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SIX MONTH REPORT

MALARIA IMMUNITY AND VACCINATION

Report Summary

1. Malaria Immunity and Vaccination, AID/ta-C-1440
2. Robert G. Brackett, Ph.D.; Parke, Davis & Co., Box 118, Detroit Michigan 48232
3. August 15, 1977 - February 15, 1978 - Contract Period
4. August 15, 1977 - February 15, 1978 - Reporting Period
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Plasmodium falciparum, FVO Asian and FRC-3 African strains, has been grown in culture by the candle jar and flow flask methods. These cultures have proven infective for Aotus monkeys and have been successfully retrieved from liquid nitrogen storage. We have found both the Rowe and the glycerol phosphate buffer methods useful for liquid nitrogen preservation of parasites. Parasite cultures have been found to attain increased levels of parasitemia in flow flasks by increasing the media flow rate relative to the number of red cells perfused. Carbon dioxide levels of 3% *vs* 5%, and gas delivery via Tygon® *vs* silicone tubing were found to be equally effective for the growth of *P. falciparum* in culture. Parasites were also found to grow as well in FEP-Teflon culture bags as in petri dishes, and at a variety of O_2 levels. pH changes in culture due to lactic acid accumulation were found to limit parasite growth while glucose levels were still adequate. The critical pH was found to be approximately 7.0, below which merozoite development was inhibited. Pretreatment of red blood cells with adenosine at 15 μM was found to have a favorable effect on parasite cultures. A degree of synchrony of parasite development in culture was achieved by drug treatment, but was found to be short-lived. Cellulose powder columns were not found to be useful for the concentration of schizonts obtained directly from the flow flask cultures. Gradient centrifugation has not proven as useful for our purposes as differential centrifugation in the purification and concentration of merozoites. Culture supernatants are our material of choice for

Report Summary (cont'd)

obtaining antigen. These antigens are being used to raise antibodies in rabbits for use in the development of an *in vitro* parasite neutralization test. *In vivo* subpassages of *P. falciparum* have resulted in a virulent strain which is suitable for monkey challenge. Our Aotus monkeys have been found to be of a single subspecies based on karyotype and to be uniformly susceptible to falciparum infection.

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Annual Research Report

I. General Background

One of the main obstacles to the development of a *P. falciparum* vaccine has been the lack of a source of antigen suitable for mass production. A highly significant advance toward mass production of antigen was made when Drs. Trager and Jensen (1) succeeded in the long term cultivation of *P. falciparum in vitro* in human red blood cells.

Varying degrees of success have been achieved by several investigators with vaccines prepared from erythrocytic stages of *Plasmodium knowlesi*. Partially successful vaccination of rhesus monkeys has been achieved with two intramuscular injections of plasmodial antigens derived from the erythrocytic stages and emulsified in Freund's adjuvant (2, 3, 4). After intravenous challenge with *P. knowlesi*, increased survival of the animals and a significant decrease in parasitemia were observed. *In toto*, Dr. Schenkel (personal communication, 1975) reported that 22 (67%) of 33 vaccinated animals survived, 8 (24%) died, but with evidence of protection, and 3 (9%) died without evidence of protection.

Similarly, Mitchell and Cohen (5, 6) have shown that vaccination with purified merozoites in Freund's complete adjuvant protected rhesus monkeys against homologous and heterologous variant infection with erythrocytic stages of *P. knowlesi*.

They not only demonstrated protection in all 24 immunized monkeys, but also showed a merozoite vaccine induced a greater degree of protection than a schizont vaccine. They have also demonstrated that adjuvanted merozoite vaccines protected rhesus monkeys against a sporozoite challenge and formol treated and freeze-dried adjuvanted vaccines protected rhesus monkeys against an erythrocytic stage challenge with P. knowlesi (7, 8).

Clearly, the feasibility of immunizing against P. knowlesi with the erythrocytic forms of the parasite has been demonstrated. Similar data has begun to appear in the literature relative to immunologic protection of Aotus trivirgatus (Owl) monkeys by P. falciparum vaccines. Siddiqui's recent successful immunization of Aotus monkeys with an adjuvanted mixture of erythrocytic forms of P. falciparum (9) established that this parasite is also vulnerable to a vaccine approach. This work was confirmed by Mitchell, et al who demonstrated an adjuvanted P. falciparum vaccine made from merozoites obtained from the blood of infected humans protected Owl monkeys against P. falciparum challenge (10).

It is now possible through the aegis of in vitro propagated P. falciparum to conduct similar studies on merozoite-induced immunity against this human malaria.

While the exact nature of immunity to malaria is not understood, it is generally believed that humoral antibody is a

primary mechanism. This has been elucidated by a series of investigations by Miller (11), Mitchell and Cohen (12, 13) and Diggs (14). Studies by Miller et al (11) indicated that protective antibody does not affect intracellular parasites, but interrupts invasion of red blood cells by merozoites. After agglutination with specific immune serum, merozoites attached to erythrocytes *in vitro*, but usually were unable to invade the cells. The authors speculate that the surface coat on the merozoite has unique antigenic determinants for induction of functional immunity. Merozoite agglutination was caused by immune aggregation on the entire surface coat and not preferentially by aggregation at their anterior end. Free merozoites in immune serum were seen to invade erythrocytes normally, which perhaps indicates they were not neutralized by a mechanism other than agglutination. Thus, Miller *et al* postulate agglutination of merozoites, mediated by antibody to this surface coat, is crucial for reduced invasion of erythrocytes *in vitro*.

Mitchell and Cohen present evidence (12, 13) that *in vitro* inhibitory antibody probably directed against merozoites correlates with the immune status of the donor rhesus monkey and its action is independent of complement or phagocytic cells and is thus analogous to viral neutralizing antibody.

Diggs and Osler (14) interpret their data and that of others as strongly suggesting that protective antibody exerts its influence on the schizonts and/or merozoites.

Thus, the literature has established that vaccines can be made which are protective and humoral antibody plays an important role in this protection.

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II. Project Objective

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

III. Continued Relevance of Objectives

At present, our research does not indicate a need for modification of the project objective.

IV. Accomplishments: August 15, 1977 to February 15, 1978

During this first six month period of the Project, we have investigated the following aspects of *Plasmodium falciparum* immunization:

A. History of *in vitro* culture

B. Preparation of Frozen Stabilates

C. Enhanced Parasitemia in Flow Flask Culture

D. Culture in FEP-Teflon-film Culture Vessels and Polystyrene Culture Dishes

E. Adenosine pre-treatment of Human Erythrocytes used in Cultures

F. Attempts to Develop Synchrony in Cultures

G. Use of Cellulose Powder Columns for the Isolation of Schizont-Infected Erythrocytes

H. Purification and Concentration of Merozoites by Gradient Centrifugation and Differential Centrifugation

I. Rabbit Immunization for Antibody Production

J. Infectivity of *in vitro* grown parasites for *Aotus trivirgatus griseimembra*.

A. History of in vitro Culture

We have grown two strains of P. falciparum in in vitro culture during the period covered by this report. The strains are the FVO Asian (FRC-1) and the FRC-3 (FMG) African. Both were obtained from Drs. Trager and Jensen as fresh in vitro cultures. They were grown in vitro by by the candle jar and Flow Flask methods described in Appendices 1 and 2.

FVO history: The FVO strain, which had been in culture for 396 days when obtained on January 20, 1977, was then passaged 29 times at 3-7 day intervals over a period of 168 days when it was subsequently lost on July 8th, the total time in culture being 564 days. On June 24, 1977, a second passage frozen stabilate was retrieved, and from June 24, 1977 to October 17, 1977, the series was passed 23 times in a period of 115 days. These passages were primarily made in candle jar cultures.

During the last three in vitro passages (19 days) the strain barely maintained itself in candle jar culture and would not grow in Flow Flask culture. We could not discern any deleterious variable imposed on the parasite before or during this period of no growth and we discontinued this serial passage line.

We inoculated the FVO strain in one Aotus trivirgatus monkey on May 3, 1977 (see Section J of this report) at

a time when the strain was growing well in culture. When the strain was lost in culture, we isolated the FVO in candle jar culture from the blood of this monkey during a recrudescence, and after a two week period of slow growth *in vitro*, it attained a vigorous growth pattern. The strain was then propagated in flow flask culture and frozen away. This stabilate has proven viable in culture.

FRC-3 history: We obtained the FRC-3 strain of *P. falciparum* as an ongoing *in vitro* culture and have maintained the strain in continuous serial *in vitro* passage since October 19, 1977. Drs. Trager and Jensen had carried the strain *in vitro* since January 10, 1976. Assuming they maintained the culture *in vitro* continuously since then, up to February 15, 1978 the strain has been in culture for 766 days.

We carried the strain serially in candle jar culture for 12 passages and in flow flask culture for an additional 32 passages for a total of 119 days. In candle jar culture, the FRC-3 strain attained 2% to 5% parasitemia* with a passage being made every 3 to 4 days. Flow flask cultures have been split routinely every 2 to 3 days from 3-7 percent parasitemia to 1-2 percent parasitemia.

*Parasitemia was recorded as percent of erythrocytes infected, not as number of parasites per 100 red blood cells.

From January 22, 1977 to February 15, 1978, we used 61 units of human AB, Rho positive, HBs antigen-negative sera to grow P. falciparum in vitro. We also used 37 units of human AB, Rho negative, HBs antigen-negative red blood cells in the cultures. Sterility tests are carried out on all blood and sera prior to use in culturing.

B. Preparation of Frozen Stabilates

During our first year of experience with P. falciparum in culture, we had significant difficulty in retrieving viable cultures of FVO and FRC-3 strain parasites from frozen stabilates. Only once was a viable culture retrieved from nine lots that were frozen and thawed by the procedure of Diggs et al*. Since then, two other procedures, the Rowe and the glycerol phosphate buffer (GPB), described in Appendix 3, were used successfully on ten occasions to preserve frozen stabilates of both in vitro cultured and monkey whole blood-infected P. falciparum.

Once we froze and retrieved aliquots of the same lot of cultured parasites by the Diggs and the GPB method, and the Diggs stabilate was not viable in vitro; the GPB stabliate was viable.

*Diggs, C. et al 1975 Am. J. Trop. Med. Hyg. 24 (5): 760-763, and Diggs, C. et al 1977 Bul. W.H.O. 55 (2-3): 297-301.

Twice aliquots of red blood cells from the same infected monkeys were frozen and retrieved by the Diggs and GPB method, and only the GPB stabilates were viable.

Aliquots of the same three lots of *in vitro* cultured parasites were frozen and retrieved by the modified Rowe or GPB methods and although both methods yielded viable cultures, the Rowe method stabilates reached higher parasitemias, faster, in candle jar cultures after retrieval than did the GPB stabilates.

Aliquots of infected blood from two Aotus monkeys were frozen and retrieved by the Rowe and GPB methods and both were equally viable in candle jar culture.

Conclusion: We will use the Rowe method for freezing *in vitro* cultured parasites because it yields higher parasitemias when retrieved into candle jar culture than does the GPB procedure, and will use the GPB procedure for infected monkey blood because it is simpler than the Rowe procedure.

C. 1. Effect of Relative Flow Rate of Medium on Percent Parasitemia in Cultures

Manipulation of the inoculum and the culture perfusion rate has enabled us to produce greater numbers of merozoites for vaccine production.

Methods: Routine maintenance of cultures is done in 75 cm^2 polystyrene tissue culture flasks containing a settled red cell layer which was initially added to the flask as 9 ml of a 25% suspension of red cells in media (Appendix 2). Perfusion of the culture was at a rate of 2 ml of medium per hour. By using 25 cm^2 flasks which were initially charged with 3.0 ml of a 12.5% red cell suspension and then perfused after cells had settled at 2 ml of medium per hour, a six-fold increase in the flow rate per unit volume of red cells was achieved. The effect of these conditions on subsequent development of parasitemia was examined. Additionally, we attempted to attain higher parasitemias in the 25 cm^2 flasks by either starting the cultures with inocula which were adjusted to elevated starting parasitemias (3-5%), or by delaying the time between splits with fresh red cells to allow the cultures to attain higher parasitemias before harvest.

Results: Parasitemias of 10-20% were achieved in 1 to 4 days, depending on the starting parasitemia, in 25 cm^2 flow flasks charged with 3.0 ml of a 12.5% red cell suspension and perfused at 2 ml of medium per hour. Routine cultures in 75 cm^2 flow flasks seldom reached 8% parasitemia. These data are presented in Table 1.

Discussion: Our results indicate that we can enhance the percent parasitemia in flow flask cultures by increasing the media flow rate per unit volume of cultured red cells. The validity of extrapolating these results to larger (75 cm^2 and 150 cm^2) flow flasks is being tested.

It should be noted that the increased percent parasitemia achieved in the 25 cm^2 flask relative to the 75 cm^2 flask has not resulted in an increased total parasite yield per unit volume of medium utilized.

Table 1. The percent parasitemia observed in 25 cm^2 flow flasks inoculated with various levels of parasitemia

Expt. #	Flask #	Day 0	1	2	3	4
I.	1	1.0	5.3	-	10.8	-
	2	1.0	6.1	-	13.4	-
	3	1.0	5.1	-	9.7	16.7
	4	1.0	5.2	-	11.3	12.7
II.	1	ca 1.0	3.0	5.8	13.5	-
	2	ca 1.0	2.1	5.6	17.5	-
III.	1	3.8	13.9	16.5	-	-
	2	3.8	16.2	21.0	-	-
IV.	1	4.5	9.5	-	-	-
	2	4.5	11.0	-	-	-

C. 2. Effect of Carbon Dioxide tension on Parasite Growth in Culture

Carbon dioxide levels have been reported to be critical for parasite growth in culture with 3% CO_2 purportedly giving better results than 7% CO_2. Silicone rubber

tubing of the type routinely used for gas delivery to cultures is gas-permeable, and may result in loss of carbon dioxide from the gassing mixture to the atmosphere, making the amount of CO_2 delivered to cultures unknown. The use of gas-impermeable Tygon® tubing would prevent such losses, allowing delivery to the cultures of gas mixtures of known analysis.

Methods: We compared the growth of P. falciparum in our flow flask cultures (Appendix 2) when gassed with 3% or 7% CO_2 delivered to the cultures through silicone or Tygon® tubing. The cultures were serially diluted three times with fresh red blood cells at two or three day intervals. Each subculture was maintained under constant gassing conditions, and the percent parasitemia was monitored.

Results: The percent parasitemias of the four cultures are presented in Table 2.

Discussion: There was no indication that either CO_2 concentration used had a differential effect on the growth of P. falciparum in flow flask culture. Nor did the use of gas-permeable silicone rubber tubing have a measurable effect on parasite growth when compared to gas-impermeable Tygon® tubing. Thus,

under the flow flask culture conditions, we are assured that delivery of either of these gas mixtures through silicone or Tygon® tubing is equally effective for the growth of P. falciparum in vitro.

Table 2. Effect of Three Percent vs Seven Percent CO_2 Delivered Through Tygon or Silicone Tubing on Flow Flask P. falciparum Cultures

Expt.	% CO_2	Tubing	Passage	% Parasitemia on Days After Inoculation 0	1	2	3
1	7	Tygon	1	1.2	2.4	3.8	NT**
			2	1.0	NT	NT	7.1
			3	1.0	4.1	5.0	NT
			4	1.0	NT	3.4	NT
2	7	Silicone	1	1.2	3.4	5.0	NT
			2	0.8	NT	NT	7.0
			3	1.0	3.1	5.3	NT
			4	1.1	NT	6.2	NT
3	3	Tygon	1	1.2	2.6	7.4	NT
			2	1.1	NT	NT	7.2
			3	1.0	2.2	4.9	NT
			4	1.0	NT	6.1	NT
4	3	Silicone	1	1.2	2.6	7.1	NT
			2	1.0	NT	NT	7.4
			3	1.0	3.7	5.0	NT
			4	1.0	NT	5.7	NT

*NT: Not Tested

D. Culture in FEP-Teflon-film Culture Vessels and Polystyrene Culture Dishes

Reports in the literature concerning the continuous in vitro culture of falciparum malaria parasites differ substantially in the proportions of oxygen and carbon dioxide used to gas the cultures. The presently used

systems for cultivation of P. falciparum utilize polystyrene or glass culture vessels with or without flowing nutrient medium. Precise control of the oxygen tension at the level of the settled infected erythrocytes is not possible under these conditions due to the very limited solubility of O_2 in the culture medium and the relative impermeability of the culture vessels. Strong O_2 gradients would readily occur between the medium surface and the settled cell layer. Thus, a critical evaluation of optimal gassing conditions might be better accomplished using highly gas-permeable fluoro-ethylene-propylene copolymer (FEP-Teflon) film culture bags, which allow the settled red cells to be gassed through the bottom of the container.

Methods: Culture medium can be added or removed by the use of a 25 ga. needle and plastic syringe.

Cultures were incubated at 37° C in dessicator jars in an atmosphere of 17, 12, 7 or 2 percent O_2 and 3 or 5 percent CO_2. Cultures were either refed by changing the culture medium daily or not refed.

Results: When cultures of P. falciparum-infected erythrocytes were incubated at 17, 12, 7 or 2 percent O_2, no difference in the rate of parasite multiplication was observed; but 2% O_2 did not appear to maintain the erythrocytes in as good a condition as the higher O_2 levels.

As illustrated in Table 3, the parasites were found to grow and multiply in FEP-teflon at a rate comparable to those grown in petri dishes, i.e., 2-3 fold. The cultures achieved higher parasitemias when refed daily than when not refed. The longevity of the cultures in both FEP-teflon bags and Falcon petri dishes was approximately 3-4 days when not refed, after which the parasites died out. The parasitemias obtained and the parasites observed indicated that the age or condition of the cultured erythrocytes may have been the limiting factor in the refed cultures, whereas acidification of the medium accompanied by failure of the merozoites to develop into ring forms was limiting in the unfed cultures.

Table 3. Parasite Development in Refed vs Unfed FEP-Teflon Culture Vessels

	Refed				Unfed			
Day	% IRBC*	M*	T*	S*	% IRBC*	M*	T*	S*
0	1.0	1	99	0	1.0	1	90	0
1	1.3	0	93	7	1.3	3	81	16
2	2.5	1	82	17	1.5	20	67	13
3	1.0	1	82	17	0.7	100	0	0

*M = Merozoite, T = Trophozoite, S = Schizont,
% IRBC = % Infected Red Bloods Cells

In refed cultures, the parasites continued to mature although the percent parasitemia declined, whereas the unfed cultures progressed to all immature merozoite forms which, although associated with erythrocytes, failed to develop. These forms appear to have attached to, but

failed to penetrate the host cells. The presence of such merozoites was coincidental with a medium pH of less than pH 7.0. When the cultures reached pH 7.0, glucose levels in the medium still contained 50% or more of the starting concentration. Lactic acid levels increased in proportion to the fall in pH of the culture. The pH fell more rapidly, and more lactic acid was produced, in infected erythrocyte cultures.

Preliminary experiments in petri dishes utilizing culture medium adjusted to pH 6.8, 6.9, 7.0, 7.1 or 7.2 suggest that pH and not lactic acid *per se* interferes with the penetration and subsequent development of merozoites. The critical starting pH level appears to be at or very near to pH 7.0 in these experiments. See Table 4.

Table 4. The Effects of pH During Incubation at 37°C of Candle Jar Cultures of P. falciparum (n = 3)

pH	Day	M	R	YT	OT	YS	OS
6.8	0	1.33	25.33	47.67	18.67	6.67	0.33
6.9	0	1.33	37.33	38.00	18.33	4.00	1.00
7.0	0	1.33	25.67	37.00	26.67	9.00	0.33
7.1	0	0.67	29.00	40.67	25.00	3.33	1.33
7.2	0	0.67	28.33	39.00	22.67	8.00	1.33
6.8	1	33.67	5.00	9.33	29.33	18.00	4.67
6.9	1	31.67	5.33	9.33	36.33	12.00	5.33
7.0	1	5.33	12.33	24.33	35.33	17.33	5.33
7.1	1	3.00	26.00	24.00	27.00	14.33	5.67
7.2	1	1.00	21.33	29.67	28.33	13.67	6.00

Discussion: In summary, these data indicate that the mass culture of *P. falciparum* parasites should be possible in the automated IL-410 Cell Culture System which utilizes FEP-Teflon multilayered culture containers, a controlled atmosphere and medium pH, and automatic medium refeeds triggered by the buffer capacity of the medium.

E. Adenosine Pre-Treatment of Human Erythrocytes Used in Cultures

Methods: Plasmodia are known to utilize intraerythrocytic ATP, and ACD-stored red cells are known to become depleted of ATP. Therefore, the effects on the parasite/erythrocyte cultures of pre-treatment of the human red cells with adenosine at 15 μM and 37°C for 120 minutes in order to replenish their intracellular ATP levels prior to their use was examined. The level of parasitemia in petri dish cultures in candle jar utilizing adenosine pre-treated erythrocytes *vs* untreated erythrocytes was studied. Also, the effects of adenosine pre-treatment of ACD-stored red cells used in flow flask parasite cultures was examined. These cells were incubated with 15 μM adenosine at 37°C for 30, 45, 90 or 120 minutes prior to use. The cultures were maintained without replenishment of red cells and were monitored for parasite growth. The cultures are compared to a control culture which was not pre-treated with adenosine or replenished with fresh red cells during the continuous culture.

Results: The level of parasitemias in petri dish cultures in candle jars was found to be slightly greater for the erythrocytes pre-treated for 120 minutes with adenosine than for the untreated control cultures. Additionally, the morphologic condition of the adenosine-pretreated red cells was found to be superior to the untreated red cells after three days of culture. Fewer abnormal cells such as echinocytes and spherocytes were observed among the adenosine treated cells.

When maintained in continuous flow flask cultures, human AB erythrocytes which had been pre-incubated in 15 μM adenosine for 30 minutes supported peak parasitemias of P. falciparum which did not exceed that supported by untreated control erythrocytes (5.21% vs 6.55% respectively). Those erythrocytes which had been pre-treated for 45 minutes produced a peak parasitemia of 9.95%, those pre-treated for 90 minutes produced a peak parasitemia of 11.50% and those pre-treated for 120 minutes produced a peak parasitemia of 10.0%. The erythrocytes which had been pre-treated for 45, 90 or 120 minutes retained more normal morphology in culture longer than those which had been treated for 30 minutes or the untreated controls.

Discussion: Adenosine is known to replenish the diminished intracellular ATP levels of erythrocytes which have been stored for several weeks in ACD solutions. Intraerythrocytic ATP content is important to the erythrocyte's

ability to maintain normal cell morphology and membrane fluidity characteristics.[1] These factors seem to be important to parasite penetration and growth,[2] as only rarely is a malarial parasite observed in a grossly spherocytic or echinocytic erythrocyte. Pre-incubation of ACD stored erythrocytes in adenosine should therefore provide a greater number of suitable host target cells for emerging merozoites than unconditioned erythrocyte suspensions, thus allowing for enhanced levels of parasitemia. Our experimental results support this hypothesis: adenosine pre-treatment of ACD-stored host erythrocytes allows for higher parasitemias and longer life of erythrocytes in culture.

F. Attempts to Develop Synchrony in Cultures

During routine culture of *P. falciparum* in human erythrocytes *in vitro*, parasite development either in flow flasks or candle jars appears to be asynchronous. Either all growth stages of asexual parasites are present at any given time, or any evidence of synchronous development is unpredictable. Since synchronous development of parasites in a controlled, predictable manner would allow for harvest of parasites at a desired stage of development, i.e., merozoites or mature schizonts for use as antigens, an attempt to accomplish this end is in progress using antimalarial drugs.

[1]Oski, F.A. in The human red cell in vitro p. 45-47, Greenwalt & Jamieson eds. 1973, Grune and Stratton, N.Y.

[2]Eaton, J.W. and Brewer, G.J. Nature 222: 389-390, 1969.

Method: The FRC strain of P. falciparum growing in vitro cultures in candle jars have been treated with either chloroquine or pyrimethamine in an attempt to establish a degree of synchrony in parasite development. Cultures are initiated in 35 mm plastic petri dishes which contain 1.5 ml of a 12.5% suspension of human AB^+ erythrocytes in GM-5. From 0.25 to 0.50% of erythrocytes are parasitized at the time cultures are started (time 0) and parasitemia generally increases to 3 to 5% by day 3 or 4 in untreated (control) cultures. Cultures are incubated at 37° and medium is decanted from settled erythrocytes daily and replaced with fresh GM-5. Cultures are exposed to either chloroquine or pyrimethamine on day 1 or day 2 when the parasite is in a stage of rapid growth. Cultures are exposed to drug for periods of 18 to 30 hours and drugs are removed from cultures by decanting the supernatant growth medium and replacing it with drug-free medium. Chloroquine as the diphosphate salt is added at levels of 25, 50 and 100 µg (base) per liter of culture medium. Pyrimethamine is added as the free base at 10 or 20 µg per liter. Thin smears were made daily and stained by the Giemsa procedure for parasite evaluation, including overall growth, morphological effects and stages present.

Results: Exposure for 30 hours to 25 or 50 µg/l of chloroquine did not affect growth or produce striking morphological

changes in growing parasites. On day 4, cultures treated for 30 hours on days 1 and 2 had higher parasitemias than controls.

The addition of 100 μg/liter on day 2 and removal 24 hours later resulted in morphological changes in parasites, but failed to suppress day 4 parasitemia when compared with untreated controls. Chloroquine-induced changes include premature clumping of pigment, compact, darkly stained parasites and an increased number of parasites free of erythrocytes. Damage to parasites resulting from 24 hours exposure to chloroquine appears to be reversible since overall parasitemia was not suppressed. Chloroquine appears to inhibit development of parasites at all stages under the conditions employed and therefore does not seem to be useful in the establishment of synchrony in *in vitro* culture of *P. falciparum*.

Pyrimethamine added to cultures on day 1 and removed 18 to 24 hours later resulted in a selective suppressive action on growing parasites. Overall depression of growth rate was seen when either 10 or 20 μg pyrimethamine/liter was employed. The most striking effect seen after 24 hours exposure to pyrimethamine was the accumulation of parasites at the late trophozoite stage. No damage to parasites in early stages of development was evident, but

the few schizonts seen 24 to 48 hours after exposure to pyrimethamine were distinctly abnormal. Chromatin in these schizonts, instead of dividing into well-formed nuclei, appeared to split into fragments that varied markedly in size and shape and were generally indistinct with a "washed out" appearance. Up to 90% of the parasites present after 24 hours exposure were in the late trophozoite stage. The degree of synchrony attained in developing parasites treated with pyrimethamine largely disappeared during five to six days in culture in the absence of drug.

Discussion: Additional manipulations are planned in our effort to establish synchrony since even at short intervals (one or two generations) would allow for harvest of parasites at the desired stage of development. Pyrimethamine appears to offer more promise in this respect since it selectively inhibits the nuclear division stage in parasites.

G. Use of Cellulose Powder Columns for the Isolation of Schizont-Infected Erythrocytes

Method: Two attempts were made to repeat the cellulose column procedure reported by Cohen *et al* for the separation and incubation of *P. falciparum* schizont-infected erythrocytes and the subsequent collection of naturally released merozoites. Two cultures of *P. falciparum* infected human

AB erythrocytes were collected from flow flasks and filtered through CF 11 cellulose powder columns. Triplicate samples were collected before and after the procedure from each column, and giemsa-stained smears were made and examined. Differential counts were done according to the stage of parasite maturation, and the three sets of data were averaged for each experiment.

Results and Discussion: Schizonts were not retained by the CF 11 columns in these experiments in contrast to those reported by Cohen* et al in which schizont-infected erythrocytes were retained by CF 11 cellulose columns. The reason for this unfavorable result is not readily apparent. In Dr. Cohen's experiments, the parasitized cells were obtained from infected humans and subsequently incubated in culture medium for 30-50 hours before filtration, whereas our preparations are parasitized human erythrocytes which have been maintained continuously in culture. Dr. Cohen's medium contains 5% human AB serum and 5% fetal calf serum, whereas ours contains 10% human AB serum. Perhaps the greatest difference lies in the fact that Dr. Cohen incubated his cultured cells in conical flasks under an atmosphere of 5% CO_2 in air. We incubate our cultures under 5% CO_2 with 7% oxygen in nitrogen, thus a considerable difference exists in the

*Cohen, S. et al, Lancet, June, 1977, 1335-1338.

oxygen levels, with ours being one-third that present in Cohen's experiments. Oxygen levels are known to be important in P. falciparum culture development.

H. 1. Purification and Concentration of P. falciparum Merozoites - Gradient Centrifugation

Methods: In vitro flow flask cultures (Appendix 2) of P. falciparum, cultures partitioned by low speed centrifugation, and the effluents obtained during the growth of these cultures, were examined by density gradient centrifugation on ficoll in an effort to isolate fractions rich in merozoites and/or schizonts. The major culture components separated by low speed centrifugation were (1) the red cell pellet, (2) the "gray layer" which consisted of various developmental stages of free parasites, hemozoin and cellular debris, and (3) the supernatant culture medium. Parasites, when centrifuged on a ficoll density gradient, are known to accumulate at a ficoll density of 22-24%. Examination of the various fractions obtained was done by microscopic evaluation of Giemsa-stained smears.

Results: (1) Whole Culture Separation

When this material was centrifuged on discontinuous ficoll gradients ranging from 24% to 32%, merozoite-like parasites were banded not only at the medium/24% ficoll interface, but also at diverse interfaces

throughout the gradient. The cellular debris collected at the 24% and 28% ficoll interfaces, while the infected and uninfected erythrocytes were found in the pellet.

(2) Erythrocyte Fraction Separation

No free merozoites were found in the erythrocyte pellet obtained by low speed centrifugation. When this culture component was centrifuged through ficoll density gradients, no separation of schizonts was obtained. Merozoites were occasionally found at the 24% ficoll interface and their presence may be attributable to maturation or disruption of schizonts contained in the erythrocyte population. These merozoites showed evidence of minimal osmotic damage.

(3) "Gray Layer" Fraction Separation

When this culture component was centrifuged through a ficoll gradient, numerous merozoites were accumulated at the 22% ficoll/medium interface. Merozoites were also found at other ficoll interfaces throughout the gradient. The merozoites obtained at the 22% ficoll interface were relatively free of cellular debris and hemozoin, but appeared to be morphologically degenerate.

(4) Supernatant Fraction Separation

When the culture supernatant was centrifuged through a 22% ficoll gradient, two bands were observed at the

ficoll/medium interface, and a brown pellet was present at the bottom of the tube. The upper band contained only debris while the lower band at the ficoll interface contained minimal numbers of degenerate merozoites together with some debris. The pellet contained a considerable amount of hemozoin and some membranes.

(5) Culture Effluent Separation

When the culture effluent was centrifuged at 3000 x g the resultant pellet contained debris, but not significant numbers of recognizable parasites, and therefore ficoll gradient separations of parasites from effluent seems to be unproductive.

Discussion: When unseparated cultures were centrifuged through ficoll gradients, merozoites were obtained primarily at the 22% and 24% ficoll interfaces, but also at higher density interfaces. Infected erythrocyte material also occasionally yielded merozoites at these interfaces, probably due to maturation or rupture of infected schizonts. "Gray layer" preparations similarly yielded merozoites principally at the less dense ficoll interfaces, as did the supernatant from resuspended whole cultures. Schizont-rich layers were not found, perhaps due to the centrifugation forces employed.

In all cases, the merozoites recovered from ficoll gradients showed some degree of damage or degeneracy, with a consequent heterogeneity of merozoite density being observed. Both aging in culture and ficoll-induced osmotic damage could have contributed to these changes. The culture supernatant preparations have shown reasonable potential as an antigen source and in this case, ficoll gradient separation is not necessary. This antigen source is currently being evaluated for *in vitro* tests. Growing-culture effluents were not found to be a productive source of particulate antigens.

H. 2. Purification and Concentration of Merozoites - Differential Centrifugation

Methods: Extraerythrocytic Merozoites: Merozoites were harvested from flow flask cultures by resuspending the culture, sedimenting the erythrocytes by centrifugation at 640 x g for 10 minutes, and then centrifuging at 3000 x g for 10 minutes to pellet the free merozoites.

Intraerythrocytic Parasites: Parasites in various stages of development were harvested from flow flask cultures by sedimenting the infected erythrocytes at 640 x g for 10 minutes, removing the supernatant culture medium and resuspending the cultured cells to 10% suspension in fresh culture medium. This cell suspension

was then forced by hand pressure out of a plastic 10 cc syringe through a 23 gauge needle in order to disrupt the parasitized red cells by violent decompression and shear forces. The disrupted suspension was then centrifuged for 10 minutes at 80 x g to sediment unbroken cells. The parasite-containing supernatant was then centrifuged for 10 minutes at 3000 x g to sediment the parasites.

Results: Extraerythrocytic merozoites: A single flow flask culture contained in a 75 cm^2 vessel yielded approximately 9 ml of supernatant fluid which contained an average of 125 μg/ml of total protein as determined by the Lowry procedure. Giemsa-stained smears of the harvested parasites were examined by light microscopy and found to contain almost exclusively a population of small parasites, presumably merozoites.

Intraerythrocytic parasites: A single flow flask culture yielded approximately 2.5 ml of packed cells. When crude parasite material was prepared by needle disruption and differential centrifugation, less than half as much total protein was recovered as from the corresponding culture supernatant. Giemsa stained smears of the crude parasite product, when examined by light microscopy, contained parasites of various developmental stages as well as erythrocyte membranes and debris.

Conclusion: Parasite material was obtained from culture supernatants in greater quantity and was of better quality than parasite material obtained by disruption of parasitized cells.

I. Rabbit Immunization for Antibody Production

Young, outbred, albino rabbits were used to raise antibody against parasite material which was obtained from either the culture supernatants or the infected red cells. The animals each received an initial intramuscular dosage of 2 mg of crude parasite material emulsified in Freund's Complete Adjuvant (FCA), followed by 2 intramuscular injections of 1 mg of crude parasite protein emulsified in FCA. Sera from these rabbits will be utilized in attempts to develop an *in vitro* neutralization test after being adsorbed with human AB red cells.

J. Infectivity of *in vitro* grown parasites for *Aotus trivirgatus griseimembra*

The purpose for this series of experiments was to assess the infectivity and course of parasitemia of *in vitro* cultured *P. falciparum* in *Aotus* (owl) monkeys and to obtain infected blood of high parasitemia for storage as frozen stabilates. Both the FVO (Asian) and FRC-3 (African) strains of *P. falciparum* have been subpassaged in owl monkeys.

Initially, an intact owl monkey was inoculated intravenously with the FVO strain of P. falciparum-infected human AB^+ erythrocytes after 409 days in continuous in vitro culture. The course of infection was monitored by daily peripheral blood (thick and thin) smears which were stained by the Giemsa procedure. Parasitemia was recorded as per cent of erythrocytes infected, not as number of parasites per 100 erythrocytes. Patency was attained on day 13 after inoculation and parasitemia persisted over a period of 25 days, becoming subpatent 39 days after inoculation of parasites. The infection reached a peak parasitemia of 3.2% on the eighth day of patency and no antimalarial drug was required to control the infection. The monkey was splenectomized four days after becoming subpatent, but parasites could not be demonstrated during a 40 day follow-up period after splenectomy. However, recrudescences of short duration and low grade parasitemias were seen at days 84 and 118 after the initial challenge.

Since a high degree of parasitemia failed to develop and a chronic, low grade infection resulted in the initial owl monkey challenged with in vitro cultured P. falciparum, it was not clear whether we were using a parasite strain with reduced virulence in the owl monkey or a monkey with a low degree of susceptibility to the infection. In an attempt to resolve this question, two approaches were used.

Individual monkeys in our Aotus colony were characterized genetically to determine susceptibility to malaria infection and the parasite was serially subpassaged by blood transfer in an attempt to adapt the parasite to more virulent development in owl monkeys.

Genetic classification of Aotus monkeys: Several authors, including Brumback[1], Thorington[2], and Ma[3], have examined New World Aotus monkeys in an attempt to differentiate species according to geographic origin, phenotype and chromosome variation. Other investigators including Young[4], Wellde[5] and Schmidt[6] have studied the susceptibility of Aotus monkeys to malaria as related to geographic origin and phenotype. Since the literature does not clearly define the relationship between karyotype and susceptibility to malaria, our colony of Aotus monkeys was studied for phenotype, karyotype and susceptiblity to falciparum malaria.

[1]Brumback, R.A., Staton R.D., Benjamin S.A., et al: The chromosomes of Aotus trivirgatus Humbolt 1812 Folia Primatol 15: 264-273, 1971.

[2]Thorington, R.W., Vorek R.E.: Observations on the geographic variation and skeletal development of Aotus. Lab Animal Science 26: 1006-1021,1976.

[3]Ma, N.S.F, Jones, T.C., Miller A.C, et al: Chromosome polymorphism and banding patterns in the owl monkey (Aotus). Lab Animal Science 26: 1022-1036, 1976.

[4]Young, M.D., Baerg, D.C., Rossan, R.N.: Studies with induced malarias in Aotus monkeys. Lab Animal Science 26: 1131-1137, 1976.

[5]Wellde, B.T., Johnson, A.J., Williams, J.S. et al: Experimental infection with Plasmodium falciparum in Aotus monkeys. I. Parasitologic, hematologic, and serum biochemical determinations. Am. J. Trop. Med. Hyg. 21: 260-271, 1972.

[6]Schmidt L.H. Infections with Plasmodium falciparum and Plasmodium vivax in the owl monkey - model systems for basic biological and chemotherapeutic studies. Trans. R. Soc. Trop. Med. Hyg. 67: 446-477, 1973.

All animals closely resemble phenotype B as described by Ma[3]. The abdomen and chest are covered with light orange or yellowish hair which extends one half of the way to the elbow and knee. A few of these hairs may extend to the chin, but hair under the chin is predominantly gray or brown in color. The back is gray to brown and agouti. The face has a narrow white mantle around its periphery and the tail is gray to brown at the base, shading to black at the tip. The feet are brown.

The leukocyte culture method of Moorhead, et al[7], was used to prepare chromosomes for conventional Giemsa staining. Since there is not yet a standard karyotype (or karyotypes) established for Aotus, the system of Ma et al[3] was used. All animals are of karyotype II, III or IV (Aotus trivirgatus griseimembra). The results from our colony of twenty animals are summarized on the next page.

[7]Moorhead, P.S., Nowell, P.C., Mellman, W.J., et al: Chromosome preparations of leukocyte cultures from human peripheral blood. Exp. Cell Res. 20: 613-616, 1960.

Summary of Aotus karyotypes

No. of Animals	Diploid No.	Karyotype
8	54	5 pairs of meta- and submetacentric chromosomes 21 pairs of subtelo- and associated chromosomes
8	53	5 ½ pairs of meta- and submetacentric chromosomes 19 pairs and 2 unpaired subtelo- and associated chromosomes
4	52	6 pairs of meta- and sub-metacentric chromosomes 19 pairs of subtelo- and acrocentric chromosomes

To date, all 14 of our animals challenged with blood forms, regardless of karyotype, have been susceptible to *P. falciparum* infection.

Adaptation of P. falciparum by Serial Passage

The FVO strain was subpassaged in a series of three owl monkeys. The results are summarized in Table 5. Monkey B-2 was splenectomized and inoculated intravenously with approximately 4.0×10^6 parasitized human AB^+ erythrocytes from *in vitro* culture. Peripheral blood smears were made daily and stained by the Giemsa procedure. Parasites were first detected in smears taken on day 17 after inoculation. A peak parasitemia of 0.35% was recorded on day 26. The resulting self-limiting infection remained patent through day 46, after which no parasites were detected

in blood smears over a period of 35 days. Two recrudescences of low grade parasitemia with short intervals of patency were seen at 83 and 142 days after the initial challenge.

Infected blood was transferred from monkey B-2 to B-14 on day 5 of the initial patency in B-2. Monkey B-14 was intact (not splenectomized) and received approximately 10×10^6 parasitized erythrocytes intravenously. Patency was obtained on day 2 after challenge, reached a peak of 2.9% on day 10 and became subpatent by day 22 without antimalarial drug intervention. Low grade parasitemias were again recorded on day 32 and on days 42 through 49.

Monkey B-18, an intact animal, was inoculated intravenously with about 10×10^6 parasitized erythrocytes from B-14 on the 19th day of his infection. Patency was first observed on day 6 after challenge and a peak parasitemia of 0.6% was recorded on day 6. A low grade parasitemia persisted through day 22 after which the infection became subpatent. Parasites were seen again in a single blood smear taken on day 105 after the initial challenge. No further subpassages of the FVO strain of _P. falciparum_ were made in Owl monkeys and no frozen stabilates of parasites in Owl monkey blood were obtained.

In summary, each of the four Owl monkeys challenged with the FVO strain were susceptible to infection. All four monkeys developed self-limited infections with low grade parasitemias that resulted in chronic infections as evidenced by low level recrudescences of parasitemia over extended periods of time. At this point, one problem was evident: we did not have a reliable challenge system to assess immunity to P. falciparum in vaccinated Owl monkeys.

Table 5. Results of Serial Passages of in vitro Culture Plasmodium falciparum (FVO strain) in Aotus trivirgatus griseimembra

Monkey	Days to Patency	Peak Parasitemia/ Days After Challenge	Subinoculated (Days After Exposure)	Result of Infection
B-2*	17	0.35%/26 days	21	self-limited, chronic
B-14	2	2.9%/10 days	19	self-limited, chronic
B-18	6	0.6%/12 days	N.D.**	self-limited, chronic

*Splenectomized immediately prior to challenging with in vitro cultured P. falciparum in human erythrocytes.

**Not Done

Rather than continue with the adaptation of the Asian strains (FVO) to virulent growth in the Owl monkey, we considered the development of virulence in the African strain (FRC-3) of P. falciparum more appropriate. The latter strain had been isolated from man and maintained

in human erythrocytes in *in vitro* over a period of 14 months prior to receipt by us. After maintenance *in vitro* in human erythrocytes in our laboratory over a period of 15 days, a series of passages of the FRC-3 strain was initiated. The purposes for passages in Owl monkeys of the FRC-3 strain are the same as previously indicated for the FVO strain.

The results of a series of six subpassages of the FRC-3 strain of *P. falciparum* in Owl monkeys are summarized in Table 2. The infection was initiated in a splenectomized monkey (B-9) by the intravenous injection of 5.0×10^6 parasitized human AB^+ erythrocytes from *in vitro* culture. Patency was attained on day 12 after challenge and parasitemia rose to 29% on day 32, at which time the infection was controlled by subcurative pyrimethamine treatment. Four daily doses of pyrimethamine given orally at 2.5 mg/kg/day resulted in clearance of peripheral blood parasites by day 41. However, a chronic infection ensued with three recrudescences recorded over the 100 day period after initial clearance of parasites.

Infected blood was transferred from monkey B-9 to B-4 during the acute stage of rising parasitemia (day 29 after challenge) prior to treatment with pyrimethamine. Glycerolized blood was preserved in liquid nitrogen at the

same time by a slight modification of the method of Rowe (see Appendix 3). Monkey B-4, an intact animal, became patent on day 4 after inoculation and reached a peak parasitemia of 2.5% on day 17. No chemotherapy was required to control the infection and blood smears became negative for parasites on day 33. A chronic infection resulted with recrudescences of parasitemia. Infected blood was transferred from Monkey B-4 to B-3 on day 28 after the initial challenge.

Monkey B-3 (intact) received approximately 10×10^6 parasitemized red blood cells intravenously and patency was observed on day 5 after challenge. Peak parasitemia of 5% was recorded on day 15 after which the infection declined to subpatent level by day 23 without drug treatment. Minor recrudescences of parasites were seen on days 45 through 49 and again on day 97. Parasitized blood was transferred on day 19 to Monkey B-19. Monkey B-19 (intact) received about 10×10^6 parasitized erythrocytes and developed patent parasitemia on day 5 after challenge. A peak parasitemia of 30% was recorded on day 22, at which time 7.5 ml of blood was drawn for freeze preservation. The monkey was moribund prior to drawing blood and died on the same day without receiving drug treatment. However, blood was transferred from Monkey B-19 to B-13 on day 16 after challenge.

Monkey B-13, an intact animal, received approximately 10×10^6 parasitized erythrocytes and developed patent parasitemia by day two after challenge. Parasitemia rose to 23% by day 10 at which time 8.0 ml of blood was drawn for freezing and chloroquine treatment initiated. The monkey died on day 13 after challenge with a parasitemia of 43% in spite of four days treatment with chloroquine, given intramuscularly, 20 mg(base)/kg/day. Infected blood was subinoculated from B-19 on day 7 after challenge, when parasitemia was 5.5% and rising rapidly, into monkey B-16.

Monkey B-16, (intact), received 10×10^6 parasitized erythrocytes and was patent for parasites on day 2 after challenge. Parasitemia rose rapidly to 19% by day 7, at which time the animal was bled out and 19 ml of blood was glycerolized and frozen in 0.5 ml aliquots in liquid nitrogen.

Table 6. Results of Serial Passages of *in vitro* cultured *Plasmodium falciparum* (FRC-3 strain) in *Aotus trivirgatus griseimembra*

Monkey	Days to Patency	Peak Parasitemia/ Days After Challenge	Subinoculated (Days After Exposure)	Results of Infection
B-9*	12	29%/32 days	29	chemotherapy - chronic
B-4	4	2.5%/17 days	28	self-limited - chronic
B-3	5	5.0%/15 days	19	self-limited - chronic
B-19	5	30%/22 days	16	death - acute
B-13	3	42%/13 days	7	death - acute
B-16	2	19%/7 days	N.D.**	bled out - acute

*Splenectomized immediately prior to challenging with *in vitro* cultured *P. falciparum* in human erythrocytes.

**Not done. Blood frozen on day 7 after challenge.

These results indicate that *P. falciparum* from *in vitro* culture in human erythrocytes underwent an adaptation during a series of six repeated passages in Owl monkeys. Originally, the parasite produced a self-limiting, chronic infection in Owl monkeys. During a period of adaptation during successive blood-induced passages in Owl monkeys, acute infections were produced which terminated in death of the monkey. This virulent *Aotus*-adapted strain of *P. falciparum*, preserved as a frozen stabilate

in liquid nitrogen, is now available for use in assessment of the degree of immunity to infection in *Aotus* monkeys vaccinated against *P. falciparum*.

K. Significance, Side Effects and Design of Research

Our research thus far has enabled us to fully implement the existing technology for the production of *P. falciparum* antigens in culture. Additionally, we have been able to develop further insights into the growth and development of *P. falciparum* in culture which have enabled us to improve our yields and the reliability of our techniques. The possibility of *in vitro* culture of *P. falciparum* in the automated IL-410 culture system may make possible the production of very large quantities of merozoite antigens in a way which is closely monitored and controlled, and which requires minimal labor.

V. Dissemination and Utilization of Research Results

There has been none to date.

VI. Work Plan for the Coming Six Months

The work plan for the next reporting period includes the following objectives.

A. We plan to continue to optimize the harvesting of merozoites from *in vitro* culture of *P. falciparum*. Emphasis will be placed on methods to synchronize the culture and the accumulation of merozoites by preventing infection of red blood cells.

B. New methods of merozoite collection will be developed by which their morphologic and immunogenic integrity is maintained.

C. We will continue to immunize rabbits with adjuvanted and unadjuvanted merozoite vaccines.

D. Development of serologic tests will begin. The first to be worked on will be a P. falciparum neutralization test in in vitro culture.

VII. Involvement of Minority Personnel and Women

Four women were involved in project activities during the reporting period.

VIII. Financial Statement

Expenditure	Period 8/15/77 to 2/14/78	Period 2/15/78 to 8/14/78
Salaries and wages including Fringe Benefits	$62,067	$48,900
Consultant Fees	-	400
Travel and Transportation	203	400
Other Direct Costs	-	-
Overhead	38,824	30,611
Research and General Administration	3,765	2,828
Supplies	10,866	18,438
TOTAL COST TO GOVERNMENT	$115,725	$101,577

METHOD FOR THE IN VITRO CULTIVATION OF PLASMODIUM FALCIPARUM IN TISSUE CULTURE IN PETRI DISH-TYPE VESSELS IN CANDLE JAR

Petri Dish: Coverslips (#1, 5x22 mm) are washed in 70% alcohol, rinsed in distilled water and air dried. A daub of Dow Corning silicone grease is applied to one side and the coverslip is placed in a petri dish and autoclaved. Coverslips are arranged greased side down on the bottom surface of a plastic petri dish by aseptic transfer. An overnight exposure to ultraviolet is used to insure sterility. Compartmented petri dishes are similarly used.

Tek Lab Chambered Slides: The use of these tissue culture chambered slides was described in our July 1, 1976 Report.

Tissue Culture Inoculation: An appropriate concentration of the tissue culture cells in tissue culture growth medium is placed in the culture vessel and incubated at 37°C until the cells have adhered to the coverslips and started to spread.

P. falciparum Inoculation: The source of the P. falciparum inoculum is either a stock flow cell culture or a candle jar culture (see Appendices C and D). The harvest from the stock culture is centrifuged at 1500 rpm for 10 minutes, the supernatant removed and the red blood cell pellet is resuspended at a 0.5% to 1.0% parasitemia in a 12.5% suspension of normal AB human red blood cells in medium GM-5, see Appendix H. The growth medium for the tissue culture cells is aspirated from the vessel and, if a primary cell containing significant amounts of debris, the vessel is rinsed once with tissue culture growth medium. The vessel is inoculated with 1.5 ml per 35 mm petri dish or 3.0 ml per segment of a compartmented 100 mm petri dish or 0.35 ml per Tek Lab chamber of the infected red blood cell suspension. The vessel is incubated in a candle jar.

Microscopic Evaluation: Daily, two smears are made of the red blood cells in the vessel and stained with Giemsa or May-Grunwald-Giemsa. The Giemsa stained smear is used for

determination of the percent parasitemia; the May-Grunwald-Giemsa stained smear is used as a control on the staining of the tissue culture coverslips or slide and is therefore processed with them. See Appendix I for staining methods.

A coverslip or slide is removed daily and stained with May-Grunwald-Giemsa.

In addition to the P. falciparum infected tissue culture vessel, a P. falciparum infected control vessel containing coverslips, or a chamber of the slide, with no tissue culture cells is run in parallel with the tissue culture vessel. The control is also monitored daily for percent parasitemia to indicate if the tissue culture cells are affecting the growth of P. falciparum in the red blood cells.

A petri dish or segment of a compartmented petri dish or a compartment of the slide containing only tissue culture cells (a tissue culture control) is included in the screening test.

METHOD FOR THE CULTIVATION OF P. FALCIPARUM IN FLOW FLASKS

Refer to the schematic and photograph of the Flow Flask Apparatus for Plasmodium cultivation on pages 4 and 5.

Flow Flask: A sterile Corning 25 cm^2 plastic tissue culture flask is prepared by cutting holes in the top of the flask at appropriate sites with a hot cork borer. White rubber stoppers are inserted in the holes. The gas and medium lines are connected to the flask via cannulated 3/0 silicone rubber stoppers after the flask has been inoculated with P. falciparum infected red blood cells.

P. falciparum Inoculum: The source of the P. falciparum inoculum is either a stock flow flask culture or a candle jar culture (Appendix C). The harvest from the stock culture is centrifuged at 2100 rpm for 10 minutes, the supernatant removed and the red blood cell pellet resuspended at a 0.5% to 1.0% parasitemia in a 12.5% to 50% suspension of normal AB human red blood cells in Medium GM-5, see Appendix H. Alternately, the contents of an on-going flow flask culture with an adequate parasitemia is split by removing the desired fraction of the contents of the flow flask and replacing it with fresh normal red blood cells suspended to the appropriate concentration in GM-5 Medium.

The red blood cell layer in the flask is sampled with a Pasteur pipette via the flask mouth. The multiplication of the P. falciparum is quantitated by determining the percent parasitemia from stained smears.

Medium Feeding: (Afferent) The Medium, maintained at 5°C, is pumped out of a resevoir to the flask at a rate of 2 ml per hour (or other rates) through silicone rubber tubing connected to a cannula. We are currently using a Manostat pump to deliver Medium to and remove it from the flask. It can handle 10 to 20 tubes at flow rates ranging from 0.25 ml to 350 ml per hour. The afferent cannula is positioned through a stopper at the end of the flask distal to the mouth. The end of the cannula is immersed in the medium-red blood cell layer in the bottom of the flask.

When the same medium is being used in a multiple-flask experiment, a single resevoir fitted with multiple cannulae and afferent lines feeds each flask, thus reducing the number of resevoirs needed and ensuring the uniformity of medium supplying all the flasks.

Spent Medium Removal (Efferent): The efferent flow line is connected to a cannulated silicon stopper inserted into the flow flask over its shoulder near the mouth. The end of the cannula almost touches the shoulder. The depth of the medium is maintained at about 4 mm. Through silicone rubber tubing connected to the cannula the spent medium is pumped into a graduated Erlenmeyer flask which also serves to roughly monitor the throughput.

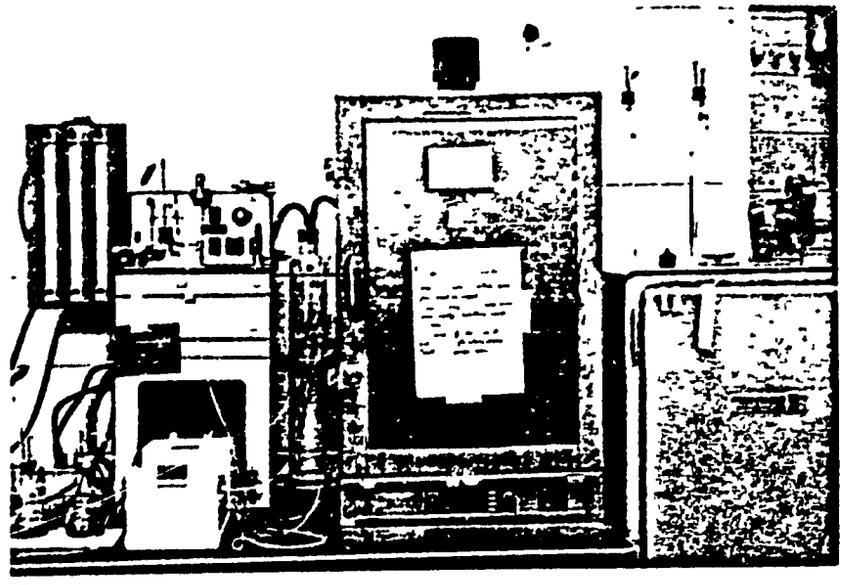
Intermittently during each experiment or passage of P. falciparum the affluent and effluent volumes are measured to determine the exact volume of medium used, disregarding evaporated loss.

Afferent Gas: A compressed gas cylinder containing 5% oxygen, 7% CO_2 and 88% nitrogen is connected via silicone tubing to a humidifier (a graduated cylinder containing water through which the gas is sparged), a 0.2 μ Millipore filter to sterilize the gas, and into the flow flask via a cannulated silicone stopper. The stopper is located at the distal end of the flask.

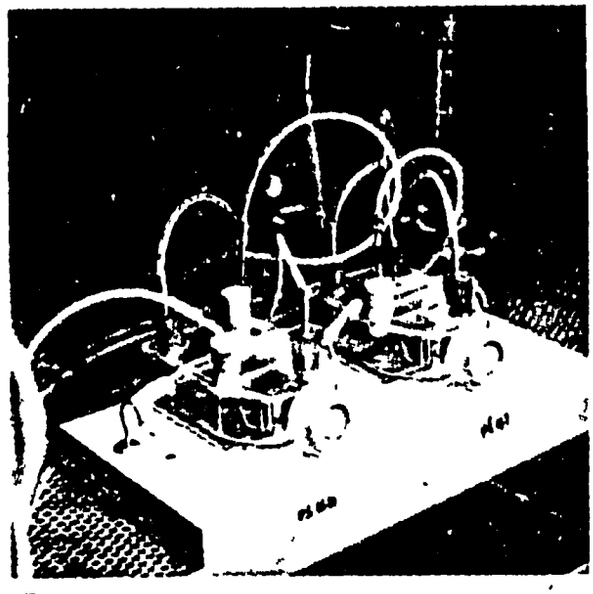
Efferent Gas: The gas exits from the flask via a cannulated silicone rubber stopper located near the mouth of the flask. Tubing connected to this cannula is led to a gas trap consisting of an empty flask connected to a graduated cylinder containing about two inches of Phemerol (a quaternary ammonium disinfectant) through which the gas is bubbled. The gas trap prevents backflow of nonsterile air into the system upon the infrequent occasion of a blockage in one of the lines leading to formation of a vacuum in the system. The trap also provides an indicator that the gas is flowing and there are no leaks in the system. Further it causes a slight back pressure in the system, ensuring an even distribution of gas. It also serves to reduce potential contamination of the incubator and room by adventitious microorganisms.

Multiple flow flasks are connected in tandem during tissue culture experiments or when P. falciparum culture conditions are being compared and a common gas supply for the cultures is desired. When connected in tandem, the efferent gas from one flask becomes the afferent gas for the next flask in line.

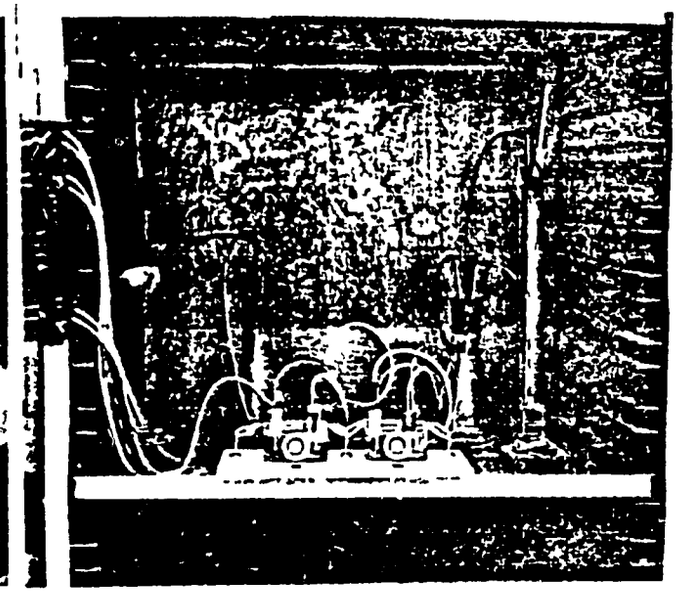
The above flow flask cultivation method for P. falciparum is based on the procedure described by Drs. Trager and Jensen in their Science publication and in personal communications. Our design, which contains minor modifications of theirs, has some important advantages. It is extremely flexible, so that one can readily study the effect of modification of the flask design altering flow rate and type of medium as well as gas and scale up. It uses commercially available flasks which are disposable, relatively inexpensive compared to specially designed glass flasks, and are available in a size that can contain six times more culture than we are presently using without altering the depth of medium. Presumably, new molds could be made which would increase this capacity if needed for production of vaccine.



A.



B.



C.

D.

Cryopreservation of P. falciparum in erythrocytes

The Glycerol-Phosphate Buffer (GPB) Method

Freezing:

1. To 1 volume of whole blood add 0.5 volume of dilutent* with mixing (swirl). Allow to equilibrate at room temperature for 10 minutes. If an *in vitro* culture in human red blood cells is to be stabilized instead of whole animal blood, the first step in the freezing procedure is modified: The culture is centrifuged 850G for ten minutes. The packed red blood cells are resuspended in a volume of GM-5 medium equal to the packed cell volume. Add to this volume 0.5 volume of dilutent with mixing (swirl). Allow to equilibrate at room temperature for ten minutes.

2. Dispense into ampoules (glass or plastic) and seal. Normally 0.75 to 1.5 ml of mixture is added per ampoule.

3. Freeze by immersion into liquid nitrogen.

Thawing:

Remove ampoule from freezer and rapidly warm to 37°C.

Thawed material may be injected intravenously into animals without further processing or glycerol may be removed by the Rowe procedure for *in vitro* parasite culture.

*Dilutent solution is prepared by mixing 30 ml glycerol with 70 ml sodium phosphate buffer, 0.067 M, pH 7.3. Solution may be sterilized by filtration or autoclaving.

Modified Rowe Method

(Rowe, A. W. *et al*, Liquid Nitrogen Preservation of Red Blood Cells for Transfusion. A low glycerol rapid freeze procedure (1968) Cryobiology 5 (2): 119-128

Freezing:

1. Centrifuge whole blood or *in vitro* culture at 850G x 10 minutes. Discard plasma or medium.

2. Add to packed red blood cells an equal volume of Protective Solution[2] and mix thoroughly.

3. Dispense into ampoules and seal. Normally 0.5 to 1.5 ml of mixture is added per ampoule.

4. Freeze by immersion into liquid nitrogen.

Thawing:

1. Remove ampoule from freezer and rapidly warm to 37°.

2. Centrifuge at 1800G for 7 minutes.

3. Discard supernatant.

4. Gently resuspend the cells in one volume of the 1st wash solution[2] equal to the volume of discarded supernatant.

5. Centrifuge at 850G for five minutes.

6. Repeat steps 3, 4 and 5, but use 0.9% saline instead of first wash solution.

7. Repeat steps 3, 4 and 5, but use RPMI-1640 instead of first wash solution.

8. Resuspend the red blood cells in GM-5.

[1]Solution consists of 28% glycerol, 3% Mannitol and 0.65% NaCl

[2]Solution (1st wash) = 16% Mannitol in 0.9% saline