritin conjugate, diluted 1:10 in P.B.S. The cells were then washed three times in P.B.S. The preparations were then fixed in ice cold 1.25% glutaraldehyde in M/10 phosphate buffered water + 4% sucrose for 1 hour and washed three times in M/10 phosphate buffered water + 4% sucrose.

These preparations were sent to Dr. Thomas Seed, Argonne National Labs., for embedding, sectioning and examining by transmission electron-microscopy. The results of this examination can be summarized as follows: (1) the negative control specimen, involving the use of normal non-immune rabbit serum, did not show a generalized specific ferritin labeling of normal red cells, infected red cells or free merozoites although there was some trapping of ferritin; (2) the positive control specimen, involving the use of rabbit serum immune to the whole "merozoite" preparation recovered from culture supernatants, showed specific heavy ferritin labeling of normal red cell membranes, infected red cell membranes and all stages of malarial parasites, including merozoites; (3) the test specimen, involving the use of rabbit serum raised against the GLP antigen, showed specific heavy ferritin labeling of the surface coat of merozoites. The vast majority of normal red cell ghosts and "knob"-bearing ghosts from infected red cells were not labeled, nor were young extracellular trophozoites. There were technical problems present in this experiment caused primarily by strong agglutination in the two immune

samples which resulted in uneven ferriting staining of the specimens and excessive damage to the free merozoites. Therefore, these experiments are being repeated with modifications of the procedure designed to improve the quality of the specimens.

C. The *In Vitro* Parasite Inhibition Test (P.I.T.) for the Evaluation of Malaria-immune Sera

The results of this phase of our work are presented as a manuscript prepared for publication (Appendix 1). The test is being further evaluated by challenge experiments conducted in *Aotus* monkeys previously recovered from *P. falciparum* infection.

There are several points pertinent to this test which should be emphasized. These are:

(1) This test was developed because of our commitment to do so in our original research proposal for the dual purposes of antigen identification and evaluation.

(2) The test method is based upon the Trager-Jensen *in vitro* culture technique and is conducted similarly to other investigators' growth and reinvasion tests but with the additional requirement

that the parasite population be synchronized at the outset. However, the interpretation of the test is a departure from that used by other investigators. Standard growth and reinvasion tests measure the increase (or decrease) in parasite populations after one or several asexual reproductive cycles in the presence of the test serum, usually at low concentrations (although the work of Phillips et al. with Gambian sera was at 40% concentration). Our P.I.T. also measures growth and reinvasion of parasites after one asexual reproductive cycle. The test differs in that it also assesses the fate of parasites within one generation by quantitating those parasites whose reinvasive capacity was inhibited due to the presence of specific immune serum. This effect of immune antibody is manifested by the appearance of fully mature schizonts whose merozoites have been inhibited from dispersal for reinvasion. The percentage of ring-form trophozoites whose asexual reproductive cycle has been interrupted in this manner can be easily calculated. This inhibition is dependent upon the concentration of the immune

serum being tested, being most effective at the highest serum concentration used.

(3) The outcome of standard growth and reinvasion tests is determined by parasitemias of the cultures. However, slightly reduced reinvasion may be due to unexplained variation within the experiment rather than specific immune effects. The presence of inhibited schizonts in cultures has a highly significant correlation with the presence of specific malaria-immune serum. Similar forms have been noted by other investigators but have not been previously quantitated.

(4) The quantitation of inhibited schizonts for the evaluation of *in vitro* growth and reinvasion tests should be considered as an *additional* criterion for the assessment of the test. It has the advantage of estimating the fate of parasites within one generation in an easily calculated fashion.

D. Characterization of *P. falciparum* Clones

Although antigenic variation is a proven feature of *P. knowlesi*, the situation with *P. falciparum* remains

unclear. The presence of "knob-producing" and "knobless" variants within the FCR-3/FMG strain indicate that such a possibility exists. Therefore, we have isolated several cloned cultures to be available for immunization trials in order to reduce the likelihood of interference by multiple antigenic variants.

The FCR-3/FMG African strain of P. falciparum has been cloned by limiting dilution techniques, as reported in the AID/ta-C-1440 Annual Report for the period of 8/15/78 to 8/14/79. Three primary clones, chosen on the basis of their growth rates in culture, were then cloned twice more, resulting in the tertiary clones FCR-3/PD-2 (C3,3°); FCR-3/PD-2 (C5,3°) and FCR-3/PD-2 (C6,3°). These clones have been examined morphologically by electronmicroscopy. Also the ability of gelatin to separate mature stages was determined, their growth rates in culture were compared, and their sensitivity to pyrimethamine and chloroquine was assessed in vitro. The results of these tests are summarized in Table 1.

Table 1

Characterization of Tertiary Clones of *P. falciparum in vitro*

	C3,3°		C5,3°		C6,3°
E.M. features	infected cells w/knobs		infected cells w/knobs		infected cells knobless
Gelatin separation/ enrichment	good separation		good separation		no separation
Growth in culture	C3,3°	<	C5,3°	<	C6,3°
Pyrimethamine and chloroquine sensitivity	C3,3°	=	C5,3°	=	C6,3°

E. Procedure for Karyotyping of *Aotus* Monkeys

Complete details in Appendix 2.

V. Dissemination and Utilization of Research Results

A presentation of research results concerning antigen isolation and characterization was made at the 1979 Annual Meeting of the American Society for Tropical Medicine and Hygiene in Tucson, Arizona.

VI. Work Plan for the Coming Six Months

A. The P.I.T., an integral part of our immunization scheme, is being more fully developed. To date the P.I.T. has only been used in retrospective studies in *Aotus* monkeys. The test clearly has the ability to detect anti-malarial antibodies present in specific immune sera and to do so in a comparative manner. The P.I.T.'s greatest value,

however, would reside in its ability to assess an individual's resistance to live parasite challenge, should the test be capable of such estimates. Data assessing the predictive value of the P.I.T. are now being generated. Five Aotus monkeys with a prior history of P. falciparum infection have been re-challenged with 10^6 infected Aotus erythrocytes. Sera obtained before challenge and following recovery from patent parasitemia will be evaluated by the I.F.A.T. and the P.I.T. These data may allow estimates of immune status to be made.

B. Our most promising candidate antigen, the GLP merozoite subunit antigen, will be tested for immunogenicity in rabbits and non-immune Aotus monkeys. Rabbits will be immunized with purified GLP antigen emulsified in Freund's complete adjuvant given intramuscularly or intraperitoneally. Three injections will be given at 2-week intervals and the resultant serum will be tested by the fluorescein- and ferritin-labeled indirect antibody tests as well as the in vitro parasite inhibition test. Following verification of the purity and specificity of the GLP antigen in rabbits, Aotus monkeys will be immunized with the GLP antigen emulsified in Freund's complete adjuvant. Three injections

will be given intramuscularly at 2-week intervals and the resultant serum will be tested by the same methods as the rabbit sera. One month after the last immunization the monkeys will be challenged by 10^6 P. falciparum-infected Aotus erythrocytes and carefully monitored for parasitemias. If necessary, drug rescue will be employed to prevent mortality. The immunization and challenge of the Aotus monkeys will be conducted with P. falciparum parasites obtained from a cloned population, probably FCR-3/PD-2 (C5,3^{o}). Appropriate adjuvant and non-immune control monkeys will be employed in the test.

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 8/15/79 through 2/14/80 and 2/15/80 through 8/14/80 (estimated) are presented as follows:

Expenditure	8/15/79-2/14/80	2/15/80-8/14/80
Salaries and Wages, including Fringe Benefits	$92,273.	$80,300.
Consultant Fees	360.	1,550.
Travel and Transportation	1,555.	1,525.
Other Direct Costs	0.	0.
Overhead	61,546.	49,786.
Research and General Administration	4,712.	4,093.
Supplies	12,537.	9,023.

Serum Inhibition of Merozoite Dispersal From *Plasmodium falciparum* Schizonts: An Indicator of Immune Status

Abstract. Specific immune serum was found to agglutinate the merozoites of individual schizonts before they could disperse, thus interfering with their subsequent reinvasion of new host erythrocytes. This phenomenon is viewed as a protective mechanism against malaria which can be measured *in vitro* and reflects the immune status of the donor.

The evaluation of protective capacity of malaria-immune sera has been difficult. Indirect hemagglutination and indirect fluorescent antibody tests have been widely applied to determine the occurrence of serological reactions to plasmodia but the results of neither test correlate well with the immune status of individuals. The enzyme-linked immunosorbent assay, although exquisitely sensitive, is likewise an imprecise indicator of immune status, as is also radioimmunoassay. The schizont-infected cell agglutination test for antibodies to host cell antigens of *P. knowlesi* origin does not work with *P. falciparum* infected cells. *In vitro* tests of inhibition of reinvasion by *P. falciparum* have shown some promise as indicators of the immune status of individual serum donors. We present here a new test for the evaluation of the immune status of individuals to *P. falciparum*. This test is based on inhibition of dispersal of merozoites from schizonts. We have termed the nondispersed schizonts "inhibited schizonts", and used their frequency in the evaluation of parasite reinvasion *in vitro*. Our method of stationary culture in candle jars is patterned after that described by Trager and Jensen (1). *Aotus* monkeys which had recovered from induced *P. falciparum* infection were used as the immune serum donors. Seronegative *Aotus* monkeys with no history of malaria infection were used as the non-immune controls. *Aotus* sera used in the test were included in the RPMI-1640 culture medium at concentrations of 10% or 50%. All sera were heat inactivated at 56°C for 30 minutes and adsorbed with human red cells before incorporation into the medium.

Parasitized red cells were pretreated with sorbitol by the method of Lambros and Vanderberg (2), which spares ring-form trophozoites but destroys older stages. The washed parasitized cells were then adjusted to a hematocrit of 6% in the complete medium at a parasitemia of 1 ± 0.5%. The cultures were placed in either 35 mm plastic petri dishes at 1.5 ml volumes or to conserve serum in 17 mm wells in multiwell plastic plates at 0.5 ml volumes. All tests were performed in duplicate. After 24 hours of culture the majority of parasites were in late trophozoite and schizont stages of the life cycle. After 48 hours the parasites cultured in the presence of normal non-immune serum had reinvaded new host cells and were found as ring-form trophozoites. Less reinvasion occurred in cultures maintained for 48 hours in the presence of immune serum than in control cultures containing normal serum. The proportions of inhibited schizonts in normal or immune serum containing cultures were quantitated. These schizonts characteristically contain fully mature merozoites which have failed to disperse and have a typically fragmented membrane, and contain hemazoin. The gross membrane damage may be in part an artifact of blood film preparation. There is a strong positive correlation with the presence of these "inhibited schizonts" and a strong negative correlation with the numbers of ring-form trophozoites resulting from reinvasion of erythrocytes by merozoites and the concentration of immune serum in the cultures. In the data presented, parasitemias were calculated from the total number of parasites present in 1000 erythrocytes, whereas the

"inhibited schizonts" were counted as the number of these forms present per 10,000 erythrocytes.

Figures 1a, b and c are photomicrographs of typical "inhibited schizonts" from 48 hour cultures containing immune serum, while figure 1d shows the ring-form trophozoites which are found in comparable control cultures containing non-immune serum. Figure 2 compares the relative inhibition of parasite reinvasion in cultures containing 10% and 50% immune serum and control cultures containing normal serum. Virtually all of the viable parasites observed in erythrocytes at 48 hours were ring-form trophozoites. In no case was inhibition of reinvasion complete. Figure 3 presents data based upon the relative abundance of "inhibited schizonts" after 48 hours of culture in the presence of immune vs non-immune sera at either 10% or 50% concentration in the culture medium. The data are derived by determining the percentage of ring-form trophozoites which were arrested upon maturity as "inhibited schizonts". The numbers of "inhibited schizonts" in the cultures were determined by direct light microscopic examination of Giemsa stained blood films taken at 24 hour intervals and were made at the time of medium replacement. As each "inhibited schizont" is a mature parasite developed from an individual ring-form trophozoite of the same generation, the degree of inhibition by immune serum can be calculated without consideration of the reinvasion efficiency of merozoites or the average number of merozoites per schizont. Assuming that any schizont prevented from dispersing its merozoites constitutes a

unit of protection, the test can be applied to the problem of serological evaluation of immune status. From examination of the data in Figure 3, it is apparent that the bers of "inhibited schizonts" in the cultures are related to the immune serum concentration in the culture.

Several important findings have emerged from this study. First, it is apparent that the ability of immune serum incorporated in the culture medium to interfere with merozoite invasion of red cells is concentration dependent. There was no significantly greater inhibition of reinvasion in the presence of 10% immune serum than in the presence of non-immune serum ($p = .166$); however, at 50% immune serum concentration there was a significantly greater inhibition of reinvasion in the immune than in the non-immune groups ($p < .001$). Second, interpretation of an individual test on the basis of reinvasion alone could be difficult since parasitemias in some cultures rose somewhat during 48 hours of incubation whereas others fell, even though as a group reinvasion in media containing 50% immune serum was less than with non-immune serum. The data shown in Figure 2 demonstrate this point. Third, the presence of "inhibited schizonts" in a 48 hour culture is strongly correlated with the concentration of immune serum in the culture. The average percentage of a given generation of parasites arrested as "inhibited schizonts" in media containing 10% non-immune sera is 0.3%, whereas in the same concentration of homologous FCR-3 immune <u>Aotus</u> sera the average value is 5.0% ($p < .01$) and in heterologous

FCR-1/FVO immune Aotus sera the value is 1.8%. The average percentage of a single parasite generation arrested as "inhibited schizonts" in media containing 50% non-immune sera is 3.2%, whereas in the same concentration of homologous FCR-3 immune sera the average value is 41.9% ($p < .001$) and in heterologous FCR-1/FVO immune Aotus sera the percentage is 21.5%. Although the ranges of activity of homologous and heterologous sera overlap, there appears to be a lesser effect associated with heterologous sera. It is of considerable interest that antisera against the FCR-1/FVO Asian strain of P. falciparum appears to possess significant protective activity against the heterologous FCR-3 African strain of parasites in vitro.

It has been demonstrated that bivalent antibodies are necessary to prevent reinvasion of malaria parasites in vitro (3), a finding that is consistent with the suggestion that merozoite agglutination is associated with reduced reinvasion (4). Complement seems to be unimportant in the protective immune response to malaria (3, 5) and its addition had no effect on our tests. Phagocytosis of the agglutinated parasites seems to be the method of their elimination (6). We believe that immune antibody is acting in vitro just before or at the time of schizont rupture by entering the cell and agglutinating the merozoites, thereby interfering with their dispersal for reinvasion. This mechanism requires that specific anti-merozoite antibody is present in sufficient concentration to accomplish this action before dispersal can occur. We have demonstrated immune antibody to be associated

with inhibited schizonts by the indirect fluorescent antibody technique (unpublished data). If such inhibition occurs *in vivo* resulting in diminished numbers of merozoites successfully reinvading new host erythrocytes, then the presence of inhibited schizonts *in vitro* can be considered as a direct indicator of protection. Our work has been done with antibody raised against *P. falciparum* in *Aotus* monkeys. Phillips et al (7) noted the presence of "increased numbers of abnormal segmenters which appeared to be lysed" in their *in vitro* reinvasion studies utilizing *P. falciparum* immune human sera. We believe the abnormal segmenters observed by Phillips et al (7) in cultures containing immune human serum are similar to the "inhibited schizonts" we have observed in these studies and we believe that their numbers can be used as a simple effective measure of the protective potency of immune serum.

Theodore J. Green
Martha Morhardt
Robert G. Brackett

Clinical Immunology
Pharmaceutical Research & Development
Warner-Lambert/Parke-Davis
Detroit, Michigan 48232

References

1. Trager, W. and J. B. Jensen, Science 193: 673-675 (1976)

2. Lambros, C. and J. P. Vanderberg, J. Parasitol. 65 (3): 418-420 (1979).

3. Cohen, S. and G. A. Butcher, Immunology 19: 369-383 (1970).

4. Miller, L. H., M. Aikawa and J. A. Dvorak, J. Immunol. 114 (4): 1237-1242 (1975).

5. Diggs, C. L., E. T. Wellde, J. S. Anderson, L. Luzzatto, R. M. Weber and E. Rodriguez, Jr., Proc. Hel. Soc. Wash. 39: 449-456 (1972); Atkinson, J. P., R. H. Glew, F. A. Neva and M. M. Frank, J. Infect. Dis. 131: 26-33 (1975) Williams, A.I.O., F. S. Rosen and R. Heff, Annals. Trop. Med. Parasit. 69: 179-184 (1975).

6. Chow, J. S. and J. P. Kreier, Exptl. Parasitol. 31: 13-18 (1972); Green T. J. and J. P. Kreier, Infect. Immun. 19: 138-145 (1978); Brooks, C. B. and J. P. Kreier, Infect. Immun. 20: 827-835 (1978).

7. Phillips, R. S., P. I. Trigg, T. J. Scott-Finnigan and R. K. Bartholomew, Parasitology 65: 525-535 (1972).

8. This work was supported by AID Contract ta-C-1440.

Figure 1.

Photomicrographs of Giemsa-stained blood films of *Plasmodium falciparum* FCR-3 (African strain) infected human erythrocytes from 48 hour cultures. RPMI-1640 culture medium containing 50% Aotus monkey serum immune to *P. falciparum* FCR-3 was used in cultures a, b and c; note "inhibited schizonts" (arrows). RPMI-1640 culture medium containing 50% normal non-immune Aotus monkey serum served as control in culture d. Note the examples of normal ring-form trophozoites (arrows). Scale line equals 10 μm.

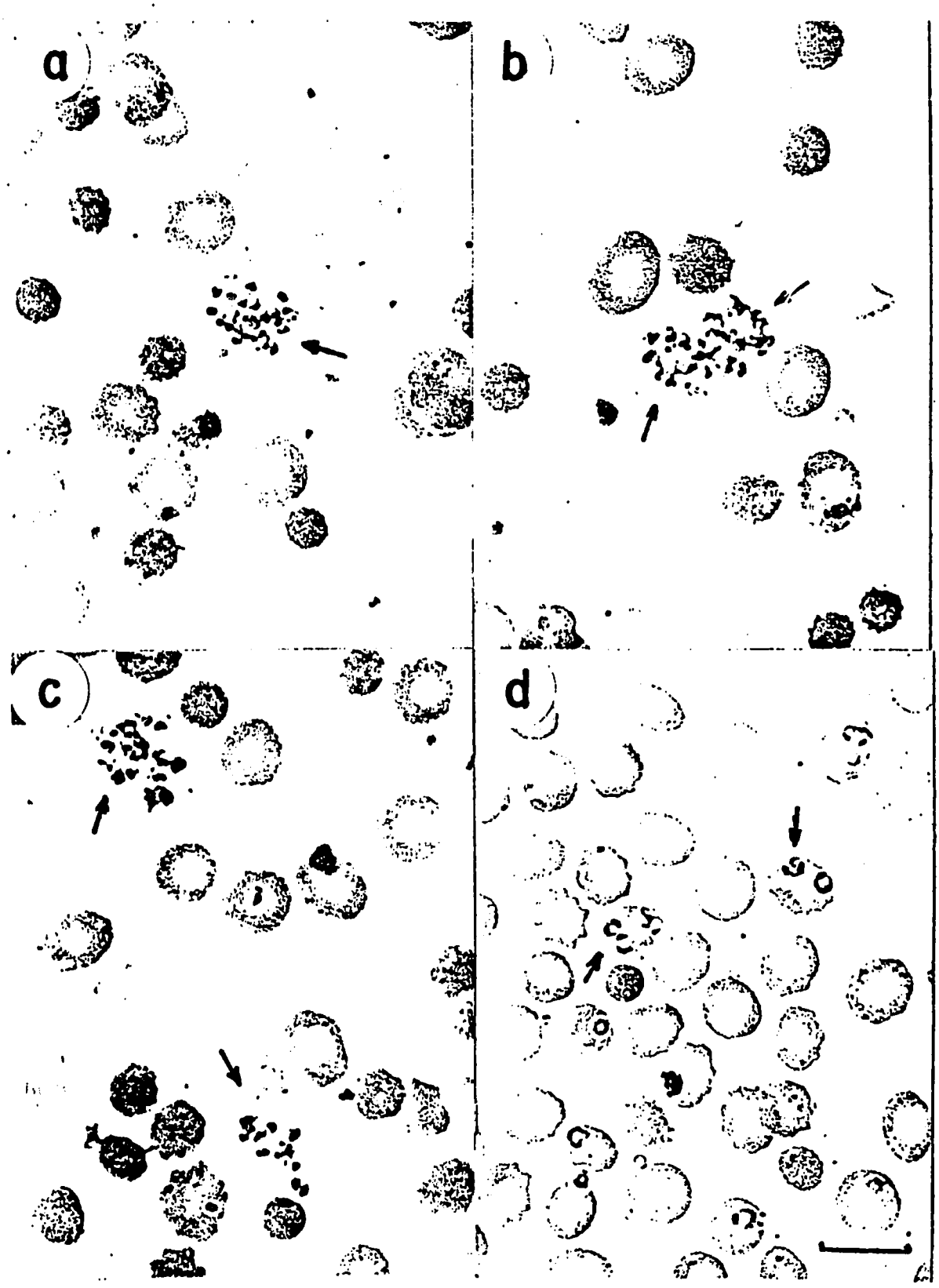

Figure 1

Figure 2

Reinvasion test data. Parasitemias are of *Plasmodium falciparum* FCR-3 (African strain) grown for 48 hours in *in vitro* candle jar cultures in RPMI-1640 culture medium containing 10% or 50% Aotus monkey serum. Each data pair consists of the starting parasitemia and the final 48 hour parasitemia after reinvasion. Parasites were pre-synchronized by the sorbitol method so that the data represent predominantly ring-form trophozoites for both sampling times. The sera used were non-immune Aotus ●, *P. falciparum* FCR-3 (African) immune Aotus ■ or P. falciparum FCR-1/FVO (Asian) immune Aotus ◆ . All data points are averages for duplicate experiments. The data were derived by determining the total number of parasites present per 1000 erythrocytes on Giemsa-stained blood films from the cultures.

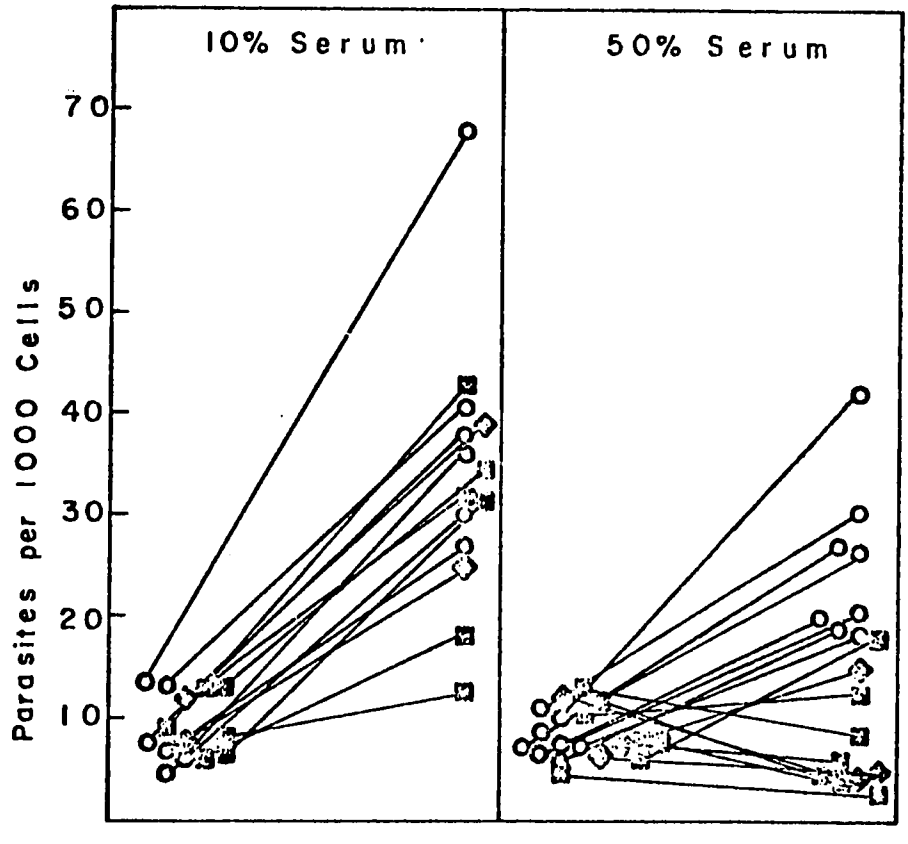

Figure 2

Figure 3

Schizont inhibition data. *Plasmodium falciparum* FCR-3 (African strain) parasites were grown for 48 hours in *in vitro* candle jar cultures in RPMI-1640 culture medium containing 10% or 50% Aotus monkey serum. Each data point is the mean of duplicate experiments, and was derived by determining the percentage of ring-form trophozoites which were arrested upon maturity as inhibited schizonts using the formula:

$$\% \text{ Inhibition} = \frac{\text{number of inhibited schizonts/10,000 cells}}{\text{number of ring-form trophozoites/1000 cells}} \times 10$$

Inhibited schizonts failed to disperse their merozoites and therefore were still present in 48 hour cultures, when merozoites which successfully reinvaded had differentiated to ring-form trophozoites. The sera used were non-immune Aotus ○ , *P. falciparum* FCR-3 (African) immune ■ , or *P. falciparum* FCR-1/FVO (Asian) immune ◆ . The data presented in Figures 2 and 3 were obtained from the same sets of specimen slides and represent the same experiments.

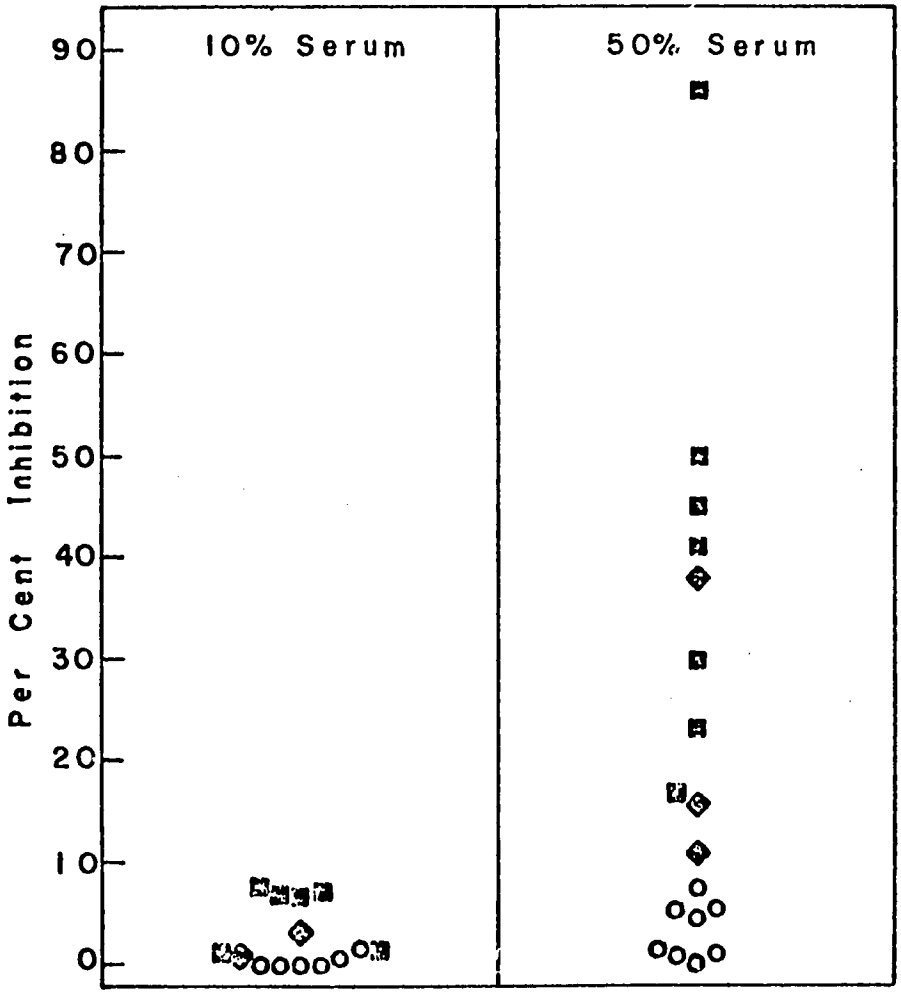
10% Serum
50% Serum
Per Cent Inhibition
90
80
70
60
50
40
30
20
10
0

Figure 3

Appendix 2

PREPARATION OF LEUKOCYTE CULTURES AND SLIDES FOR KARYOTYPING AOTUS MONKEYS

COLLECTION AND CULTIVATION OF SPECIMEN

1. Bleed monkeys with a needle and syringe (heparin optional), remove needle and transfer the whole blood to plastic leukocyte culture flasks containing 5 cc of medium. One cc of whole blood is divided among 3 flasks to yield 0.3 cc - 0.4 cc/flask.

2. Shake flasks gently to mix and incubate at 37°C with caps tightened and in an upright position for 3 days.

HARVESTING AND FIXATION OF CELLS

3. Add 0.05 cc of a 10 μg/ml stock of Colcemid to each culture flask to give a final concentration of Colcemid of 0.10 μg/ml.

4. Incubate at 37°C for 4 hours.

5. Shake the culture flask slightly and transfer the flask contents to a 15 ml centrifuge tube.

6. Centrifuge at 2640 RPM (1500 X g) for 10 minutes. Use a centrifuge with a swingout head since with a slant head centrifuge cells will be distributed along the length of the tube on one side and will be lost on removal of the supernatant fluid.

7. Draw off the supernatant to about 1.0 ml above the packed cells.

8. Resuspend the pellet with the vortex mixer set on 5, or use a 9 inch pasteur pipette being careful to keep the suspension in narrow part of pipette.

9. Add warm (37°C) hypotonic solution (0.075 M KCl) a drop at a time as you vortex for about 20 drops. Then fill the tube with about 10 ml of hypotonic solution, cap and vortex.

10. Incubate in the 37°C waterbath for 10 minutes.

11. Centrifuge at 2640 RPM (1500 X g) for 10 minutes.

12. Remove the supernatant to about 1.0 ml above the packed cells.

13. Resuspend the pellet with the vortex mixer set on 5.

14. Slowly add fixative (3 parts methanol + 1 part glacial acetic acid) drop by drop as you vortex for about 20 drops, then up to 8 ml, cap and vortex. Fixative should be made fresh every 30-40 minutes due to water absorption of fixative solution.

15. Incubate at room temperature for 10 minutes.

16. Centrifuge at 2640 RPM (1500 X g) for 5 minutes.

17. Remove supernatant and add fixative dropwise while vortexing for about 20 drops. Pool cultures from the same animal making 2 tubes out of three original tubes. Add fixative up to 6 ml, cap and vortex.

18. Incubate at room temperature for 5 minutes.

19. Centrifuge at 2640 RPM (1500 X g) for 5 minutes.

20. Remove the supernatant and add fixative up to 4 ml as in Step 14.

21. Incubate at room temperature for 5 minutes.

22. Centrifuge at 2640 RPM (1500 X g) for 5 minutes.

23. Remove the fixative supernatant and add fresh fixative dropwise while vortexing for about 20 drops. Pool cultures from the same animal making one tube from two. Add fixative up to 2 ml, cap, and vortex.

24. Incubate at room temperature for 5 minutes.

25. Centrifuge at 2640 RPM (1500 X g) for 5 minutes.

26. Remove the supernatant and add 0.5 cc of fixative and resuspend the pellet with a pasteur pipette. Volume of fixative used depends on the size of the pellet.

PREPARATION OF AIR-DRIED SLIDES

27. Draw some of the above suspension into a pasteur pipette.

28. With the other hand dip a precleaned slide into a beaker of cold distilled water. Those slides that do not wet uniformly are discarded. Use dry ice to chill the water or make distilled water icecubes.

29. Shake off the excess water from the slide.

30. Drop several drops of suspension onto the slide held horizontal or at a slight angle.

31. Blow on the slide several times and then allow to air dry at a slant.

STAINING OF SLIDES, CONVENTIONAL GIEMSA

32. Acid hydrolysis: Expose slides to 5N HCl for 10 minutes in a Coplin jar (this reduces background staining of cytoplasm and other non DNA material, but is not an essential step). (Add 1.3 volumes distilled water to 1 volume concentrated HCl; store on shelf. Concentrated HCl is 11.7 N.) Caution: Handle with care in a ventilated hood or use 1N HCl at 60°C for 10 minutes.

33. Rinse slides in 2 changes of distilled water.

34. Air dry slides or dry on a warming tray. If a warming tray is not available use a hot plate. Control temperature setting by placing a thermometer in a beaker of water on the hot plate and maintain a temperature of 45-50°C.

35. Prepare 100 ml of Giemsa stain for each set of 6-8 slides (4 slides/dish, 50 cc/dish).

    91 ml distilled water

    3 ml 0.15 M ammonium hydroxide

    6 ml Giemsa blood stain concentrate

36. Place slides in a Coplin jar. Add staining solution and stain for 6-8 minutes.

37. Flood staining dish with distilled water so that the overflow flushes away the flocculant surface film which has formed.

38. Remove each slide and rinse in 2 changes of distilled water in a large staining dish, scraping edge of slide on bottom of dish to dislodge precipitate.

39. Shake the excess water and slant slides to dry overnight or on the warming tray.

40. For permanent mounting using #1 thinness coverglass, dip slide into xylol, add 1-2 drops of mountant fluid and use the blunt end of the forceps to press firmly on the coverglass so as to eliminate small bubbles and extrude surplus mountant fluid.

41. Examine slides microscopically at 10X or 16X and select cells with little or no overlapping of chromosomes. Count and photograph metaphases at 63X or 100X. Use High Contrast Copy film (green filter) and develop in D76 (Kodak Developer) for 8 min @ 68°F. Contact print film strips on one 8 X 10. Enlarge best metaphases to 5 X 7 or 8 X 10 and karyotype.

"The preparation of high quality slides is most important. This is even more crucial for slides to be used for G-banding procedures. Excellent metaphase chromosome preparations demand attention to certain points: 1) selection of cell cultures at

optimal mitotic activity; 2) controlled timing of Colcemid pretreatment; 3) adjustment of the hypotonic pretreatment to the specific cell; 4) use of high purity, reagent grade chemicals and clean glassware; 5) good technique in minimizing loss of cells during the procedures; 6) achieving individual cell suspensions at all steps in the procedures. Of these the selection of cultures at their peak of mitotic activity is the most important single factor."*

*Laboratory Protocols, Dr. Paul S. Moorhead, Karyology of Cultured Cells, Dec. 1 - Dec. 5, 1975. Alton Jones Cell Science Center.

SUPPLIES FOR KARYOTYPING

Colcemid
CIBA Pharmaceutical Co., Summit, New Jersey 07901

**Hanks Balanced Salt Solution 10X Calcium and Magnesium Free
Grand Island Biological Company, Grand Island, New York 14072

**Gurr Buffer Tablets pH 6.8 Searle Diagnostic
Bio/medical Specialties, Box 1687, Santa Monica, Calif. 90406

**Gurrs Improved R66 Giemsa Stain Searle Diagnostic
Bio/medical Specialties, Box 1687, Santa Monica, Calif. 90406

Gurrs Neutral Mounting Medium Searle Diagnostic
Bio/medical Specialties, Box 1687, Santa Monica, Calif. 90406

Giemsa Blood Stain
Matheson Coleman and Bell, Norwood, Ohio 45212

Methanol Spectroquality
Matheson Coleman and Bell, Norwood, Ohio 45212

Corning Micro Slides Single Frosted 75 X 25 mm No. 2948
Corning Glass Works, Corning, New York 14830

Corning Cover Glass No. 1 24 X 50 mm
Corning Glass Works, Corning, New York 14830

Corning 15 ml/Centrifuge Tube with Screw Cap #25310
Corning Glass Works, Corning, New York 14830

Glacial Acetic Acid, Baker Analyzed Reagent
J. T. Baker Chemical Co., Phillipsburg, New Jersey 08865

30 ml Tissue Culture Flasks #3012
Falcon Plastics, Los Angeles, California 90045

**Trypsin-EDTA (10X) Lyophilized #540L
Grand Island Biological Co., Grand Island, New York 14072

**Trypsin 2.5% (1:250) Lyophilized #509L
Grand Island Biological Co., Grand Island, New York 14072

McCoy's 5a (modified) medium
Grand Island Biological Co., Grand Island, New York 14072

Fetal Calf Serum Rehatuin N.F.S.
Reheis Chemical Company, Chicago, Illinois

Heparin Sodium Injection, U.S.P. 5,000 units/ml
The Upjohn Company, Kalamazoo, Michigan 49001

Bacto-Phytohemagglutinin P
Difco Laboratories, Detroit, Michigan

Gentamicin
Schering Corporation, Port Reading, New Jersey 07064

**Used for banding procedures.

PREPARE STOCK MATERIALS

Colcemid

Stock solution 10 µg/ml in distilled H_2O.
Filter to sterilize. (Membrane) (0.22 µM Swinnex & 50 cc syringe)
Freeze in 1 ml amounts
Store at -20°C
Prepare stock 1 mg/100 ml of H_2O
.5 mg/50 ml of H_2O or .0005 gm

Hypotonic 0.075 M KCL

0.56 gm KCL/100 ml H_2O
0.28 gm KCL/50 ml H_2O

Fixative 3:1 Absolute Methanol:Glacial Acetic Acid

Add one volume of glacial acetic acid to three volumes of methanol. This results in thorough mixing without pipetting.

Do not mix more than 30 minutes before use. For continuous use it must be freshly prepared every 30 to 40 minutes.

Phytohemagglutin P

Supplied lyophilized and the contents of one vial is dissolved in 5 ml of sterile water. The reconstituted material can be frozen and thawed repeatedly without losing its effectiveness for at least 6 months. 50 mg/ml

0.15 M Ammonium hydroxide

Supplied in a solution that makes one liter of 1 Normal (1 Molar). For one liter of 0.15 M add 150 cc of 1 Normal to a volumetric flask and q.s. to one liter.

Leukocyte Culture Medium

For microculture of leukocytes from whole blood prepare the following medium in 100 ml volumes and freeze in 20 ml sterile scintillation counting vials or 30 ml plastic tissue culture flasks in 5 ml volumes. Store at -20°C.

Per 100 ml

85 ml	McCoy's 5a (modified medium)
15 ml	Fetal Calf Serum
.1 ml	Phytohemagglutin P (stock solution 50 mg/ml)
.08 ml	Heparin Sodium (stock solution 5000 USP units)
.2 ml	Gentamicin (stock solution 50 mg/ml)