AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

# BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY
Batch 95 ARDA

1. SUBJECT CLASSIFICATION
A. PRIMARY: Serials — Y-NS00-0000-0000
B. SECONDARY: Health--Tropical diseases

2. TITLE AND SUBTITLE
The In vitro cultivation of malaria parasites in cell cultures; progress report, Jan.-June,1978

3. AUTHOR(S)
(101) Parke,Davis and Co.,Detroit,Mich.

4. DOCUMENT DATE: 1978
5. NUMBER OF PAGES: 35p.
6. ARC NUMBER: ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS
Parke-Davis

8. SUPPLEMENTARY NOTES (Sponsoring Organization, Publishers, Availability)
(Research summary)

9. ABSTRACT

Reports on an ongoing research project to modify tissue culture cells so that they can better support the multiplication of plasmodium falciparum, a malaria causal agent. The tissue culture chosen was obtained from the Vero line of African Green Monkey kidney cells. It is hoped that results obtained with Vero cell cultures will be useful in further studies on vaccine substrate tissue culture cells. This report includes a background summary of prior experiments, a list of accomplishments for the report period (January 1-June 30, 1978), micrographs and tables displaying laboratory results, and material concerning specific research contract requirements and budget information.

10. CONTROL NUMBER
PN-AAG-218

11. PRICE OF DOCUMENT

12. DESCRIPTORS
Malaria

13. PROJECT NUMBER
931-15300

14. CONTRACT NUMBER
AID [illegible]-1158 Res.

15. TYPE OF DOCUMENT

AID [illegible] (4-74)

81

CONTRACT AID/ta-C-1258

January 1 to June 30, 1978

SIX MONTH REPORT

# THE *IN VITRO* CULTIVATION OF MALARIA PARASITES IN CELL CULTURES

## Six Month Report Summary Sheet

The *In Vitro* Cultivation of Malaria Parasites in Cell Cultures, AID/ta-C-1258

Principal Investigator and Contractor: Robert G. Brackett, Ph.D.
Parke, Davis & Co., P. O. Box 118, Detroit, MI 48232

Contract Period: Jan. 1, 1976 to June 30, 1978

Reporting Period: Jan. 1, 1978 to June 30, 1978

Total AID funding of contract to date: $668,519

Total Expenditures and Obligations through previous contract year: $196,814.

Total Expenditures and Obligations for Current Six Months: $100,794

Estimated Expenditures for next six months: $116,550

## Summary

Summary of Accomplishments and Utilization: *P. falciparum* readily associates with about 1% of the Vero cells (African Green monkey kidney line) in co-culture. Ovomucoid, a protein protease inhibitor, markedly increased this association to as high as 30% of the Vero cells and increased the number of parasites per Vero cell. The associated parasites are mostly merozoites or young ring stages. Whether these parasites are interior or exterior to the Vero cells has not been determined. By means of this activity of ovomucoid, we now have highly parasitized Vero cells available for our continuing effort to enhance maturation and growth of *P. falciparum* in tissue culture. These efforts have been started using a variety of chemical modifiers of cell metabolism.

TABLE OF CONTENTS


| | Pages |
|---|---|
| I. Background | 1 |
| II. Statement of Project Objective as Stated in The Contract | 5 |
| III. Continued Relevance of the Objective | 5 |
| IV. Accomplishments: January 1, 1978 to June 30, 1978 | 5 |
| A. Attempt to adapt *P. falciparum* to Vero (African Green Monkey Kidney Line) by Serial Passage | 5 |
| B. Effect of Protease Inhibitors on the Association of *P. falciparum* with Vero Cells | 6 |
| 1. Introduction | 6 |
| 2. Method | 7 |
| 3. Experimental Results | 8 |
| a. Alkylating protease inhibitors | |
| (1) TLCK | 8 |
| (2) TAME | 8 |
| b. Polypeptide protease inhibitors | 8 |
| (1) antipain | 8 |
| (2) leupeptin | 9 |
| c. Protein protease inhibitor - ovomucoid | 9 |
| 4. Discussion | 12 |
| a. Alkylating and polypeptide protease inhibitors | 12 |
| b. Ovomucoid | 12 |
| 5. Research Design | 14 |
| 6. References | 16 |
| V. Dissemination and Utilization of Research Results | 18 |
| VI. A. Statement of Expenditures and Obligations and Contractor Resources | 18 |
| B. Involvement of Minority Personnel and Women | 19 |
| VII. Work Plan and Budget Forecast for the Coming Six Months | 19 |
| Appendix A. Attempted Adaptation of *P. falciparum* to Vero Cells | |
| Appendix B. Tables | |
| Budget Statements: January 1, 1978 to December 31, 1978 | |

# I. BACKGROUND

Prior to this report, we reported in detail 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. 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.

*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.

We also 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. Speer reported Leydig cells did not interiorize these beads nor killed parasites. We also studied mouse bone marrow according to 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 in human AB and O red blood cells in petri plates in candle jars and in a continuous feeding Flow Flask System.

Both the Flow Flask and petri plate culture methods afford very nice methods 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.

Twenty-one tissue cell cultures were evaluated for their effect on the growth of P. falciparum in co-culture. Normal appearing parasites were associated with primary human umbilical vein endothelial, primary Rhesus monkey kidney, liver and bone marrow, Cynomolgus monkey kidney and the Vero line of African Green monkey kidney cells.

Two different mechanisms for cell-parasite association appear to be operative. The first involves adsorption of infected red cells by the tissue culture cell followed by phagocytosis and the second adsorption of free merozoites with or without interiorization.

Interiorization of an infected red cell and subsequent destruction of the red cell (observed as a loss of color) results in an essentially normal-appearing parasite in the phagosome occupied by the red cell. Normal developmental forms are seldom seen within phagosomes in the absence of red cell material.

A second mode of association, characterized by free merozoites either adhering to the plasma membrane or interiorized in the cell, is observed more frequently. Essentially normal merozoites or very immature trophozoites (rings) are seen on or within the plasma membrane of certain cells in the culture. No evidence has been obtained that these cell-associated parasites mature; however, this type of association results in the temporal association of the potentially infective stage of the parasite, the merozoite, with tissue culture cells.

Both of these types of parasite association are observed in Vero cell culture and we are attempting to improve the association, maturation and multiplication of P. falciparum in Vero cell culture by manipulation of the cultural conditions by:

1. Adaptation, i.e., serial passage of infected red cells in tissue culture;
2. Alteration of the tissue culture cell surface to increase adsorption and penetration of the parasite; and
3. Treatment of tissue culture cells with selective drugs to decrease destruction of cell-associated parasites.

There are several approaches which we intend to explore to accomplish the second and third goals, namely:

1. Incorporation of protease inhibitors in the culture medium;
2. Treatment of tissue culture cells with chemicals known to be intracellularly active inhibitors of eukarvotic protein synthesis;
3. Treatment of cells with glucocorticoid steroids which may - depending on dosage, type of steroid and cell - inhibit or stimulate growth of the tissue culture cell, increase cAMP levels, increase or decrease protein synthesis, or increase peripheral microfilaments involved in phagosome-lysosome interactions;
4. Use of Concanavalin A to inhibit fusion of endocytic vacuoles with lysosomes and the subsequent digestion of P. falciparum or to increase interiorization of the parasite since Con-A stimulates pinocytosis and phagocytosis;
5. Disruption of microfibrils by treatment of the cells with Cytochalosin B, resulting in loss of mobility of the phagosome to fuse with the lysosome; and
6. Inhibition of DNA and RNA synthesis and/or processing in the eukaryotic cell by antimetabolites, thereby rendering the cell unable to degrade interiorized parasites.

This six month report will describe preliminary results obtained by protease inhibitor treatment of Vero cells inoculated with *P. falciparum*, and by serial passage of *P. falciparum* in Vero cell culture.

## II. 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.

## III. CONTINUED RELEVANCE OF THE OBJECTIVE

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

## IV. ACCOMPLISHMENTS: January 1, 1978 to June 30, 1978

### A. Attempt to adapt P. falciparum to Vero (African Green Monkey Kidney Cell Line) by Serial Passage

Details of the Methods and Results of this experiment are reported in Appendix A.

An increase in the number of Vero cells with associated parasites was observed after nine serial passages of infected red cells in Vero cells. However, when parasitized blood was removed and

the Vero cells continued in culture, no growing parasites were observed throughout the passage series. It was concluded that further work in this area might be worthwhile.

## B. Effect of Protease Inhibitors on the Association of *P. falciparum* with Vero Cells

### 1. Introduction

The protease inhibitors that we are testing to improve the association, maturation and growth of *P. falciparum* in Vero cells fall into three groups: (1) alkylating protease inhibitors; (2) polypeptide protease inhibitors; (3) protein protease inhibitors.

The first group consists of alkylating chloromethyl ketones of various enzymatic specificities and modes of action. Based on a review by Schnebli[1] two compounds were selected: TLCK (N-α-tosyl-L-lysyl chloromethyl ketone) and TAME (N-α-tosyl-L-arginyl methyl ester). Their activity may be extracellular on enzyme substrates such as trypsin and chymotrypsin-like enzymes which may interfere with *P. falciparum* adsorption/interiorization in the cell or, as suggested by Collar and Smets[2], their alkylating activities may intracellularly block cell metabolism. Different modes of action are inferred since TAME is a substrate analog expected to competively inhibit the natural proteases and esterases while TLCK is a substrate analog that acts irreversibly on proteases. Furthermore, Chou et al[3] have demonstrated inhibition of protein synthesis in intact cells by alkylating protease inhibitors.

In addition, we are testing two members of a group of polypeptide protease inhibitors extracted from Actinomycetes[4], namely, leupeptin and antipain. Leupeptin has been demonstrated to be intracellularly active in eukaryotic cells[5] and has *in vivo* activity against muscular dystrophy in chickens[6].

Representative of the third group is ovomucoid, one of the egg albumin proteins. It has extracellular protease inhibitory activity[7] which might aid adsorption and interiorization of *P. falciparum* by forming a poorly dissociated macromolecular complex with proteases of the trypsin family.

## 2. Method

The protease inhibitors were tested at minimally toxic and sub-toxic concentrations or at concentrations determined by availability or solubility. After treatment with the drugs for two hours at 37°C, the Vero cell cultures were inoculated with *P. falciparum* in GM-5 medium (RPMI-1640 supplemented with 10% human serum) in a 3% red blood cell suspension. Incubation of the cultures was at 37°C in a candle jar. These co-cultures were treated with fresh drug on days one and two after *P. falciparum* inoculation. Daily, coverslips were stained with May-Grunwald-Giemsa for evaluation of Vero cell-parasite inter-relationships and a Giemsa-stained thin smear made of the erythrocyte culture for determination of parasitemia.

Vero cell and erythrocytic parasitemias are expressed as the percentage of cells infected, irrespective of whether there is one or more than one parasite per cell.

## 3. Experimental Results

Tables are presented in Appendix B.

### a. Alkylating Protease Inhibitors

#### (1) TLCK

The three tested concentrations of TLCK were antimalarial. This activity precluded a satisfactory evaluation of drug effect and presumably is the cause of reduced association of parasites with the Vero cells (Table 1).

In a separate test, TLCK was found to partially suppress Vero cell growth at concentrations of 78 and 39 μg/ml.

#### (2) TAME

At the one test concentration, TAME was antimalarial, thus precluding satisfactory evaluation and presumably was the explanation for the reduced association of parasite with the Vero cells (Table 2).

As determined in a separate toxicity test, TAME did not inhibit Vero cell growth.

### b. Polypeptide Protease Inhibitors

#### (1) Antipain

Antipain is a polypeptide protease inhibitor from Actinomycetes which was kindly supplied to us by Dr. Umezawa (Institute of Microbial Chemistry, Tokyo). The tested concentration was chosen

according to the amount available and the protease inhibiting concentrations reported in the literature. It was neither toxic to Vero cells nor antimalarial (Table 3). On day 3, but not on days 1 and 2, antipain increased the percentage of Vero cells that had merozoite-like parasites associated with them than is usually observed in the absence of drug treatment; however, day three's control coverslip was accidentally broken.

(2) Leupeptin

This is another of the Actinomycetes polypeptide protease inhibitors obtained from Dr. Umezawa. The test concentration was chosen because it is active in eukaryotes and because of limited availability of the drug. Leupeptin was not toxic to the Vero cells, but was anti-malarial at the test concentration (Table 4). Presumably the antimalarial activity caused the reduction in the number of Vero cells associated with parasites.

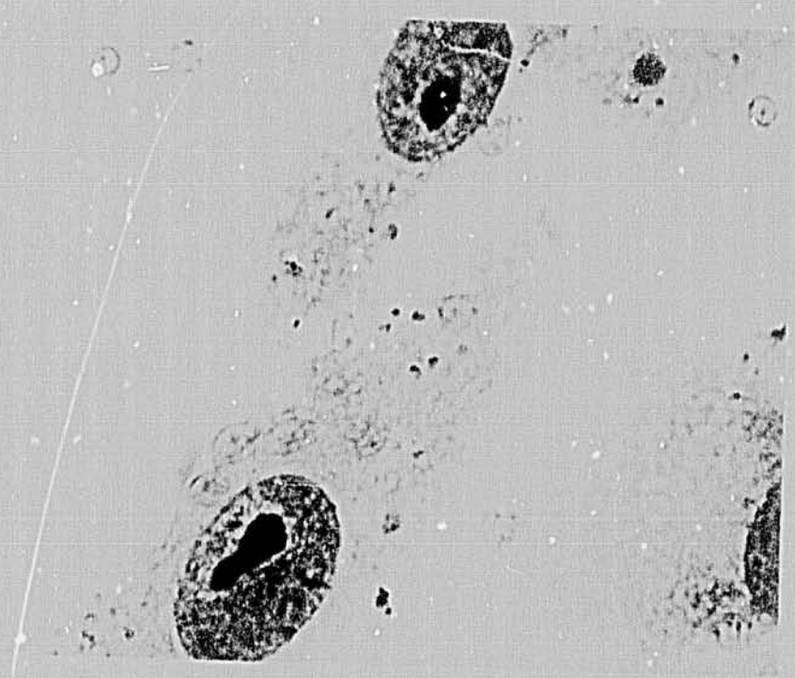
C. Protein Protease Inhibitor - Ovomucoid

Ovomucoid increased the percentage of Vero cells associated with parasites (Table 5) and increased the number of parasites associated with each cell. The parasites associated with the Vero cells were predominantly merozoite or very young ring stages as depicted in Figure 1.

The test concentration of 3000 μg/ml, which is about the maximum soluble concentration, was not toxic to the Vero cells and was not antimalarial.

Figure 1

Photomicrograph of merozoite-like parasites associated with Vero cells two days after treatment of the culture with ovomucoid and inoculation with *P. falciparum*. (May-Grunwald-Giemsa Stain)



Because of these promising results, a confirmatory test was performed according to the methods used in the first experiment. Also incorporated into this second test were attempts to obtain ancillary information by changing the experimental approach; these will be described below.

Treatment with 3000 μg/ml of ovomucoid again markedly increased the association of normal P. falciparum with Vero cells. (Table 6). It was again observed that it was the merozoite, or young ring stage, that was preferentially increased on or in the Vero cells. Also, a larger number of "merozoites" were again associated with treated cells than with untreated cells.

Differential counts were done on the erythrocytic parasitemia in the ovomucoid (3000 μg/ml) treated and untreated P. falciparum inoculated tissue cell cultures on day 2 of the first experiment and on days 2 and 3 of the second experiment. The percentage of parasites at each stage in the cultures and the percent parasitemia were essentially the same in the treated and untreated cultures. Therefore, a difference in the available parasite stages in treated vs untreated cultures would not explain the greater number of merozoites associated with Vero cells from ovomucoid treated as compared to untreated cultures.

The data presented in Table 7 indicate that as little as 30 μg/ml of ovomucoid enhanced the association of P. falciparum with Vero cells. However, 30 μg/ml, and to a lesser extent, 300 μg/ml, were less efficient than 3000 μg/ml in enhancing the percentage of Vero cells associated with parasite. Also, there were fewer

parasites associated with each Vero cell at the lower concentrations of ovomucoid.

When the association of merozoites with ovomucoid treated Vero cells was high on day 3, the ovomucoid, or the infected erythrocytes, or both, were washed out of some cultures. These cultures, and ovomucoid treated cultures of Vero containing infected red blood cells, were then maintained in candle jars at 37°C.

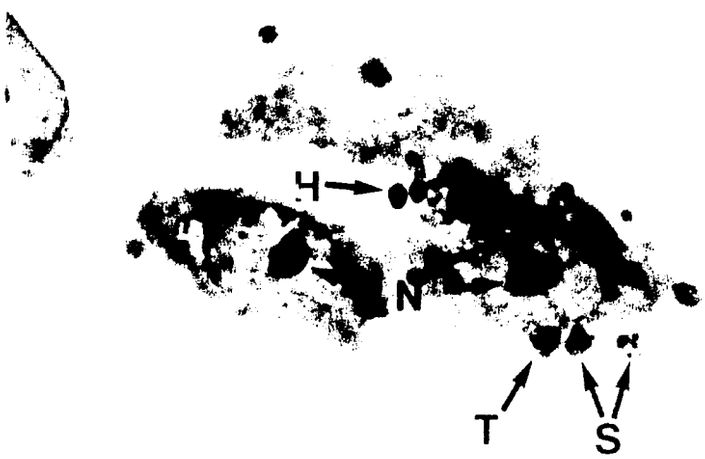
On days 3, 4, 5 and 6 the appropriate cultures were fed with GM-5 or ovomucoid (3000 μg/ml) or reinfected daily with P. falciparum or combinations of these. The data presented in Table 8 indicate removal of only ovomucoid from the culture had no effect on the enhanced association of parasites with the Vero cells, even through day 7, four days after removal of ovomucoid. However, removal of the erythrocytic culture of P. falciparum resulted in essentially complete cessation of association of parasites with Vero cells one day later.

By days 6 and 7, the erythrocytic parasite culture was degenerating. The degenerating P. falciparum erythrocytic cultures were characterized by the presence of few rings and the late stages were compact and dark-staining. It was these aberrant late stages that were the predominant stages associated with Vero cells on days 6 and 7. The appearance of these older stages, their relation to the Vero cells and the lack of red blood cells with these Vero cell-associated parasites is depicted in Figure 2.

Figure 2

Photomicrograph of aberrant trophozoites and schizonts associated with Vero Cells six days after treatment of the culture with ovomucoid and inoculation with *P. falciparum*. The ovomucoid was washed from the culture on day 3.

H: hemozoin; N: Vero cell nucleus; S: schizont



## 4. Discussion

### a. Alkylating and Polypeptide Protease Inhibitors

Because TAME, TLCK and leupeptin were anti-malarial at the concentrations tested, these drugs will be re-evaluated by treating the Vero cells with the drugs and removing the drugs from the culture prior to inoculation with *P. falciparum*. That this approach may be satisfactory is indicated by the fact that one of these drugs, TLCK, was reported by Schubli and Bruger[6] to be active as a tissue culture cell growth inhibitor after one hour of treatment and subsequent removal of the drug from the medium.

### b. Ovomucoid

A marked enhancement of parasite association with Vero cells is caused by treatment of the Vero cell-*P. falciparum* co-culture with ovomucoid. This enhancement primarily involves merozoite or very young ring stages, and results in an increased number of parasites per Vero cell and an increased percentage of Vero cells with associated parasites. The enhancing effect remained in treated cultures even when the ovomucoid was removed from the medium.

The nature of this enhancing effect is unclear. The erythrocytic *P. falciparum* culture was degenerating on days 6 and 7 and few ring stages were observed and segmenters were few, inferring few merozoites were being released in the culture. The enhancement with Vero cells involved aberrant trophozoites and schizonts, the

predominating stages in the red blood cells. This result indicates the Vero cell-associated parasites directly originate from parasites released from red blood cells. That the older stages associated with the Vero cells on days 6 and 7 may have originated from the merozoites associated with the Vero cells on day 4 is a possibility and would indicate maturation of the associated merozoites had occurred. However, direct evidence of maturation during the first 4 days was not observed, indicating the aberrant older stages seen on days 6 and 7 did not originate from the merozoites observed on days 1 to 4.

The location of these Vero cell-associated parasites is also unclear. Most of the merozoites are not in the plane of focus of the stained cell cytoplasm (see Figure 1). Also, the older stages observed on days 6 and 7 seem to be at the periphery of the cell (see Figure 2). These results indicate a surface adsorption phenomenon was induced by the ovomucoid, but at least some of the merozoites could be interiorized within the Vero cell membrane and still present this appearance. Electron microscopy is being actively pursued to answer this question, which will help direct our efforts to either enhance interiorization, if this is necessary, or reduce parasite destruction by the cells if the parasites are in the interior of the cell.

Although ovomucoid is said to adsorb to plastic and glass, we see no parasites on the Vero cell-free parts of the coverslip. Thus the ovomucoid effect is specific for the Vero cell-parasite

interaction and does not appear to be a non specific adsorption phenomenon.

5. Research Design

During the current reporting period we have proceeded with the project as described in "The In Vitro Cultivation of Malaria Parasites in Cell Culture" Contract AID/ta-C-1258 (Extension). We are concentrating our efforts to modify tissue culture cells so as to enhance *P. falciparum* multiplication in tissue culture to the Vero line of African Green monkey kidney cells because *P. falciparum* becomes associated with the Vero cell readily, albeit at low cell incidence, and Vero is easily maintained in the *P. falciparum in vitro* culture system.

Any breakthrough achieved with Vero will be immediately tested in other tissue culture cell cultures to see if the results obtained with Vero cell cultures can be extrapolated to probably acceptable vaccine substrate tissue culture cells.

As proposed in the Project Extension, we attempted to adapt *P. falciparum* to more successful association and growth in Vero cell cultures by serial passage of the parasite in the tissue culture.

We have also proceeded into the first experiments designed to modify the tissue culture cells to increase adsorption and penetration by altering the tissue culture cell surface with protease inhibitors.

One of the protease inhibitors, ovomucoid, markedly increased the numbers of parasites associated with each Vero cell and the number of Vero cells associated with parasites was increased up to 30%, compared to an incidence of about 1% of untreated Vero cells associated with parasites. These increases in Vero cell-parasite association primarily involved merozoites (or very young trophozoites). Therefore, we have not felt the need to increase merozoites in the culture by synchronizing the growth of the P. falciparum erythrocyte culture inoculum.

We plan to utilize the enhancing activity of ovoumucoid as we proceed to test agents in Vero cell culture inoculated with P. falciparum which may decrease the parasiticidal activities of the tissue culture cells.

## 6. References

(1) Schnebli, H. P. (1975) The Effects of Protease Inhibitors on Cells in Vitro in Proteases and Biological Control, ed. by Reich, E. et al. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 2 Cold Spring Harbor Laboratory, p. 785-794.

(2) Collard, J. G. and Smets, L. A., 1974. Effect of Proteolytic Inhibitors in Growth and Surface Architecture of Normal and Transformed Cells. Exp. Cell Res. 86: 75.

(3) Chou, I. N., Black, P. H. and Roblin, R. O. 1974. Non-Selective Inhibition of Transformed Cell Growth by a Protease Inhibitor. PNAS (USA) 71 (5): 1748-1752.

(4) Aoyagi, T. and Umezawa, H. 1975. Structures and Activities of Protease Inhibitors of Microbial Origin in Proteases and Biological Control. Eds. Reich, E. Rifkin D. B. and Shaw, E., Cold Spring Harbor Laboratory

(5) Libby, P. and Goldberg, A. L. 1978. Leupeptin, A Protease Inhibitor, Decreases Protein Degradation in Normal and Diseased Muscles. Science 199 (4328). 534-536.

(6) Stracher, A. and McGowan, E. G. 1978. Muscular Dystrophy: Inhibition of Degeneration in vivo with Protease Inhibitors. Science 200 (4337): 50-51.

(7) Talmadge, K. W., Noonan, K. D. and Burger, M. M. The Transformed Cell Surface: An Analysis of the Increased Lectin Agglutinability and the Concept of Growth Control by Surface Proteases. In Control of Proliferation in Animal Cells. ed. by Clarkson, B. and Baserga, R. Cold Spring Harbor Laboratory, ps. 313-325.

(8) Schnebli, H. P. and Burger, M. M. 1972 Selective Inhibition of Growth of Transformed Cells by Protease Inhibitors. Proc. Nat. Acad. Sci., USA 68 (12): 3825-3827.

## V. DISSEMINATION AND UTILIZATION OF RESEARCH RESULTS

Dr. R. G. Brackett participated in the AID Contractors Meeting in Albuquerque, New Mexico in June, 1978.

## VI. A. STATEMENT OF EXPENDITURES AND OBLIGATIONS AND CONTRACTOR RESOURCES

A budget statement showing the expenditures for the period January 1, 1978 to June 30, 1978 is presented as a detachable section of this report.

The major targets for the contract period January 1, 1978 to June 30, 1978 were:

To choose one or more candidate tissue culture cell types for modification so as to enhance P. falciparum association/maturation/growth in the tissue cell culture. We chose the Vero line of African Green Monkey kidney as our primary candidate and plan to use primary Cynomolgus kidney cells or other potentially acceptable vaccine substrate cells as secondary candidates.

To inaugurate the experiments designed to modify Vero cells so as to enhance P. falciparum association/maturation/growth in the tissue cell culture. We evaluated the effect of two alkylating protease inhibitors, one protein protease inhibitor and two polypeptide protease inhibitors.

B. Involvement of Minority Personnel and Women

Six women were involved in project activities during the reporting period. Four were involved as professional laboratory investigators, one as a secretary and one as a laboratory aid. One black professional laboratory investigator and one black laboratory aid were involved in project activities.

## VII. WORK PLAN AND BUDGET FORECAST FOR THE COMING SIX MONTHS

The work plan for the coming six months includes the following targets.

Test if the promising results obtained by ovomucoid modification of Vero cells are applicable to primary Cynomolgus kidney cell culture or other potentially acceptable vaccine substrate tissue culture cells.

Evaluate the modification of Vero cells by other protease inhibitors, protein synthesis inhibitors, steroids and a lectin on the enhancement of *P. falciparum* association/maturation/growth in the tissue cell culture.

Use the enhancing effect of ovomucoid (and other enhancers as they are discovered) in combination with the agents listed above. Ovomucoid is most likely producing a cell surface effect, resulting in increased association of merozoites with the tissue culture cell. The other agents listed above are chosen to increase interioriation and decrease proteolytic degradation of interiorized parasites.

A budget statement showing planned expenditures for each of the major inputs for the period 7/1/78 to 12/31/78 is presented as a detachable section of this report.

## APPENDIX A

### Attempt to Increase the Association of *P. falciparum* and African Green Monkey Kidney Cell (Vero) by Adaptation during Serial Passage

As proposed in the work plan for the third year of this contract, we have attempted to adapt *P. falciparum* to improved development in a candidate tissue cell culture. Vero cells were chosen because they culture well under *P. falciparum*-human red blood cell conditions. Also, *P. falciparum* was seen to routinely become associated with the cytoplasm of some Vero cells during two to three days culture together. The rationale for this experiment was that some variant of *P. falciparum* might be better adapted to tolerate the conditions for development when interiorized by Vero cells. Thus, we might hope for an increase in the incidence of parasite-cell association, an increase in survival time of interiorized parasites, and evidence of parasite maturation within Vero cells.

#### Method

*P. falciparum* (FCR-3 strain) in human $AB^+$ red blood cells and GM-5 was cultured serially nine times in the presence of Vero cells in flow flasks. Coverslips were pulled from day 1 through day 4 and stained by the May-Grunwald-Giemsa procedure for microscopic evaluation. Daily erythrocytic parasitemias were also determined.

During the first three passages fresh Vero cells were added at each subpassage and parasitized blood was added as a 12.5% suspension (3.0 ml/flask). No fresh Vero cells were added after

the third subpassage and parasitized blood was reduced to 3.0 ml of a 3% suspension during each of the last six passages and parasitized blood was diluted with normal blood to reduce parasitemia to 1 to 2% at each subpassage.

Results

The results of parasite-Vero cell association and parasitemia in red blood cells are summarized in Table 1.

Vero cells grew well during each passage. Starting with a light scattering of individual cells on day 0, daily increases in cell numbers were seen and varying degrees of monolayered cells were present on coverslips by days 2 and 3. Development of *P. falciparum* in red blood cells during each of the passages was consistently good (see parasitemia in Table) and no fresh parasites were added during the nine subpassages.

A variable degree of parasite-Vero cell association was seen during the different passages on days 1 through 4 (see Table). The merozoite, or young ring, forms predominated in association with Vero cell cytoplasm and accounted for 80 to 90% of the parasites seen. Schizonts were the next most frequently seen stage in association with cell cytoplasm. Very few growing trophozoites were identified. Occasionally an abnormal form with black-staining nuclear material was seen that was suggestive of a growing trophozoite. However, when parasitized blood was removed and Vero cells continued in culture, no good evidence of persisting (growing) parasites in Vero cells was seen after one or

two days throughout the series of passages. Very good parasite-Vero association was seen in the final two passages (see Table) suggesting an increase in parasite association with Vero cells during the nine passages.

Discussion

In summary, during nine subpassages of *P. falciparum* and Vero cells together *in vitro*, little evidence of increased development of the parasite in association with Vero cells was seen. It is concluded that under the conditions employed, no significant "adaptation" of the parasite to development (growth) in Vero cells was evident, but an increase in relative number of Vero cells with associated parasites was seen during the latter two passages. These results suggest that additional efforts to adapt *P. falciparum* to growth in Vero cells, or another cell line, may be worthwhile.

Vero Cell/Parasite Association and Development of

P. falciparum in RBC's During Series of Passages

| Passage No. | Percent of Vero Cells with Associated Parasites | | | | Percent Parasitemia in Red Blood Cells | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 1 | Day 2 | Day 3 | Day 4 |
| 1 | 5 | 2 | 3 | ND* | 1.8 | 2.9 | 4.7 | ND |
| 2 | ND | ND | 15 | ND | ND | ND | 1.9 | ND |
| 3 | 8 | 6 | ND | ND | 1.7 | 4.2 | ND | ND |
| 4 | 1 | ND | 5 | ND | 2.1 | ND | 9.4 | ND |
| 5 | 0.5 | 10 | ND | ND | 1.9 | 2.5 | ND | ND |
| 6 | 0.4 | 1 | ND | ND | 1.5 | 2.0 | ND | ND |
| 7 | ND | ND | ND | 5 | ND | ND | ND | 13.2 |
| 8 | ND | 10 | 10 | ND | 2.9 | 6.6 | 11.3 | ND |
| 9 | 6 | ND | 35 | ND | 2.7 | 4.6 | 8.4 | ND |

*ND = Not Done

Table 1

*P. falciparum* (FCR-3) Inoculated Vero Tissue Culture Cells Treated with TLCK

| Drug Concentration (μg/ml) | % Parasitemia, Erythrocytes* on Days | | | % Parasitemia, Vero Cells on Days | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 20 | 2.1 | 3.5 | 0.7 | 5 | 0.2 | 0.0 |
| 39 | 1.4 | 1.8 | 0.2 | 5 | 0.0 | 0.0 |
| 78 | 1.2 | ND** | 0.1 | 0.0 | 0.0 | 0.0 |
| No Drug and No Vero | 2.2<br>2.5 | 3.2<br>4.2 | 6.1<br>4.9 | NA*** | NA | NA |
| No Drug | 2.4 | 4.6 | 6.7 | ND | 3 | ND |

*% Parasitemia in *P. falciparum* inoculum was 2.4

**No Datum

***Not Applicable

Table 2

*P. falciparum* (FCR-3) Inoculated Vero Tissue Culture Cells Treated with TAME

| Drug Concentration (μg/ml) | % Parasitemia, Erythrocytes* on Days | | | % Parasitemia, Vero Cells on Days | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 1350 | 1.2 | 1.5 | 0.5 | 1 | ND** | 0 |
| No Drug and No Vero | 2.2<br>2.5 | 3.2<br>4.2 | 6.1<br>4.9 | NA*** | NA | NA |
| No Drug | 2.4 | 4.6 | 6.7 | ND | 3 | ND |

*%Parasitemia in *P. falciparum* inoculum was 2.4

**No Datum

***Not Applicable

Table 3

*P. falciparum* (FCR-3) Inoculated Vero Tissue Culture Cells Treated with Antipain

| Drug Concentration (μg/ml) | % Parasitemia, Erythrocytes* on Days | | | % Parasitemia, Vero Cells on Days | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 35 | 5.5 | 9.3 | 14.8 | 0 | 4 | 25 |
| No Drug and No Vero | 6.4<br>6.2 | 11.0<br>10.2 | 10.6<br>12.9 | NA** | NA | NA |
| No Drug | 3.4 | 7.7 | 9.0 | 2 | 10 | ND*** |

*% Parasitemia in *P. falciparum* inoculum was 2.7

**Not Applicable

***No Datum

Table 4

*P. falciparum* (FCR-3) Inoculated Vero Tissue Culture Cells Treated with Leupeptin

| Drug Concentration (μg/ml) | % Parasitemia, Erythrocytes* on Days | | | % Parasitemia, Vero Cells on Days | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 48.3 | 3.6 | 2.3 | 1.5 | 1 | 2 | 0.5 |
| No Drug and No Vero | 6.4<br>6.2 | 11.0<br>10.2 | 10.6<br>12.9 | NA** | NA | NA |
| No Drug | 3.4 | 7.7 | 9.0 | 2 | 10 | ND*** |

*%Parasitemia in *P. falciparum* inoculum was 2.7

**Not Applicable

***No Datum

Table 5

*P. falciparum* (FCR-3) Inoculated Vero Tissue Culture Cells Treated with Ovomucoid (First Experiment)

| Drug Concentration (μg/ml) | % Parasitemia, Erythrocytes* on Days | | | % Parasitemia, Vero Cells on Days | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 3000 | 6.5 | 11.8 | 17.1 | ND** | 20 | ND |
| No Drug and No Vero | 6.4<br>6.2 | 11.0<br>10.2 | 10.6<br>12.9 | NA*** | NA | NA |
| No Drug | 3.4 | 7.7 | 9.0 | 2 | 10 | ND |

* % Parasitemia in *P. falciparum* inoculum was 2.7

** No Datum

*** Not Applicable

Table 6

*P. falciparum* (FCR-3) Inoculated Vero Tissue Culture Cells Treated with Ovomucoid (Second Experiment)

| Rx | Test Day | Approximate % Parasitemia, Vero Cells - Predominant (≥80%) Stage | % Parasitemia, Erythrocytes - Average (# of culture) | Range |
|---|---|---|---|---|
| Vero + Ovomucoid (3000 μg/ml) + *P. falciparum* | 1 | 20 - "Merozoites" | 7.8 (5) | 6.2 - 8.8 |
| | 2* | 30 - "Merozoites" | 15.3 (5) | 13.9 - 15.9 |
| | 3 | 10 - "Merozoites" | 1.6 (5) | 1.4 - 1.9 |
| | 4 | 15 - "Merozoites" | 7.9 (2) | 7.8 - 7.9 |
| Vero + *P. falciparum* (No Ovomucoid) | 1 | 0.8 - QNS** | 7.2 (2) | 6.9 - 7.4 |
| | 2* | 5 - "Merozoites" | 11.6 (2) | 11.3 - 11.9 |
| | 3 | 3 - "Merozoites" | 1.3 (2) | 1.1 - 1.5 |
| | 4 | 1 - QNS | 4.0 (2) | 2.6 - 5.3 |
| *P. falciparum* (No Ovomucoid, No Vero) | 1 | Not Applicable | 7.5 (2) | 7.2 - 7.8 |
| | 2* | " " | 13.8 (2) | 10.4 - 17.2 |
| | 3 | " " | 1.2 (2) | 1.2 - 1.2 |
| | 4 | " " | 5.4 (2) | 5.0 - 5.7 |

*Erythrocytic cultures were diluted with normal red blood cells to about 1% parasitemia on day 2.

**There was an insufficient number of Vero cells associated with parasites to make an evaluation of the predominant stage.

Table 7

Titrations of Ovomucoid in *P. falciparum* Inoculated Vero Cell Tissue Culture

| Ovomucoid Concentration (μg/ml)[a] | Day of Test | % Erythrocytic Parasitemias[b] | % Vero Cells Associated with Parasite[c] |
|---|---|---|---|
| 3000 | 1 | 7.8 | 20 |
| | 2 | 15.3 | 30 |
| | 2(after split) | 1.4 | |
| | 3 | 1.6 | 10 |
| | 4 | 7.9 | 15 |
| 300 | 1 | 8.0 | QNS[d] |
| | 2 | 13.3 | 10 |
| | 2(after split) | 0.8 | |
| | 3 | 1.9 | 6 |
| | 4 | 6.0 | 5 |
| 30 | 1 | 8.4 | QNS |
| | 2 | 14.5 | 10 |
| | 2(after split) | 0.9 | |
| | 3 | 2.3 | 5 |
| | 4 | 6.8 | 5 |
| None | 1 | 7.2 | 0.8 |
| | 2 | 11.6 | 5 |
| | 2(after split) | 0.6 | NA |
| | 3 | 1.3 | 3 |
| | 4 | 4.0 | 1 |

[a] Vero - *P. falciparum* cultures were treated with ovomucoid on days 0, 1, 2 and 3.

[b] Erythrocytic parasites were in good to very good conditions throughout test. The erythrocytic culture was split 1:10 on day 2.

[c] Merozoites were the predominating parasite stage (>80%) associated with Vero cells.

[d] QNS: Insufficient Vero Cells in culture for evaluation.

Table 8

The Effect of Withdrawal of Ovomucoid/P. falciparum Infected Erythrocytes from Treated Cultures

| Rx | Test Day* | Approximate % Parasitemia, Vero Cells - Predominant (≥80%) stage | Parasitemia, Erythrocytes: Percent | Parasitemia, Erythrocytes: Parasite Condition |
|---|---|---|---|---|
| Vero + Ovomucoid (3 mg/ml) + *P. falciparum* x 3 da. *P. falciparum* and rbc washed from culture on da. 3 | 3 | 10 - Merozoites | 1.4 | Good |
| | 4** | 1 - Degenerate merozoites and schizonts | NA*** | NA |
| Vero + Ovomucoid (3 mg/ml) + *P. falciparum* x 3 da. Ovomucoid washed from culture on day. 3 | 3 | 10 - Merozoites | 1.5 | Good |
| | 4 | 25 - Merozoites | 6.0 | Fair |
| | 6 | 20 - Aberrant troph. | 3.1 | Poor |
| | 7 | 15 - Degenerate troph. | 2.3 | Poor |
| Vero + Ovomucoid (3 mg/ml) + *P. falciparum* x 3 da. *P. falciparum*, red blood cells and ovomucoid washed from culture on da. 3 | 3 | 10 - Merozoites | 1.8 | Good |
| | 4** | < 1 - Degenerate troph. | NA | NA |
| Vero + Ovomucoid (3 mg/ml) + *P. falciparum* x 7 days | 3 | 10 - Merozoites | 1.6 | Good |
| | 4 | 15 - Merozoites | 7.9 | Good |
| | 6 | 20 - Aberrant troph. | 4.6 | Poor |
| | 7 | 3 - Aberrant troph. | 2.2 | Poor |
| Vero + *P. falciparum* x 7 days | 3 | 3 - Merozoites | 1.3 | Good |
| | 4 | 1 - Too few parasites | 4.0 | Good |
| | 6 | < 1 - Too few parasites | 1.6 | Poor |
| | 7 | 0 - Too few parasites | 0.8 | Poor |

*Data from these cultures on days 1 and 2 are presented in Table 7.

**No datum from these cultures is presented for days 6 and 7 because the Vero cells had overgrown and could not be evaluated.

***NA: Not Applicable.