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JANUARY 1 to DECEMBER 31, 1977

ANNUAL REPORT

THE IN VITRO CULTIVATION OF MALARIA PARASITES IN CELL CULTURES

Report Summary

1. The In Vitro Cultivation of Malaria Parasites in Cell Cultures AID/ta-C-1258
2. Robert G. Brackett, Ph.D.; Parke, Davis & Company, Box 118, Detroit, Michigan 48232
3. January 1, 1977 - December 31, 1977
4. January 1, 1976 - December 31, 1977
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We investigated the growth of P. falciparum in 24 tissue culture cells: bone marrow from Rhesus monkey, Squirrel monkey and baboon; frozen primary Cynomolgus monkey kidney, lung, spleen; primary Rhesus monkey lung and spleen; primary frozen Rhesus monkey kidney, lung and liver; the Vero African Green monkey kidney cell line; primary chimpanzee kidney and spleen; frozen primary chimpanzee kidney, lung and spleen; a line of human kidney; four human embryonic diploid lung lines; primar frozen owl monkey spleen; and primary human umbilical vein endothelial. Neither multiplication nor maturation of P. falciparum was observed in any of these cell cultures. However, association of identifiable parasites was observed with the frozen Cynomolgus monkey kidney and th human umbilical vein endothelial cells. We were unable to repeat the original observation of normal merozoites or rings associated in large numbers with Cynomolgus monkey kidney cells, but since original test conditions were not reproduced, additional work is planned. About 1% of the human endothelial cells contained identifiable stages of P. falciparum. We plan to pursue this possible lead. The FRC-3 strain of P. falciparum, recently obtained from Drs. Trager and Jensen was intro duced into our testing program. It grows to higher parasitemias in our in vitro Flow Flask culture than does the FVO strain and thereby enables us to increase the number of parasites in the cell culture inoculum. We studied mouse bone marrow cell culture and mouse Leydig tumor cell culture inoculated with P. berghei by the exact methods of Dr. Speer and did not demonstrate maturation nor multiplication of the parasite in these cells.

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Annual Research Report

A. General Background

Malaria remains a serious public health problem in tropical Africa and in several areas of Asia, Central and South America. It is estimated that this disease causes about 150 million clinical cases each year and more than one million children under 14 years of age die (1, 2).

Of the human malarias, the disease caused by P. falciparum is the most serious, causing high fatality rates in endemic areas and the chronic form of the disease reduces the level of normal living and working activities. Control measures, thus far, have been effective in only a few tropical countries, and we are sorely in need of other approaches to the control of this illness.

Immunization offers hope for the control of malaria. Varying degrees of success have been achieved in primate models 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 (3, 4, 5). 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 (6, 7) 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 also demonstrated that adjuvanted merozoite vaccines protected Rhesus monkeys against a sporozoite challenge and formol treated and freeze-dried adjuvanted vaccines protected Rhesus against an erythrocytic stage challenge with P. knowlesi (8, 9).

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 (10), Mitchell (11) and Diggs (12). Studies by Miller et al (10) indicated that protective antibody did not affect intracellular parasites, but interrupted 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 speculated 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 indicated 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 (11, 12) present evidence that *in vitro* inhibitory antibody probably directed against merozoites correlates with the immune status of the donor rhesus and its action is independent of complement or phagocytic cells and is analogous to viral neutralizing antibody

Diggs and Osler (12) 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.

One of the main problems for the development of a *P. falciparum* vaccine was 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 succeeded in the long-term cultivation of *P. falciparum in vitro* in human red blood cells (14). However, a vaccine prepared from pooled red blood cells and serum from thousands of individuals presents

serious theoretical and actual concerns regarding its safety. The risk of contamination with adventitious agents is formidable, e.g., hepatitis, herpes, Epstein Barr, adeno and measles viruses.

Exquisite purification of the malaria antigens from the human red blood cell-serum substrate may render this source of antigen practical. However, a tissue culture cell with an established record of safety in the production of killed and live virus vaccines offers a promising alternative for a malaria vaccine.

The work of Speer and Silverman with the erythrocytic stages of P. berghei in mouse Leydig testicular cell cultures (15) and mouse bone marrow cell cultures (16), if confirmed, would support the contention that it is possible for P. berghei to mature in cell cultures. However, we have been unable to confirm these observations.

Siddiqui's recent successful immunization of Aotus monkeys with an adjuvanted mixture of erythrocytic forms of P. falciparum (17) 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 (18). What remains is to find a suitable substrate for P. falciparum which will allow for the production of a safe and effective vaccine in man.

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B. Statement of Project Objective as stated in the Contract

The original objective of the Project has not been modified. It is the development of an *in vitro* tissue culture method for the propagation of the asexual stages of the malaria parasite in order to provide sufficient antigen for the preparation of vaccines for mass immunization programs.

C. Continued Relevance of Objectives

At present, there are no circumstances nor does our research indicate a need for modification of the project objective.

D. Accomplishments to Date

In the annual report of January 1, 1977 and the six month report of July 1, 1977, we reported in detail, and will not do so in this report, on the following:

Attempts were made to propagate *Plasmodium berghei*, NK-65, in 15 tissue culture cell types including primaries from organs of mice, rabbits and rats, and mouse and human cell lines. One medium and one set of standard conditions were used, except that modifications of medium and/or culture conditions were used with mouse L cells (enucleation) and mouse erythroleukemic cells (dimethyl sulfoxide differentiated). Morphologic evidence and lack of persistence of infectious *P. berghei* in cultures beyond the control indicate we were not successful in propagating the parasite in these cells. Three cell types interiorized the parasite efficiently and are likely candidates for further experimental work.

P. berghei infection in mouse Leydig testicular tumor cells was studied by our standard methods. We found Leydig cells: (1) efficiently interiorized P. berghei; (2) showed no morphologic evidence of maturation or growth of the parasite in the cells, nor did infectivity for mice persist in tissue culture for a longer time than in cell-free controls; (3) interiorized UV killed P. berghei as efficiently as live P. berghei; and (4) efficiently interiorized polystyrene beads.

The P. berghei inoculum for tissue cultures was modified by: (1) removing white blood cells on cellulose columns; and (2) decreasing the number of uninfected red blood cells and increasing the proportion of schizont infected red blood cells by gradient fractionation.

P. vinckei chaubaudi was found not to be a satisfactory source of synchronized parasites as tissue culture inoculum.

We investigated the applicability of the Trager-Jensen technology of cultivation of P. falciparum in human red blood cells to the growth of P. berghei in mouse red blood cells without success.

We investigated the ability of Leydig mouse testicular tumor cells to support the growth of P. berghei using the exact methods of Dr. Speer. We did not observe normal P. berghei within the Leydig cells. Under these conditions, the cells were able to interiorize ultraviolet killed parasites and polystyrene beads. Dr. Spaer reported Leydig cells did not interiorize these beads

nor killed parasites. We also studied mouse bone marrow *a la* Dr. Speer's methodology and did not observe normal parasites in these cells nor were cultures infective for mice at intervals in which parasite control cultures were infective. Bone marrow cells interiorized polystyrene beads and UV irradiated *P. berghei*.

On 1/21/77, we acquired *P. falciparum* from Drs. Trager and Jensen and have maintained the culture continuously in human AB red blood cells in petri plates in candle jars and in a continuous feeding Flow Flask System.

We found the Flow Flask affords a very nice method for long term evaluation of tissue cultures in a dynamic complex system in which merozoites are continuously released from infected red blood cells, while the tissue culture cells continue to multiply and the percent parasitemia in the red blood cells increases.

Nine cell cultures were evaluated for their ability to propagate *P. falciparum*. These included MRC-5 and WI-38 (human diploid lung strains); Rhesus monkey kidney, liver and lung; Cynomolgus monkey kidney, spleen and lung and the Vero (African Green monkey kidney) cell line. Normal appearing parasites were associated with certain clones of Cynomolgus kidney cells. The nature of the cell-parasite association was uncertain and there was no evidence of maturation of the associated parasites. One to two percent of Vero cells became associated with abnormal appearing parasites. Two other cell types interiorized the parasite, but

only degraded forms were seen. Parasites did not become associated with the human diploid cell strains. The remaining three types of cells did not interiorize parasites.

P. falciparum was infective for an *Aotus trivirgatus* monkey after it was in red blood cell culture for 509 days.

The items investigated from July 1, 1977 to December 31, 1977 involved the following:

I. *P. falciparum* (FVO) Inoculated Macaca macaque (Rhesus) Bone Marrow Tissue Culture Cells

II. *P. falciparum* (FVO) Inoculated Squirrel Monkey (Saimiri sceureus) Bone Marrow Tissue Culture Cells

III. *P. falciparum* (FVO) Inoculated *Papio sp.* (Baboon) Bone Marrow Tissue Culture Cells

IV. *P. falciparum* (FVO) Inoculated Primary Frozen Cynomolgus Monkey Kidney, Lung and Spleen and Human Diploid Lung (MRC-5) Tissue Culture Cells

V. *P. falciparum* (FVO) Inoculated Chimpanzee Kidney and Spleen and Rhesus Lung and Spleen Tissue Culture Cells

VI. *P. falciparum* (FVO) Inoculated Chimpanzee Kidney, Lung, Spleen and Human Kidney (TCL-598) Tissue Culture Cells

VII. P. falciparum (FRC-3) Inoculated Human Umbilical Vein Endothelial, Human Embryonic Lung Diploid (HEL-208) and Aotus trivirgatus Spleen Tissue Culture Cells

VIII. P. falciparum (FVO) Inoculated Aotus trivirgatus Spleen and Human Embryonic Lung Strains HEL 208 and 191 Tissue Culture Cells

D. Accomplishments July 1, 1977 to December 31, 1977

I. P. falciparum (FVO) Inoculated Macaca macaque (Rhesus) Bone Marrow Cells

Methods

Cells: The bone marrow cells were obtained by aspiration of the bone marrow from the femur of an intact adult female Rhesus monkey. The cells were harvested into MEM containing 2% fetal calf serum. The bone marrow aspirate contained many red blood cells. The number of red blood cells in the aspirate was reduced by harvesting the top 2 ml of the 10 ml bone marrow aspirate after an 800xG centrifugation. Few nucleated bone marrow cells were lost by this procedure and the cultured suspension now contained more nucleated bone marrow cells than red blood cells.

Cell Culture: The experiment was conducted in four-chambered plastic petri dishes (quad plates). Each quad contained six to eight coverslips stuck to the surface of the plate with silicone grease. Adherent cells were cultured on these coverslips. Each quad was inoculated with $9x10^6$ nucleated

bone marrow cells suspended in 3 ml of tissue culture medium G-2 (see Appendix H in the July 1, 1977 Report) supplemented with 30% fetal calf serum. The quad plates were incubated overnight at 37°C in an ambience of 5% CO_2, 95% air.

P. falciparum (FVO) Inoculation

The next day (day 0) the non-adherent cells were harvested from the quads and the harvest divided into eight equal aliquots. Each aliquot was mixed with P. falciparum infected human red blood cells from in vitro culture. Each aliquot was centrifuged at 1000 rpm for 10 minutes, the supernatant discarded and the cell pellets resuspended in 2 ml of media as described in Table 1. Each aliquot was inoculated into a quad which contained adherent cells on coverslips. Each aliquot contained 12.5% human red blood cells with a parasitemia of 0.21%.

Sampling and Staining: Two coverslips were pulled from each quad on the days recorded in Table 1 and stained with May-Grunwald-Giemsa or Lepehne-Giemsa (see Appendix I of the July 1, 1977 Report). The May-Grunwald-Giemsa stained preparations were used to determine cell morphology and the interaction of the adherent cells with the red blood cells and P. falciparum. The Lepehne stained coverslips were used to determine if the adherent cells contained hemoglobin.

The non-adherent cells were harvested *in toto* with the red blood cells in each quad on each day. The total nucleated bone marrow cells were determined on an aliquot of the culture by hemocytometer count after lysis of the red blood cells with 2% acetic acid. The culture was centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded. Ten µl of fetal calf serum was added to the pellet and three smears were made. About one µl of cell suspension was used to make each smear. Thus, each day about 20% of the suspended cells were used to make smears. The smears were stained with May-Grunwald-Giemsa for detection and evaluation of nucleated bone marrow cells and their association with parasites and for determination of parasitemia and with Lepehne stain for detection of hemoglobin-containing nucleated cells (See Appendix I in the July 1, 1977 Report for staining methods).

Results

P. falciparum association with nucleated bone marrow cells

Adherent cells: May-Grunwald-Giemsa stain: Fibroblast type cells predominated in these cultures, especially from two days after inoculation with *P. falciparum*. The fibroblasts were not associated with parasites nor red blood cells during the six days after inoculation with *P. falciparum*.

Large mononuclear cells were present on the coverslips in about equal numbers as the fibroblasts for the first 2 days after inoculation with P. falciparum, but after day 2 mononuclear cells formed 3 to 20% of the bone marrow cell culture population. These cells were highly phagocytic and contained increasing amounts of hemozoin, debris and degenerating red blood cells. In no instance were intact or even degenerate parasites observed in these phagocytes.

Adherent cells: Lepehne-Giemsa Stain: No hemoglobin containing adherent cells were seen.

Non-adherent cells: May-Grunwald Giemsa Stain: Intact parasites were not observed in non-adherent nucleated bone marrow cells. About 50% of these cells were phagocytic, as evidenced by accumulation in the cells of hemozoin and debris. Only in a few instances were degenerate parasites observed. The parasites did not mature in these cells.

Non-Adherent cells: Lepehne-Giemsa Stain: The area on each slide that contained about 10,000 red blood cells was examined for hemoglobin-containing (Lepehne positive) nucleated red blood cells on day 0, which is the day the cultures were inoculated with P. falciparum, and days 1, 2, 3 and 5. Also, the bone marrow cell suspension that was inoculated into the quad plates on day 0 was examined and found to contain 10 Lepehne positive nucleated cells.

Few hemoglobin positive nucleated cells were seen during the test (2 to 10) and their incidence followed no pattern relative to medium or time.

Parasitemia: The tabulation of developing parasitemia in these cultures primarily reflects medium effects (Table 1). The only medium which maintained the parasite in good condition and supported normal multiplication was GM-5 (RPMI-1640 containing 10% human AB serum)

Medium effects on tissue culture cells

Total cell counts (Table 1) indicated the non-adherent white blood cell count declined during the experiment and none of the medium prevented this decline. Adherent cells multiplied in all media, but the phagocytic types were lost from the cultures as the test proceeded and as they phagocytized greater amounts of material from the culture. As this occurred, fibroblasts took over as the predominating cells on the coverslips.

Discussion

An experiment preliminary to this one to culture Rhesus monkey bone marrow cells in these media indicated no medium was significantly better than another. Because attempts by us to grow *P. berghei* in cultured bone marrow cells were not successful, we decided to attempt the cultivation of *P. falciparum* in both adherent and non-adherent bone marrow

cells cultured in a variety of medium chosen (1) to enhance the growth of the parasite in red blood cells (RPMI-1640 containing 10% human serum), (2) medium that are frequently used to culture bone marrow cells (e.g. MEM containing 30% fetal calf serum), (3) in medium containing serum that was heterologous to the host cells (fetal calf serum and human serum) and (4) medium containing serum hemologous to the host cells (Rhesus monkey serum).

None of these combinations allowed the parasite to develop or be preserved normally in the Rhesus monkey bone marrow cells.

Aotus bone marrow cells may have been a better theoretical choice than Rhesus monkey bone marrow cells for this experiment, but because of the shortage of Aotus monkeys this was not attempted.

Table 1

Total Nucleated Rhesus Monkey Bone Marrow Tissue Culture Cell Counts and Percent Parasitemia

Medium	Days After P. falciparum Inoculation	TC Cells Per Quad ($x10^{-6}$)	Percent Parasitemia	Condition of Parasite
GM-5 (Appendix H, July 1, 1977 Report)	2	4.2	0.2	Fair
	3	NT*	0.4	Fair
	4	2.6	1.2	Good
	6	2.2	1.3	Good
RPMI-1640 + 2% human serum + 28% fetal calf serum	2	4.0	0.1	Fair
	3	NT	0.2	Fair
	4	3.2	0.3	Fair
	6	2.2	0.2	Fair
MEM + 10% human serum	2	4.6	0.2	Fair
	3	NT	0.2	Fair
	4	2.2	0.2	Poor
	6	1.4	0.1	Poor
MEM + 2% human serum + 28% fetal calf serum	2	5.2	0.2	Fair
	3	NT	0.3	Fair
	4	2.8	0.5	Good
	6	1.7	0.3	Fair
RPMI 1640 + 10% Rhesus monkey serum**	2	4.2	0.4	Fair
	3	NT	0.4	Fair
	4	3.8	0.5	Fair
	6	2.2	0.5	Fair
MEM + 30% fetal calf serum	2	3.8	NT	-
	3	NT	0.3	Fair
	4	3.0	0.4	Fair
	6	1.9	0.1	Poor
RPMI-1640 + 30% fetal calf serum	2	5.0	0.2	Fair
	3	NT	0.2	Fair
	4	3.4	0.5	Fair
	6	2.6	0.3	Fair
RPMI-1640 + 2% Rhesus monkey serum** and 28% fetal calf serum	2	3.4	0.2	Fair
	3	NT	0.5	Fair
	4	2.8	0.5	Fair
	6	1.9	0.3	Fair

*NT: Not Tested

**The Rhesus monkey serum was adsorbed with a 50% suspension of human AB red blood cells before it was added to the medium. This adsorption removed human AB red blood cell agglutinins in the Rhesus serum.

II P. falciparum (FVO) Inoculated Squirrel Monkey (Saimiri sceureus) Bone Marrow Tissue Culture Cells

Method

Cells: Two intact femurs of a 720 gram adult male squirrel monkey were received from Dr. McClure of the Yerkes Regional Primate Research Center, Emory University, Atlanta, GA. The bone marrow cells from one femur were washed out of the shaft of the femur with MEM that contained 2% fetal calf serum. The cells were washed once by centrifugation at 1000 rpm x 10 minutes and resuspended in MEM that contained 30% fetal calf serum. A trypan blue exclusion viable count of the nucleated cells indicated the yield was 4.5×10^8 viable bone marrow cells plus 5.4×10^7 dead cells. The total cell count of the bone marrow cell suspension in which the red blood cells were lysed with 2% acetic acid indicated the bone marrow suspension contained 4.1×10^8 cells.

Cell Culture: Quads in plastic divided petri dishes that contained coverslips stuck to the bottom of the quads with silicone grease were inoculated with three ml of cell suspension that contained 9×10^6 cells per quad. The quad plates were incubated at 37°C in a flowing gas mixture of 5% CO_2 - 95% air.

The next day the suspended cells (non-adherent cells) were harvested from two quads, packed by centrifugation and resuspended in RPMI-1640 tissue culture medium that contained 28% fetal calf serum and 2% human AB serum.

P. falciparum Inoculation: The bone marrow cell suspension was mixed with P. falciparum from an in vitro culture (12.5% red blood cell final concentration) and 2 ml of the suspension inoculated into two quads that contained coverslips. Each quad received 6.6 x 10^6 viable cells (trypan blue exclusion test) or 8.2 x 10^6 total white blood cells.

Two empty quads were inoculated with 2.5 ml of 12.5% infected red blood cell suspension. These parasite control quads did not contain tissue culture cells.

The quad plates were incubated at 37°C in a candle jar.

Sampling and Staining: Adherent Cells: Two coverslips were pulled from the quads on days 0, 1, 4, 5, 6 and 7 after inoculation with P. falciparum and stained with May-Grunwald-Giemsa or Lepehne-Giemsa (see Appendix I of the July, 1977 Report) for determination of parasite association with the cells, cell morphology and condition and to determine if adherent cells were hemoglobin positive.

Sampling and Staining: Non-Adherent Cells: On days after inoculation, 0, 1, 3, 4, 5, 6 and 7, the non-adherent tissue culture and red blood cells were resuspended, centrifuged, the supernatant discarded, the cell pellet resuspended in the residual medium and three thin smears made. The smears were stained with May-Grunwald-Giemsa for determination of parasite association with the nucleated bone marrow cells

and to determine cell morphology and condition; Giemsa stain for determination of percent parasitemia; and Lepehne-Giemsa to determine the presence of hemoglobin positive nucleated red blood cells (See Appendix I of the July 1, 1977 Report for staining methods).

The remainder of the cell suspension was resuspended in 5 ml of medium, divided between the two quads and incubated in the candle jar.

Results

P. falciparum association with tissue culture cells

Adherent cells: May-Grunwald-Giemsa Stain: From the day after inoculation of the tissue cultures (day 0) through day 6, two types of adherent cells were seen, a rounded, highly phagocytic cell which predominated and a fibroblast type cell which reached significant numbers by day 5 (15-20% of the nucleated bone marrow cells) and never was associated with red blood cell parasites nor hemozoin. By day 7, the tissue culture cells were degenerate and presen in too low numbers for evaluation.

Red blood cells "rosetted" on the phagocytic nucleated bone marrow cells and were interiorized. Parasites could not be identified within any nucleated bone marrow cell throughout the test period.

Non-Adherent Cells: May-Grunwald-Giemsa Stain: Through day 6 after inoculation with *P. falciparum* 200 to 500 nucleated cells were examined. On day 7, there were too few tissue culture cells available in the culture. Large mononuclear phagocytic cells, non-phagocytic round mononuclear cells, some putatively containing hemoglobin (normoblasts and megaloblasts - see Lepehne staining results) were observed. Infrequently, the phagocytic cells were seen to contain degenerative parasites, but never contained normal parasites. The other nucleated cells never contained parasites.

Adherent cells: Lepehne-Giemsa Stained: Nucleated adherent cells did not contain hemoglobin during the observation period.

Non-adherent cells: Lepehne Giemsa Stain: Prior to inoculation with the infected red blood cells, the bone marrow suspension contained 3% nucleated hemoglobin-positive cells (normoblasts/megaloblasts) of the total red blood cells. The day after inoculation with *P. falciparum* infected red blood cells, the tissue culture contained 1.5% nucleated hemoglobin positive cells. Through day 7 after inoculation this percentage ranged between 2.4% and 3.9% of the total cells (red blood cells and white blood cells).

Results

Parasitemia: The data presented in Table 2 indicate parasite viability was short-lived in these cultures. The G-2 medium containing fetal calf serum was chosen for optimum maintenance of the bone marrow cells and was obviously deficient for maintenance of the parasite infection in the red blood cells. However, there was sufficient infective parasites in the tissue culture during the first four days of the experiment to assure adequate exposure of the nucleated cells to infective parasites.

Nucleated bone marrow cell maintenance: The data for the total non-adherent nucleated bone marrow cell counts during the test are presented in table 3. Although a gradual decline in total cell count occurred, there were sufficient tissue culture cells for adequate evaluation throughout the test and these cells were seen to be in good condition in the May-Grunwald-Giemsa stained smears throughout the test period.

Discussion: Preliminary experiments with Rhesus monkey bone marrow tissue culture cells indicated RPMI-1640 that contained 2% human serum and 28% fetal calf serum maintained the adherent and non-adherent bone marrow tissue culture cells and maintained the parasite fairly well. When used in this squirrel monkey bone marrow tissue culture experiment, tissue culture cell maintenance was good, but a hemolytic

effect by the fetal calf serum lot caused a decrease in red blood cells during the test. Parasites looked normal during the first four days of the test and therefore were considered capable of normal infection and growth in the tissue culture cells if potentially able to do so.

The tissue cultures contained hemoglobin positive erythroblasts (normoblasts and megaloblasts), probably erythrocyte stem cells (deduced from the fact that nucleated red blood cells were present throughout the test), non-phagocytic cells and phagocytic cells presumably of the monocyte series.

The squirrel monkey was shown to be susceptible to P. falciparum [Young, M.D. et al, Experimental Parasitology 38: 136-152 (1975)]. Therefore, these cultured erythroid bone marrow cells have a reasonable expectancy of susceptibility to infection. P. falciparum infection of cultured squirrel monkey bone marrow cells did not occur. We had earlier observed this in mouse bone marrow cell cultures inoculated with P. berghei. Even the almost mature normoblast which we have seen infected with P. falciparum in terminal Aotus trivirgatus infections was was not seen to be infected in these squirrel monkey bone marrow cell cultures.

It would be of interest to study A. trivirgatus bone marrow cultures inoculated with an A. trivirgatus adapted strain of P. falciparum.

Red Blood Cell Parasitemia in Spider Monkey (Saimiri sceureus) Bone Marrow Cell Culture in Quad Plates*

Culture	% Parasitemia on Days After Inoculation					
	1	3	4	5	6	7
Tissue Culture	1.5	1.4	1.6	0	0	0
Parasite Control	1.1	3.0	0.2	0	0	0

*Inoculum contained 1.2% parasitemia.

Table 3

Total Nucleated Squirrel Monkey Bone Marrow Tissue Culture Cell Counts

Days After Inoculation with P. falciparum	Total # wbc per quad
0	8.2×10^6
3	8.2×10^6
4	4.1×10^6
5	2.4×10^6
6	2.5×10^6
7	2.0×10^6

III. *P. falciparum* (FVO) Inoculated *Papio sp.* (Baboon) Bone Marrow Cells

Method

Cells: A femur was sent to us by Dr. McClure of Yerkes Regional Primate Center, Emory University, Atlanta, GA. The cells were obtained by flushing out the bone marrow from the shaft of the femur with MEM that contained 2% fetal calf serum. Twenty-six x 10^6 viable and 40.7 x 10^6 total nucleated cells were harvested.

Cell Culture: The bone marrow cells were centrifuged at 1000 rpm for 10 minutes and the pelleted cells resuspended in GM-5 tissue culture medium (See Appendix H of the July, 1977 Report). Three quads of a four-chambered plastic petri dish were inoculated with 9 x 10^6 nucleated cells in 2 ml per quad. One quad contained bone marrow cells and was inoculated with *P. falciparum*; one quad was inoculated only with bone marrow cells (tissue culture control). One quad was inoculated with bone marrow cells and normal, uninfected, red blood cells (a bone marrow microscopic control); and one quad was inoculated only with *P. falciparum* (parasite control).

P. falciparum (FVO) Inoculation: The test quad (contained bone marrow cells) and the parasite control quad was inoculated with *in vitro*-grown *P. falciparum* in a final concentration of 12.5% red blood cell suspension containing 0.9% parasitemia.

Sampling and Staining: The suspended cells in each quad were harvested on days 1, 2, 5, 6, 7, 8 and 9 and centrifuged at 850 x G for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in residual medium. Three thin smears were made: One was stained by May-Grunwald-Giemsa to determine tissue culture cell morphology, cell condition and association of the nucleated cells with parasites and red blood cells. The second smear was stained with Giemsa to determine the percent parasitemia and the condition of the parasites. The third smear was stained with Lepehne-Giemsa to detect hemo positive nucleated red blood cells (normablasts and megaloblasts).

To determine nucleated bone marrow cell counts, an aliquot of each harvest from a quad plate inoculated with bone marrow cells was mixed with 2% acetic acid to lyse the red blood cells and the count made in a hemocytometer.

Results

Parasitemia: The tabulation of developing parasitemia in these cultures is presented in Table 4. These data indicate a red blood cell parasitemia occurred throughout the test that assured the nucleated cells were exposed to adequate numbers of infective parasites.

Description of the cultures: The total nucleated cell counts are presented in Table 5. A general decline in cell numbers occurred on days 0 to 7 with a precipitous decline on day 7 when the cultures were split. As the experiment progressed, major changes in the tissue culture cell population were seen. Prior to *P. falciparum* inoculation, the bone marrow suspension contained about 50% nucleated hemoglobin positive normoblasts and megaloblasts. This was about ten times as many as were seen in the Rhesus and Squirrel monkey bone marrow suspensions. The remainder of the nucleated bone marrow cells were the expected non-hemoglobin containing mononuclear cells and a variety of leukocytes. By the first day after inoculation with *P. falciparum*, the proportion of nucleated hemoglobin-positive cells had declined, but were present in moderate to large numbers which persisted through day seven. These are theoretically the more likely cells capable of support of *P. falciparum* growth.

Phagocytes were evident by day one and were obvious because of their enormous propensity for accumulating materials from the cultures. They persisted in fair numbers through day eight and a few were found on day nine.

A non-phagocytic mononuclear, non-hemoglobin containing cell was present throughout the first eight days of the culture. These were probably stem cells or maturing cells of the erythroid or monocyte series.

The other nucleated cells (leukocytes and lymphocytes) were few by day 2 and disappeared from the culture by day five. This evolutionary hematologic picture was seen in all three baboon bone marrow cultures.

P. falciparum association with nucleated bone marrow cells: Two hundred to 500 nucleated cells were examined in May-Grunwald-Giemsa stained smears each day. The only nucleated cell which became associated with P. falciparum was the phagocytyte. Only on day 9 after inoculation were identifiable parasites seen; these were two trophozoites and a schizont. This was when the tissue culture cells were obviously degenerating and little significance is attached to this observation. Evidence for intracellular parasite development was never seen.

Discussion

P. falciparum development in baboon bone marrow tissue culture cells did not occur during the 9 days after inoculation with P. falciparum. Nucleated bone marrow cells, both those that contained hemoglobin and those that did not, were present in sufficient number and in good condition throughout the first eight days of culture. Red blood cell parasitemia was generally high because the medium used, GM-5, was chosen to insure adequate P. falciparum multiplication.

Table 4

Red Blood Cell Parasitemia in Papio (Baboon) Bone Marrow Cell Cultures in Quad Plates

Culture	% Parasitemia on Days After Inoculation*						
	1	2	5	6	7**	8	9
Bone Marrow	1.3	1.8	2.3	4.5	7.8	2.4	2.6
Parasite Control (no tissue culture cells)	1.7	2.1	ND***	9.2	12.1	2.6	ND

*Inoculum parasitemia was 0.9%

**Cultures were fed with fresh red blood cells on day 7 at a 1:3 split ratio

***No datum available

Table 5

Total Nucleated Baboon Bone Marrow Tissue Culture Cells

Culture	Total Cells per Quad (+ 1 x 10^6) on Day After Inoculation with P. falciparum:						
	1	2	5	6	7	8	9
BM* + P. fal.	16.0	9.0	3.1	1.2	0.2	0.2	0.3
BM	5.8	3.0	2.3	1.2	0.2	0.2	0.3
BM + Normal rbc	9.0	5.9	2.9	1.2	0.3	0.4	0.3

*Bone marrow cells inoculated into each quad on day 0:

Total = 13.6 x 10^6

Viable = 9.0 x 10^6

IV. *P. falciparum* (FVO) Inoculated Primary Frozen Cynomolgus Monkey Kidney, Lung and Spleen and Human Diploid Lung (MRC-5) Tissue Culture Cells

Method

These cells were tested in Flow Flask cultures in the same experiment. The methods are presented in Table 6 and Appendix F of the July 1, 1977 Report. A similar test of these same cells was reported in the July 1, 1977 Report, Sections VII and VIII. They were repeated because of a deficiency in the earlier experiment.

Results

Parasitemia: The data are presented in Table 7. The parasitemias in the tissue cultures were similar to that of the parasite control during the first three days after inoculation. On day six after inoculation, parasitemia significantly decreased in all 5 flasks and the parasites were in poor condition. However, the parasites were satisfactory during the first three days of the experiment and the parasitemia was sufficient to insure the cells were adequately exposed to infectious *P. falciparum*.

Cynomolgus Kidney Cell Cultures

Microscopic: Coverslip cultures were available for evaluation on days 1 (50 cells) and 2 (50 cells) after inoculation with *P. falciparum*. On day one, the phagocytic activity

of these cells, described in the July 1, 1977 Report, was evidenced by inclusion of red blood cells in vacuoles in the cytoplasm of some cells. On day 2, red blood cells were seen in many cells and three cells contained infected red blood cells in which the parasites appeared degenerate.

Discussion

Maturation of parasites in the cells could not be evaluated because of the short period of observation in this test. Interiorization of parasites and red blood cells occurred, as previously reported. This test must be repeated with an extended observation period and a higher level of parasitemia.

Cynomolgus Lung Cell Culture

Microscopic: Twenty-five to 50 cells were examined on days 1 and 6 after the culture was inoculated with *P. falciparum*. No parasites were observed within the cells; some cells contained red blood cells and debris.

Discussion

Because of the few cells available on the coverslips, a repeat test with a more complete evaluation of this tissue culture cell is necessary.

Cynomolgus Spleen Cell Cultures

Microscopic: Three hundred to 500 cells were examined on days 1, 2, 3 and 6 after *P. falciparum* was inoculated. These cells were highly phagocytic to the red blood cells. No parasites were identified within the Cynomolgus spleen cells.

Discussion

Because of its high phagocytic activity, this may be a cell type that will be useful in studies designed to curtail lysosomal destruction of ingested parasites. However, no interiorized parasites were seen, indicatin destruction of the presumed interiorized parasite occurred. A cell such as Cynomolgus kidney with apparent less complete destruction of interiorized material may be better for such studies.

Human Diploid Lung(MRC-5) Cell Culture

Microscopic: During the six days after inoculation with *P. falciparum*, association of parasites with the cells was not observed. During the first three days 200 to 250 cells were examined and 60 cells were examined on day six. The cells actively adsorbed and interiorized red blood cells. Interiorized red blood cells were destroyed.

Discussion

Although the MRC-5 cells were highly phagocytic for red blood cells, neither degenerate nor normal, *P. falciparum* was seen in the cells. Because MRC-5 is an acceptable vaccine substrate, efforts will be made to decrease intracellular destruction of red blood cells and their included parasites by modification of the cells' enzymatic activities, as proposed in the Project Extension Proposal for the third year's work.

Table 6

METHODS: P. falciparum (FVO) Inoculated Primary Frozen Cynomolgus Monkey Kidney, Lung and Spleen and Human Diploid Lung (MRC-5) Tissue Culture Cells

Tissue Culture Cell	Cynomolgus Kidney Frozen Primary	Cynomolgus Lung Frozen Primary	Cynomolgus Spleen Frozen Primary	MRC-5 Human Diploid Lung Line 39th Passage
Cell Seeding Data				
Number of Cells	1.1×10^6	1.9×10^6	6.0×10^5	4.5×10^5
Volume	5 ml	5 ml	5 ml	5 ml
Growth Medium	GM-3	GM-2	GM-2	GM-9
Time Grown	7 days	7 days	7 days	4 days
Days After Inoculation				
Microscopic Evaluation Done	1, 2	1, 6	1, 2, 3, 6	1, 2, 3, 6
Parasitemia Determined	0, 1, 2, 3, 6	0, 1, 2, 3, 6	0, 1, 2, 3, 6	0, 1, 2, 3, 6
Culture Vessel	Flow Flask (Appendix F of July 1, 1977 Report)			
P. falciparum Inoculum				
Volume		3 ml		
% Red Blood Cells		12.5%		
% Parasitemia		1.41%		
Medium		GM-5		
Medium Flow Rate	2 ml per hour, continuous			
Reference to Methods	Frozen Cell Cultures (Appendix G - July 1, 1977 Report) Tissue Culture Medium (Appendix H - July 1, 1977 Report)			

Table 7

Red Blood Cell Parasitemia in Cynomolgus Kidney, Lung and Spleen and Human Embryo Diploid Lung (MRC-5) Cell Cultures in Flow Flasks

Tissue Culture Cell	% Parasitemia on Days After Inoculation*			
	1	2	3	6
Cynomolgus Kidney	1.36	1.70	2.00	0.71
Cynomolgus Lung	1.08	1.23	1.05	0.20
Cynomolgus Spleen	1.85	2.30	1.77	0.60
MRC-5	1.27	1.24	1.49	0.31
Parasite Control (No tissue culture cells)	1.29	1.8	1.32	0.20

*FVO Inoculum contained 1.1% parasitemia.

V. P. falciparum (FVO) Inoculated Chimpanzee Kidney and Spleen and Rhesus Lung and Spleen Tissue Culture Cells

Method

These cells were tested in Flow Flask culture in the same experiment. The methods are presented in Table 8 and Applendix F of the July 1, 1977 Report.

Parasitemia: The data presented in Table 9 indicate the parasitemia increase among these culture was quite variable. The parasitemia and the good condition of the parasites was sufficient at times in all cultures to assure an adequate exposure of the tissue culture cells to infective parasites.

Chimpanzee Kidney Cell Culture

Microscopic: During the six days after inoculation, no parasites were observed in association with the cells. Five hundred to 1000 cells on each coverslip were examined. These cells showed no phagocytic activity.

Discussion

We do not plan further evaluation of *P. falciparum* infection in primary chimpanzee kidnay cells.

Chimpanzee Spleen Cell Culture

Microscopic: On the two days (1 and 6) after inoculation that there were enough cells in good condition for examination, no parasites were associated with the cells. Fifty cells were examined each time.

Discussion

Parasitemia was adequate on the two days cells were examined, but too few cells were available and coverslips from other days during the test did not contain a sufficient number of cells. Therefore, this cell as a frozen primary, was retested. See Section VI of this report for the results of the retest.

Rhesus Monkey Lung Cell Culture

Microscopic: Parasites were not associated with the cells during the eight days after inoculation that coverslips were examined. Five hundred cells were examined on each coverslip. The cells were not phagocytic.

Discussion

We do not plan further evaluation of *P. falciparum* infection in primary Rhesus monkey lung cells.

Rhesus Monkey Spleen Cell Culture

Microscopic: Although the parasitemia in this culture was low, adequate evaluation was made during the first three days of culture. *P. falciparum* was not associated with the cells. The cells were not phagocytic.

Discussion

No further evaluation of Rhesus Monkey spleen cells inoculated with *P. falciparum* is planned.

Table 8

METHODS: *P. falciparum* (FVO) Inoculated Chimpanzee Kidney and Spleen and Rhesus Lung and Spleen Tissue Culture Cells

Tissue Culture Cell	Chimpanzee Kidney Primary	Chimpanzee Spleen Primary	Rhesus Lung Primary	Rhesus Spleen Primary
Cell Seeding Data				
Number of Cells	2×10^6	2×10^6	2×10^6	2×10^6
Volume	5 ml	5 ml	5 ml	5 ml
Growth Medium	GM-3	GM-2	GM-2	GM-2
Time Grown	5 days	5 days	7 days	7 days
Days After Inoculation				
Microscopic Evaluation Done	1,2,3,6	1,6	1,2,3,6,7,8	1,2,3,8
Parasitemia Determined	0,1,2,3,6,7,8,9	0,1,2,3,6,7,8,9	0,1,2,3,6,7,8,9	0,1,2,3,6,7,8,9
Culture Vessel	Flow Flask (Appendix F of July 1, 1977 Report)			
***P. falciparum* Inoculum**				
Volume	3 ml			
% Red Blood Cells	12.5%			
% Parasitemia	0.80%			
Medium	GM-5			
Medium Flow Rate	2 ml per hour, continuous			
References to Methods	Tissue Culture Medium (Appendix H, July 1, 1977 Report)			

Table 9

Red Blood Cell Parasitemia in Chimpanzee Kidney and Spleen and Rhesus Lung and Spleen Cell Cultures in Flow Flasks

Tissue Culture Cell	% Parasitemia on Days After Inoculation*						
	1	2	3	6	7**	8	9
Chimpanzee Kidney	0.6	1.0	1.2	0.6	0.1	0.6	0.1
Chimpanzee Spleen	0.8	0.6	0.6	1.1	1.2	1.0	1.4
Rhesus Lung	0.9	1.3	1.8	1.8	0.5	0.4	0.3
Rhesus Spleen	0.7	0.5	0.3	0.1	0.2	0.2	0.4
Parasite Control (No tissue culture cells)	0.9	1.0	1.2	3.4	2.2	1.0	0.5

*Inoculum contained 0.80% parasitemia

**Normal human AB red blood cells were added to the flasks on day 7

VI. *P. falciparum* (FVO) Inoculated Chimpanzee Kidney, Lung, Spleen and Human Kidney (TCL-598) Tissue Culture Cells

Method

These cells were tested for their ability to support *P. falciparum* growth in the same experiment. The chimpanzee cell cultures were derived from frozen primary cells. The human kidney cell was a continuous line originally isolated from a female. The methods are presented in Table 10 and Appendix F of the July 1, 1977 Report.

Results

Parasitemia: The data are presented in Table 11. Parasitemias in the tissue culture cell flasks were similar to that of the control throughout the 7 or 9 days of the test and were satisfactory throughout the test.

Chimpanzee Kidney Tissue Culture Cells

Microscopic: During the eight days this culture was tested, about 25 cells were available for examination on each coverslip. The cells were isolated and only in fair condition. Degenerate parasites were observed infrequently in the cells and there was no evidence of parasite development. Small amounts of hemozoin and degraded red blood cells were seen in some cells.

Discussion

Although parasitemia was satisfactory in this chimpanzee kidney cell culture, the culture medium condition was toxic to the cells. Thus, the failure to observe normal or developing parasites in the tissue culture cells was not necessarily a valid observation and must be verified.

Chimpanzee Lung Tissue Culture Cells

Microscopic: During the nine days of observation after inoculation of the culture with *P. falciparum*, at least 200 to 500 cells were examined on each coverslip. About 50% of the cells were phagocytic and contained red blood cells, debris and hemozoin. Degenerate parasites were observed in about 1 to 2% of the cells, but parasite development did not occur.

Discussion

These highly phagocytic cells did not support parasite growth. Because they interiorize parasites readily, chimpanzee lung cells are good candidates for modification to decrease lysosomal destruction of parasites.

Chimpanzee Spleen Tissue Culture Cells

Microscopic: About 500 cells were examined on each coverslip during the 9 days after the culture was inoculated with *P. falciparum*. Red blood cells were associated with about

50% of the tissue culture cells by an apparent adsorption mechanism, but few cells contained red blood cells. About 50% of the cells contained hemozoin. About 1 to 2% of the tissue culture cells contained identifiable parasites on some of the days, but they were degenerate and no parasite development was seen.

Discussion

There was some indication of differential interiorization of parasites by these cells since few red blood cells were seen in the cells, but degenerate parasites were seen. This may be a cell worthy of modification to prevent lysosomal degradation of the parasite

Human Kidney (TCL-598) Tissue Culture Cells

Microscopic: These are extremely difficult cells to examine for intracellular parasites (or anything else) because of their small, densely staining cytoplasm similar in appearance to small bone marrow monocytes. Red blood cells adsorbed to the cells, but phagocytic activity characterized by vacuolation and interiorization was not seen. Parasites, either adsorbed to the cells or interiorized, were not seen, although about 500 cells were examined on most of the 9 days they were examined.

Discussion

Because of the difficulty of examining these cells and the apparent lack of interiorization of parasites or red blood cells, we will not test these cells again.

Table 10

METHODS: *P. falciparum* (FVO) Inoculated Chimpanzee Kidney, Lung, Spleen and Human Kidney (TCL-598) Tissue Culture Cells

Tissue Culture Cell	Chimpanzee Kidney Frozen Primary	Chimpanzee Lung Frozen Primary	Chimpanzee Spleen Frozen Primary	Human Kidney TCL-598
Cell Seeding Data				
Number of Cells	3.0×10^5	3.0×10^5	3.8×10^5	1.0×10^6
Volume	5 ml	5 ml	5 ml	5 ml
Growth Medium	GM-3	GM-1	GM-1	GM-3
Time Grown	6 days	4 days	4 days	1 day
Days After Inoculation				
Microscopic evaluation done	1,2,3,6,7,8,9	1,2,3,6,7,8,9,	1,2,3,6,7,8,9	1,2,3,6,7,8
Parasitemia determined	1,2,3,6,7,8,9	1,2,3,6,7,8,9	1,2,3,6,7,8,9	1,2,3,6,7,8,9
Culture Vessel	Flow Flask (Appendix F of the July 1, 1977 Report)			
P. falciparum Inoculum				
Volume	3 ml			
% Red Blood Cells	12.5%			
% Parasitemia	1.02%			
Medium	GM-5			
Medium Flow Rate	2 ml per hour, continuous			
References to Methods	Frozen Cell Cultures (Appendix G of the July 1, 1977 Report) Tissue Culture Media (Appendix H of the July 1, 1977 Report)			

Table 11

Red Blood Cell Parasitemia in Chimpanzee Kidney, Lung, Spleen and Human Kidney (TCL-598) Cell Cultures in Flow Flasks

	% Parasitemia on Days After Inoculation*						
	1	2	3**	6	7	8	9
Tissue Culture Cell							
Chimpanzee Kidney	0.9	1.5	1.8	1.6	1.5	0.7	0.8
Chimpanzee Lung	0.9	1.6	1.9	2.3	3.0	3.1	1.0
Chimpanzee Spleen	1.2	1.4	1.7	2.9	2.9	3.0	2.5
TCL-598	1.2	1.9	2.3	3.0	3.1	1.9	0.8
Parasite Control (no tissue culture cells)	1.1	1.4	1.9	3.4	3.5	3.6	1.8

*Inoculum parasitemia was 1.0%

**1.5 ml of a 12.5% suspension of normal human AB red blood cells were added to each flask on day 3.

VII. P. falciparum (FRC-3) Inoculated Human Umbilical Vein Endothelial, Human Embryonic Lung Diploid (HEL-208) and Aotus trivirgatus Spleen Cells

Method

These cells were tested in Flow Flask cultures in the same experiment. Three other HEL cell strains were also tested in this experiment, but the human red blood cells and/or serum were toxic to these HEL and data could not be obtained. The methods are presented in Table 12 and in Appendix F of the July 1, 1977 Six Month Report.

Results

Parasitemia: The data are presented in Table 13. The parasitemia in the six tissue culture flasks was similar to that of the parasite control flask throughout the eight days of the test.

Human Umbilical Vein Endothelial Tissue Culture Cells

Microscopic: On day 1 through day 6 after the cultures were inoculated with P. falciparum, 500 or more cells were examined. On days 7 and 8, because fewer cells were present on the coverslips, only 50 cells were examined. Throughout the test period, microscopic examination revealed parasites within the endothelial cells. Because apparent degeneration of the interiorized parasites also occurred, normal forms were not frequently observed. However, in about 1% of the cells, schizonts, merozoites or rings and

more mature trophozoites were identified at various times. Interiorized parasites were not accompanied by a significant number of interiorized red blood cells. Therefore, it is possible that the identifiable stages matured from merozoites that had infected these cells. Hemozoin was first observed in the cells two days after the culture was inoculated with P. falciparum and accumulated in the cells throughout the remainder of the eight day test period.

Discussion

The human umbilical vein endothelial cell has several characteristics that make it a theoretically promising tissue culture cell for the propagation of P. falciparum. First, data in the literature indicates this nucleated cell has blood group antigens A, B and H on its surface. Thus, it can be theorized that the cell may also have P. falciparum "receptors" on its surface and may be capable of infection with P. falciparum in a manner analogous to that of red blood cells. Secondly, human umbilical cords are readily available.

Third, albeit the yield of cells from an umbilical cord vein is small, the literature indicates these cells can be cultivated serially for at least several months; thus the numbers of cells can be increased and practical amounts obtained by in vitro cultivation.

In this first test, the cell showed sufficient promise, so we plan to continue work with it. The apparent lack of active phagocytosis of red blood cells and the interiorization of parasites may indicate the parasite was preferentially interiorized. The apparent destruction of the parasite and/or failure to mature normally may be overcome by modification of cell lysosomal activity which may be responsible for parasite destruction. It is this type of approach we proposed to take with promising cell types during the third year of the contract.

Human Embryonic Lung Diploid Tissue Culture Cells(HEL-208)

Microscopic: Forty to 500 cells were examined on days 1, 3 and 6 after inoculation of the culture with *P. falciparum*. Hemozoin was detected in some cells, but no interiorized parasites were observed

Discussion

Of the 4 strains of human embryonic lung tissue culture cells that were put on test in this experiment, only HEL-208 cells survived well enough to be partially evaluated. No parasites were seen in HEL-208.

All human sera to be used in the growth of *P. falciparum* are screened for toxicity against the L 1210 mouse leukemic cell culture prior to its use. This method has been effective in eliminating human sera toxic to most cell

cultures. However, this method did not predict toxicity for human embryonic lung cultures.

Because human embryonic (diploid) lung cultures are approved for virus vaccine production, additional research effort will be applied to these cell cultures.

Aotus trivirgatus Spleen Tissue Culture Cells

The spleen was removed from an A. trivirgatus monkey that was later inoculated with P. falciparum and found to be susceptible

Microscopic: From day two to day eight after inoculation with P. falciparum, 50 to 200 cells were examined on each coverslip. Some cells were weakly phagocytic for red blood cells and a small amount of hemozoin was seen in some cells. Only on day 8 were identifiable parasites seen in the cells and these looked degenerate.

Discussion

We do not intend to study Aotus spleen cells again because of the small number of parasites found within the cells and the parasites degenerate condition.

Table 12

METHODS: *P. falciparum*(FRC-3)Inoculated Human Umbilical Vein Endothelial, Human Embryonic Lung Diploid (HEL 208) and Aotus trivigatus Spleen Tissue Culture Cells

	Aotus trivigatus Spleen Tissue Culture Cells		
Tissue Culture Cell	**Human Umbilical Vein Endothelial**	**HEL 208 Human Diploid Lung Line 5th Passage**	**Aotus Spleen Secondary**
Cell Seeding Data			
Number of cells	1.05×10^6	1.0×10^6	1.0×10^6
Volume	5 ml	5 ml	5 ml
Growth Medium	GM-10	GM-1	GM-3
Time Grown	6 days	1 day	1 day
Days After Inoculation			
Microscopic evaluation done	1,2,3,6,7,8	1,3,6	1,2,3,6,7,8
Parasitemia determined	1,2,3,6,7,8	1,2,3,6,7,8	1,2,3,6,7,8
Culture Vessel	Flow Flask (Appendix F of the July 1, 1977 Report)		
P. falciparum Inoculum			
Volume	2 ml		
% Red Blood Cells	10%		
% Parasitemia	1.26%		
Medium	GM-5		
Medium Flow Rate	2 ml per hour, continuous		
Reference to Methods	Tissue Culture Media (Appendix H of the July 1, 1977 Report)		

Table 13

Red Blood Cell Parasitemia in Human Umbilical Vein Endothelial, Human Embryonic Lung Diploid (HEL 193, HEL 195, HEL 201, HEL 208) and Aotus Spleen Cell Cultures in Flow Flasks

	% Parasitemia on Days After Inoculation*					
Tissue Culture Cell	1	2	3**	6	7	8
Human Endothelial	1.93	3.27	6.04	3.06	2.56	4.98
HEL 193	2.07	4.21	2.65	6.48	7.72	6.85
HEL 199	2.23	4.35	5.42	9.90	8.20	10.70
HEL 201	2.11	4.27	8.20	8.50	9.85	9.35
HEL 208	3.20	4.12	6.40	8.79	10.60	5.14
Aotus Spleen	1.94	3.95	5.90	5.85	7.70	7.40
Parasite Control (no tissue culture cells)	3.15	4.30	7.75	9.20	9.45	9.87

*Inoculum contained 1.45% parasitemia

**1.5 ml of a 25% normal AB human red blood cell suspension was added to each flask on day 3.

VIII. *P. falciparum* (FVO) Inoculated *Aotus trivirgatus* Spleen and Human Embryonic Lung Strains HEL 208 and 191 Tissue Culture Cells

Method

These cells were tested in Flow Flask cultures in the same experiment. The methods are presented in Table 1 and in Appendix F of the July 1, 1977 report.

Results

Parasitemia: The data are presented in Table 15. Parasitemia was adequate throughout the test period of 10 days after inoculation with *P. falciparum* in the flasks containing tissue culture cells. A medium feeding deficiency in the parasite control flask caused the parasite to not grow normally from day five to day seven. On day seven, the cultures were fed with fresh red blood cells.

Aotus trivirgatus Spleen Cell Culture

Microscopic: Two hundred to 300 tissue culture cells were examined from day one to day five after inoculation with *P. falciparum*. The cells were phagocytic for parasites, red blood cells and hemozoin. Degenerate parasites were observed in the tissue culture cells infrequently. After day five, the tissue culture cells deteriorated and valid observations could not be made.

Discussion

Because of the infrequent interiorization of *P. falciparum* by these cells and lack of normal or maturing parasites in the cells, no further work with the Aotus spleen cells is planned.

Human Embryonic Lung (HEL 208) Cell Culture

Microscopic: Two hundred to 500 HEL cells were examined on days 1 and 3 after inoculation with *P. falciparum*. Coverslips removed on days 5 to 9 had forty to fifty cells available for evaluation. By day 10, only 12 cells were found. On days 1 and 3 only a small amount of hemozoin and no parasites were seen in the cells. On day 4, three tissue culture cells contained parasites (one schizont and two degenerate parasites in phagocytized red blood cells). From day 5 onward, only degenerate parasites and hemozoin were seen in the cells.

Discussion

Parasite interiorization was infrequent in HEL 208 cells, although red blood cell parasitemia was high and afforded a good exposure of the tissue culture cells to infective parasites. No development of interiorized parasites occurred.

The culture conditions are toxic to the HEL cells, so they

maintain slightly better than HEL 208 and other HEL strains we have tested, but after 4 to 5 days in culture even MRC-5 is obviously toxified. This type of cell does not hold up well in long term culture, even under ideal culture conditions and the *P. falciparum* culture medium is not ideal. Thus, only short term experiments are possible using HEL and MRC-5 cells.

Human Embryonic Lung (HEL 191) Cell Cultures

Microscopic During the five days, good to fair tissue culture cells were available for evaluation. Intracellular parasites were infrequently seen in the 100 to 500 cells examined on each coverslip. These cells were poorly phagocytic for red blood cells and parasites. Parasite development in these cells was not observed.

Discussion

The lack of identifiable parasites in HEL 191 indicates this cell line is of no special interest for these studies.

Table 14

METHODS: *P. falciparum* (FVO) Inoculated Aotus trivirgatus Spleen, Human Embryonic Lung Strains, HEL 208 and 191 Tissue Culture Cells

Tissue Culture Cell	Aotus Spleen Unfrozen Primary	HEL 208 7th Passage	HEL 191 18th Passage
Cell Seeding Data			
Number of Cells	9.75×10^5	9.00×10^5	9.00×10^5
Volume	5 ml	5 ml	5 ml
Growth Medium	GM-2	GM-1	GM-1
Time Grown	3 days	3 days	3 days
Days After Inoculation			
Microscopic Evaluation done	1,3,4,5	1,3,4,5,6,7,10	1,3,4,5
Parasitemia determined	3,4,5,6,7,10	3,4,5,6,7,10	3,4,5,6,7,1
Culture Vessel	Flow Flask (Appendix F of the July 1, 1977 Report)		
P. falciparum Inoculum			
Volume		3 ml	
% Red Blood Cells		12.5%	
% Parasitemia		0.94%	
Medium		GM-5	
Medium Flow Rate		2 ml per hour, continuous	
Reference to Methods		Tissue Culture Media (Appendix H of the July 1, 1977 Report)	

Table 15

Red Blood Cell Parasitemia in Aotus trivirgatus Spleen, Human Embryonic Lung Strains, HEL 208 and 191 Cell Cultures in Flow Flasks

Tissue Culture Cell	% Parasitemia on Days After Inoculation*					
	3	4	5	6	7**	10
Aotus Spleen	2.9	2.5	1.2	4.2	4.8	1.2
HEL-208	2.7	6.3	3.7	4.1	4.3	5.7
HEL-191	3.1	5.7	3.4	5.8	3.2	6.9
Parasite Control (no tissue culture cell)	2.7	3.6	0.2***	0.1***	0.1***	ND****

*FVO inoculum contained 0.94% parasitemia.

**On day 7, most of the red blood cell suspension was removed and replaced with 3 ml of 12.5% normal human AB red blood cell suspension.

***See text for explanation.

****ND = No Data

D. Research Design

During the current reporting period, we have proceeded with Phase III of our contract proposal.

The FVO (Asian) and FRC-3 (African) strains of *P. falciparum* were grown *in vitro* in human red blood cells by a modification of the Trager-Jensen method. This technique was described in detail in Appendices C and D of our July 1, 1977 Report

Tissue cultures were prepared from cells of various organs of human and sub-human primates and inoculated with *P. falciparum* infected red blood cells from *in vitro* cultures. The tissue cultures were sampled periodically, stained and examined microscopically for evidence of (a) parasite attachment or interiorization, and (b) intracellular growth or development. The red blood cells were sampled periodically, stained and examined microscopically to determine the percent parasitemia present in the culture system.

Two cell cultures, human endothelial and cynomolgus monkey kidney cell culture, offered some indication of parasite interiorization. These, along with MRC-5, a human embryonic lung strain approved for virus vaccine manufacture, will be studied in a new approach described in our contract proposal submitted in July, 1977. This new design is being implemented as of January 1, 1978.

During the third year of the contract, we are taking a multi-faceted approach toward cultivating P. falciparum in mammalian tissue culture cells:

1. We will grow P. falciparum in red blood cells in the presence of MRC-5 or human endothelial or Cynomolgus monkey kidney cells. Concomitant serial splits of the P. falciparum culture and the tissue cell culture will be made. The objective is to adapt P. falciparum to propagate in the cell miliu by a process of adaptation.

2. The inoculum will be changed. We are working toward increasing the synchrony and the infectivity of P. falciparum in red blood cell culture to obtain a higher parasitemia and more merozoites to fulfill the objectives of the Malaria Immunity and Vaccination AID Contract AID/ta-C-1440. Successes in these endeavors will be applied to culture of P. falciparum in tissue culture cells.

3. The surface of tissue culture cells will be modified by chemical treatments with the hope that this will facilitate adsorption of merozoites to the cell surface and penetration (or passive interiorization) of infective parasites into the tissue culture cell

4. We have observed numerous instances of parasites being interiorized by tissue culture cells, presumably by a phagocytic mechanism. This interiorization seems to be followed by destruction of the parasite. This was not

accompanied by obvious phagocytosis of red blood cells in two instances, Cynomolgus kidney and human umbilical vein endothelial cells. An effort will be made to modify the intracellular parasiticidal activities of the tissue culture cells by interfering with these mechanisms. Specifically we plan to use: protease inhibitors, especially those that are known to enter mammalian and interfere with lysosomal proteases, such as leupeptin; DNA synthesis inhibitors, e.g. Vira A (vidarabine) or 2'-Deoxy-5-fluorouridine, compounds that interfere with RNA synthesis and/or processing, e.g. Adriamycin, Dactinomycin. Hopefully, some of these compounds will have differential effects on the mammalian cell and the protozoa.

5. If we are successful in these efforts and release of the matured parasite from the infected cell does not occur, we plan to facilitate this release by physical methods, e.g., ultrasonics or by chemical methods, e.g., surfactant rupturing or by immune lysis.

E. <u>Dissemination and Utilization of Research Results</u>

The dissemination and utilization of research results have included: On March 10-12, 1977, participation by Dr. Robert G. Brackett in the USAID/WHO Workshop on the In Vitro Cultivation of Malaria Parasites at Rockefeller Institute, New York, NY.

On March 21, 1977, we submitted a proposal in reply to RFP-ta-50078 entitled, Malaria Immunity and Vaccination. This proposal was accepted and we began work on the contract on August 15, 1977.

On July 12, 1977, we submitted a proposal for an extension of our current contract, AID/ta-C-1258. This proposal was accepted and we began work on the extension of this contract on January 1, 1978.

F. Statement of Expenditures and Obligations and Contractor Resources

The major targets for the contract period, January 1, 1977 to December 31, 1977, as related to our major inputs were:

1. Complete the evaluation of the culture of *P. berghei* in Leydig cells and in mouse bone marrow cells according to the methodology of Dr. C. Speer.

2. Convert our effort from culture of *P. berghei* in tissue culture cells to culture of *in vitro* grown *P. falciparum* in tissue culture cells.

3. Evaluate the culture of *P. falciparum* in twenty-two sub-human primate and human tissue culture cells. For a variety of reasons, eight repeat tests in some of these 22 tissue culture cells were performed. Thus, a total of 30 tests of the ability of *P. falciparum* to grow in tissue culture cells were performed.

Involvement of Minority Personnel and Women

Four women were involved in project activities during the reporting period. Two were involved as professional laboratory investigators, one as a secretary and one as a laboratory aid. The future involvement of women is expected to remain the same. One of these professional women was promoted during this past year. One Black professional laboratory investigator was involved in project activities during the year. Another Black has been added to the project as a laboratory aid.

The expenditures and obligations related to the budget plan from January 1, 1977 to December 31, 1977 and from January 1, 1978 to December 31, 1978 are presented as follows:

Expenditures	Period 1/1/77 to 12/31/77	Period 1/1/78 to 12/31/78 (est.)
Salaries & Wages including Fringe Benefits	$111,389	$133,520
Consultant Fees	-	2,000
Travel & Transportation	944	1,150
Other Direct Costs	-	500
Overhead	68,839	83,583
Research & General Administration	7,104	8,441
Supplies	8,538	17,060
Total Cost to Government	$196,814	$246,254

G. Work Plan and Budget Forecast for the Coming Year

The work plan for the coming year includes the following targets:

1. Although we did not repeat the promising results obtained with P. falciparum-inoculated Cynomolgus monkey kidney cell culture presented in the July 1, 1977 Report Section D - VIII, experimental conditions were not repeated. Especially the high percent parasitemia that occurred in the first experiment was not duplicated in the repeat test. We will attempt to repeat the original observations when the culture is inoculated with a 5% parasitemic inoculum and the parasitemia is maintained between 5 and 10%. If repeatable, the large amounts of parasites associated with this cell will make the cell an ideal candidate for some of the modifications described in Section D - Research Design - this report.

2. The modification of cells described in Section D - Research Design - this report, will be applied to the human umbilical vein endothelial cells because of the observations and the theoretical considerations presented in Section D - VII of this report.

3. Although there are considerable tissue culture problems associated with human embryonic diploid cells inoculated with P. falciparum and the lack of significant interaction between P. falciparum and these cells, the MRC-5 line will

be modified for increased adsorption and penetration of *P. falciparum*. If successful, the vaccine substrate potential of MRC-5 is so great we will actively pursue investigations of other cell-modifying procedures with MRC-5.

4. Adaptation of *P. falciparum* to a tissue culture cell will be attempted. The human umbilical vein endothelial cell and the Cynomolgus monkey kidney cell are presently the most likely candidate cells to be used in this approach.

A budget statement showing planned expenditures for each of the major inputs for the period 1/1/78 to 12/31/78 is presented in Section F of this report.