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JANUARY 1, 1978 to DECEMBER 31, 1978

ANNUAL REPORT

THE IN VITRO CULTIVATION OF MALARIA

PARASITES IN CELL CULTURES

## ANNUAL REPORT SUMMARY SHEET

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

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### SUMMARY OF ACCOMPLISHMENTS AND UTILIZATION

A significant increase in the number of Vero cells associated with parasites has been consistently observed when co-cultured with Plasmodium falciparum (FCR-3) infected erythrocytes in the presence of ovomucoid. Fifty to eighty percent of the cells became associated with erythrocyte-free merozoites or young ring stages and numerous parasites were associated with each Vero cell. The degree of association was dose-related and in the absence of ovomucoid, a lower percentage of the Vero cells were associated with parasites and fewer parasites were associated with each Vero cell. Maturation of the parasites did not occur. The association was tenuous in that a significant decline in associated parasites occurred within two hours after the infected erythrocytes were washed out of the culture. Interference, phase and transmission electron microscopy indicated that the parasites were predominantly located on the outside of the Vero cell membrane. Other efforts to increase or stabilize association of the parasites with Vero cells, interiorize the parasites, and enhance their maturation have been unsuccessful to date. Methods used have included treatment with an alkylating protease inhibitor, five polypeptide protease inhibitors, two protein synthesis inhibitors and two steroids. Future efforts will concentrate on virus- and chemical-induced fusion of the parasites with the Vero cell followed by attempts to increase parasite maturation and multiplication by modification of the Vero cell.

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## I. BACKGROUND

Prior to this report, we reported on the following -

In order to select a promising cell substrate for the propagation of malarial parasites, Plasmodium berghei (NK-65) was studied in fifteen tissue culture types including primaries from organs of mice, rabbits and rats, and cell lines of mouse and human origin. Neither morphologic evidence for maturation or growth of the parasite nor persistence of infectious P. berghei in cultures beyond the control was observed.

Since maturation and multiplication of P. berghei in Leydig cells (mouse testicular tumor cell line) and in primary mouse bone marrow had been reported by Dr. Clarence Speer when he was associated with the University of New Mexico, we studied these two cell systems both by our standard method and by that of Dr. Speer. Although both Leydig and mouse bone marrow cells interiorized the parasite, there was no morphologic evidence of maturation of the parasites and infectivity for mice was not enhanced by passage in cell culture. Furthermore, in contrast to Dr. Speer's findings, the cells efficiently interiorized both UV-killed P. berghei and polystyrene beads.

On 1/21/77, we acquired P. falciparum from Drs. Trager and Jensen of the Rockefeller Institute and have propagated it both in petri plates in candle jars and in a continuously fed Flow Flask System using human AB and O red blood cells. Both the Flow Flask and petri plate culture methods were adapted 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 cultures were evaluated for their effect on the growth of P. falciparum in co-culture. Normal-appearing parasites were associated with primary cultures of human endothelial umbilical vein; Rhesus monkey kidney, liver and bone marrow; Cynomolgus monkey kidney cells; and the Vero line of African Green monkey kidney cells.

Two different mechanisms for cell-parasite association appeared to be operative: (1) adsorption of infected red cells by the tissue culture cell followed by phagocytosis and (2) adsorption of free merozoites with or without interiorization.

The first mode of association involved interiorization of an infected red cell, destruction of the red cell (observed as a loss of color), and finally an essentially normal-appearing parasite in the phagosome formerly occupied by the red cell. Normal developmental forms were seldom seen within phagosomes in the absence of red cell material.

The second mode of association, characterized by free merozoites either adhering to the plasma membrane or interiorized in the cell, was observed more frequently. Essentially normal merozoites

or very immature trophozoites (rings) were seen on or within the plasma membrane of a limited number of 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.

Since both types of parasite association were observed in Vero cell cultures, we are attempting to improve the association, maturation, and multiplication of P. falciparum in Vero cells by manipulation of the cultural conditions.

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 DECEMBER 31, 1978

A. Introduction

Details of results obtained during the first six months of 1978 were reported in our Six Month Contract Report of July 1, 1978 and will be briefly reviewed in this report.

Ovomucoid, a protein protease inhibitor, was found to markedly increase the association of P. falciparum merozoites and young ring stages with Vero cells in co-culture. By means of this phenomenon we now have available large numbers of P. falciparum parasites in contact with or within Vero cells for our continuing effort to enhance parasite maturation and growth in tissue culture. Maturation of these Vero-associated parasites was not observed and the location of the associated parasites, intracellular or extracellular, remained to be determined.

Treatment of Vero cell-P. falciparum co-cultures with the alkylating protease inhibitors, TLCK (N-2-tosyl-L-lysyl chloromethyl ketone) and TAME (N-2-tosyl-L-arginyl methyl ester), and the polypeptide protease inhibitors derived from Actinomycetes, leupeptin and antipain, was studied. Results with TLCK, TAME and leupeptin were inconclusive because the drugs were antimalarial in vitro at the concentrations tested and for antipain were negative.

During nine serial passages of P. falciparum in co-culture with Vero cells, there was increased association of merozoites or young ring stages with Vero cells, indicating further study of

this approach may be fruitful. When parasitized blood was removed from the co-culture and the Vero cells with their associated parasites, were continued in culture, no growing parasites were observed throughout the passage series.

The accomplishments from July 1 to December 31, 1978 are reported below.

B. Characterization of the Ovomuroid-Enhanced Vero Cell-  
P. falciparum Association

1. The transitory association of P. falciparum with Vero cells

We previously reported that in cultures in which association of P. falciparum with Vero cells had been established, a significant decline in associated parasites occurred within 24 hours after the infected erythrocytes were washed out of the co-culture.

A confirmatory experiment was performed by the method described in the Appendix, modified in order to determine the minimal time of persistence of associated parasites with the Vero cells. In this experiment, the infected erythrocytes were washed out of ovomuroid treated co-cultures on day two, at which time 55% of the Vero cells were associated with the merozoite-young ring stage. These cultures, and those which contained infected erythrocytes throughout the experiment, continued to be fed with GM-5 medium that contained ovomuroid.

It can be seen from the data recorded in Table 1 that coverslips pulled from cultures two hours after removal of the erythrocytes contained a significantly diminished percentage of Vero cells associated with parasites compared to the percentage of Vero cells associated with parasites from the cultures prior to removal of the infected erythrocytes and from the co-cultures in which infected erythrocytes remained. Although a coverslip was not obtained at hour zero from the co-culture in which the erythrocytes were retained, our experience with the system indicates that the percentage of Vero cells associated with the parasites in this co-culture at hour zero would have been similar to what it was two hours before and four hours after hour zero, i.e., between 40% and 51%. The number of parasites per Vero cell in the erythrocyte-free cultures was also reduced and the remaining Vero cell-associated parasites were morphologically more degenerate.

It is apparent from these results that the association of erythrocyte-free P. falciparum with Vero cells is tenuous. Further, it seems likely that the observed Vero cell-associated parasites result from continual replenishment with merozoites that arise from mature erythrocytic segmenters.

We do not know the cause of the lack of persistence of parasites associated with the Vero cells. It could result from degeneration of associated parasites, a simple disengagement by the parasite from its sojourn with the surface of the host cell, or active

Table 1

Lack of Persistence of *P. falciparum* Association  
with Vero Cells After Removal of Erythrocytes

Vero cells - <i>P. falciparum</i> <sup>(a)</sup> Co-cultures treated with ovomucoid and	% Vero Cells Associated with <i>P. falciparum</i> on hours:							
	<u>-24</u>	<u>-2</u>	<u>0</u>	<u>+1</u>	<u>+2</u>	<u>+4</u>	<u>+22</u>	<u>+24</u>
Infected erythrocytes washed out of culture at hour 0 <sup>(b)</sup>	17	50	55	36(c)	10(c)	10(c)	2(c)	< 1
Infected erythrocytes not washed out of culture	16	40	NT(d)	NT	NT	51	22	12

(a) The *P. falciparum* inoculum contained a 4.5% parasitemia. Thereafter, on days 1, 2 and 3 after inoculation the erythrocytic parasitemia ranged from 8.2% to 14.1%.

(b) Zero hour was two days after inoculation of the Vero cell cultures with *P. falciparum* and treatment of the cultures with ovomucoid at 3 mg/ml.

(c) Morphologic degeneration of the Vero cell-associated parasites was observed.

(d) NT = Not Tested.

degradation of the parasites by the host cell. Whatever the cause, our efforts to stabilize and abet the maturation and multiplication of the Vero cell-associated parasites by modifying the culture conditions and by treatment with various drugs continue to be of considerable importance to the fulfillment of the project objective.

2. The anatomic location of P. falciparum associated with Vero cells determined by:

a. Phase and interference microscopy

Because it is very difficult to unequivocally determine if the Vero cell-associated parasites are external or internal to the Vero cell membrane by bright field microscopy of stained coverslip preparations, phase and interference microscopy were used to attempt to clarify this point.

The coverslip preparations were made as described in the Appendix, using ovomucoid (3 mg/ml) to enhance Vero cell-P. falciparum association. In addition, unfixed and unstained wet mounts were examined by phase and interference microscopy.

The erythrocytic parasitemia in these co-cultures was 10% to 12% at the time the coverslips were pulled for examination, two and three days after inoculation of the co-cultures with P. falciparum. The evaluation by bright field microscopy of the stained coverslips indicated 44% to 87% of the Vero cells were

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associated with parasites, about 90% of which were merozoites or young rings.

Although the Vero cell anatomy was seen better by phase microscopy than by interference microscopy, the visualization of parasites associated with the Vero cells was poor by both methods. The parasites appeared as round, dense bodies on the periphery of the Vero cells.

Vero cell-associated parasites could not be unequivocally identified as intracellular by these microscopic techniques because of their limits of resolution, inability to clearly define parasites from organelles within the cells, and failure to clearly demarcate the Vero cell membrane.

b. Transmission electron microscopy

Another approach we took to determine if some of the Vero cell-associated parasites were intracellular was to have transmission electron microscopy performed. The high resolution of electron microscopy on thin sections should also answer other questions as to the physical interrelations between the associated parasites and the Vero cells and whether the associated parasites appear to be normal and therefore presumably viable and infective.

Ovomucoid-treated Vero cell-P. falciparum co-cultures were prepared in plastic Petri dishes as described in the Appendix. On day two, when 40% of the Vero cells were associated with merozoites-

~~young rings and multiple parasites were seen on each Vero cell,~~  
the erythrocytes were washed out of the culture. The Vero cells were harvested from the surface of the tissue culture dish with either EDTA or EDTA-trypsin. After removal of the EDTA or EDTA-trypsin by centrifugation, portions of the cell pellet were examined by the following techniques:

(1) After resuspension in GM-5 medium, cells were smeared on a slide, stained with May-Grunwald-Giemsa, and examined by bright-field microscopy.

(2) After resuspension in Vero cell growth medium, the cells were cultured at 37°C in 5% CO<sub>2</sub> in air for four hours on coverslips in tissue culture petri dishes, at which time most of the cells had attached and spread on the coverslips. Coverslips were examined by bright-field microscopy after May-Grunwald-Giemsa staining.

(3) After fixation with glutaraldehyde by the method of Aikawa\*, cells were submitted to Dr. Thomas Seed (Argonne National Laboratories) who kindly performed the embedding, sectioning and transmission electron microscopy.

\*Aikawa, M., Huff, C. G. and Sprinz, H. 1966. Comparative Feeding Mechanisms of Avian and Primate Malarial Parasites. Military Medicine Supplement 131 (9): 969-992.

Parasites were not observed to be associated with the Vero cells that were smeared on slides and stained after they were harvested from the co-culture or after they were harvested and cultured on coverslips for four hours prior to fixing and staining. Dr. Seed did not see any parasites associated with the Vero cells examined by transmission electron microscopy.

These results lend further support to the conclusion that ovomucoid-enhanced P. falciparum association with Vero cells is tenuous, as reported in section IV-B-1 of this report. An ancillary conclusion is that most of the associated parasites may be located on the surface of the Vero cell.

In this experiment, we did not study the effect of EDTA and EDTA-trypsin on the persistence of the P. falciparum association with the Vero cells. It is possible that EDTA and EDTA-trypsin stripped the parasites from the Vero cells. It is also possible that the rounding of the Vero cells as they were harvested from the tissue culture dish surface or the washing and centrifugation prior to fixation may have been sufficiently traumatic to cause removal of the parasite from the Vero cells.

A recently introduced, modified Petri dish, the Petri Perm (W. C. Heraeus, West Germany) will give us the opportunity to submit for electron microscopy a P. falciparum associated Vero cell that has had no manipulation prior to fixing. Before submitting such a Petri Perm preparation for electron microscopy, we had to prove P. falciparum multiplied and associated with Vero

cells as readily in co-cultures in Petri Perms as it does in Petri dishes.

The Petri Perm is a plastic Petri dish with a flexible, transparent, gas-permeable membrane substituted for the rigid plastic bottom of the Petri dish. Ovomuroid-treated P. falciparum-Vero cell co-cultures were set up in Petri Perm dishes and in Petri dishes, as described in the Appendix. Petri dish co-cultures were incubated in a candle jar; Petri Perm and Petri dish co-cultures were incubated in an anaerobic incubator (National Appliance Co., Model 36401) in an atmosphere of 3% CO<sub>2</sub>, 3% O<sub>2</sub> and 94% N<sub>2</sub>\*. After incubation at 37°C, the Vero cell-P. falciparum associated culture on the Petri Perm membrane was fixed and stained by May-Grunwald-Giemsa in the same manner as were the coverslips from Petri dish co-cultures. Glutaraldehyde was found to be as satisfactory as methanol for fixing these preparations prior to staining, and is the fixative used for preparation of specimens for electron microscopy.

\*This gas atmosphere was shown to be a satisfactory mixture for propagation of P. falciparum in erythrocyte cultures in Petri Perms during a series of experiments in which the gas proportions were varied in the anaerobic incubator. (Data not presented).

The data recorded in Table 2 indicate P. falciparum grew as well in Petri Perms in the controlled gas atmosphere as in Petri dishes in either a candle jar or in the controlled gas atmosphere. In addition, the association of merozoite-young ring stages of P. falciparum with the Vero cells was similar under these culture conditions.

Using the Petri Perm technology, we will submit to Dr. Seed for electron microscopic examination a Vero cell-associated P. falciparum preparation which has been fixed in situ with glutaraldehyde. Part of the same fixed Petri Perm membrane will be stained for direct comparison by light microscopy. By this procedure, we expect to obtain unequivocal information on the physical interrelation of the Vero cells and their associated parasites. Dr. Seed recently visited our laboratories and after examining some stained Petri Perm preparations indicated that he thought he could effectively evaluate them by electron microscopy.

C. The Effect of Intermittent Drug Treatment with Protease Inhibitors on P. falciparum-Vero Cell Association

In the July 1, 1978 contract report, we recorded that TAME, an alkylating protease inhibitor, and leupeptin, a polypeptide protease inhibitor, were antimalarial and therefore the evaluation performed according to the method described in the Appendix may have been invalid. We therefore modified the test procedure in the following manner. The Vero cell culture was treated with 1.5 mg/ml of TAME or 48.3  $\mu$ g/ml of leupeptin for four hours on

Table 2

Co-Cultivation of *P. falciparum* with Ovomucoid Treated  
Vero Cells in Petri Perm and Petri Dishes

Culture Method	Percent Parasitemia (a)					
	Day 1		Day 2		Day 3	
	<u>Vero</u>	<u>RBC</u>	<u>Vero</u>	<u>RBC</u>	<u>Vero</u>	<u>RBC</u>
Petri Perm Dishes in 3% CO <sub>2</sub> , 3% O <sub>2</sub> , 94% N <sub>2</sub>	3	12.0 (b)	33	18.9	22	18.0
Petri Dishes in 3% CO <sub>2</sub> , 3% O <sub>2</sub> , 94% N <sub>2</sub>	8	11.4	41	15.5	38	21.0
Petri Dishes in Candle Jar Atmosphere	ND (c)	12.1	30	11.6	42	10.9

(a) Percent of Vero cells with parasite association and percent of red blood cells (RBC) infected with parasites.

(b) Erythrocytic parasitemia of co-culture inoculum was 5.3% on day zero.

(c) No datum.

day zero and the drugs were washed out of the culture before inoculation with P. falciparum and incubation at 37°C in a candle jar. On days 1 and 2, the infected erythrocytes were removed from the culture, the Vero cells were treated with the drugs for four hours, the drugs were washed out of the culture, and the P. falciparum-infected red blood cells inoculated back into the culture. The untreated Vero cell-P. falciparum co-culture was manipulated in the same manner as the drug treated co-cultures.

The data presented in Table 3 indicate that these drugs at the concentrations tested, did not increase association of P. falciparum with Vero cells when the cells were intermittently treated with the drugs. In fact, there was an apparent decrease in the percent of Vero cells associated with the parasite in the drug treated cultures; its cause was not determined. In addition, maturation of the associated parasites was not increased. However, this approach did eliminate the antimalarial activity of the drugs in the co-cultures.

These results indicate that intermittent treatment of the Vero cells with these drugs was not efficacious and will not be further explored.

Table 3

Effect of Intermittent Treatment of Vero Cells with  
Protease Inhibitors in Co-Culture with *P. falciparum*

<u>Vero Cells Treated With:</u>	Percent Parasitemia <sup>(a)</sup>					
	Day 1		Day 2		Day 3	
	<u>Vero</u>	<u>RBC</u>	<u>Vero</u>	<u>RBC</u>	<u>Vero</u>	<u>RBC</u>
TAME (1.5 mg/ml)	< 1	5.8 <sup>(b)</sup>	< 1	9.7	8	10.2
Leupeptin (48.3 $\mu$ g/ml)	< 1	5.0	< 1	9.0	10	12.6
No Drug	< 1	5.4	20	9.6	20	14.0

(a) Percent of Vero cells with parasite association and percent of red blood cells (RBC) infected with parasites.

(b) Erythrocytic parasitemia of culture inoculum was 3.5% on day zero.

D. The Effect of Polypeptide Protease Inhibitors on P. falciparum-Vero Cell Association

The effect of three polypeptide protease inhibitors, pepstatin, elastinal and bestatin, on the association and maturation of P. falciparum in Vero cell cultures was evaluated by the method described in the Appendix. The drugs were tested at the concentration of 50  $\mu$ M because of limited availability.

As recorded in Table 4, the data indicate pepstatin might have enhanced to a small degree the percentage of Vero cells that were associated with P. falciparum, but not to the extent achieved with ovomucoid. Elastinal and bestatin did not increase association of parasites with the Vero cells. Maturation of the associated parasites was not observed in any of the treated cultures.

Further evaluation of these drugs will not be made at this time.

E. The Effect of Protein Synthesis Inhibitors Alone, and Combined with Ovomucoid, on P. falciparum-Vero Cell Association

The protein synthesis inhibitors, puromycin and cycloheximide, were tested for their effect on the association and maturation of P. falciparum in Vero cell culture by the method described in the Appendix and by a modification of this method. The modification entailed treating the co-culture with ovomucoid on days 0 (day of P. falciparum inoculation), 1 and 2, and with the protein synthesis inhibitors on days 1 and 2. The purpose

Table 4

The Effect of Pepstatin, Elastinal and Bestatin in Co-Cultures of Vero Cells and *P. falciparum*

Co-cultures treated with:	Percent Parasitemia <sup>(a)</sup>			
	Day 1		Day 2	
	<u>Vero</u>	<u>RBC</u>	<u>Vero</u>	<u>RBC</u>
Pepstatin (50 $\mu$ M)	7	4.2 <sup>(b)</sup>	4	4.3
Elastinal (50 $\mu$ M)	< 0.2	4.1	1	3.1
Bestatin (50 $\mu$ M)	3	4.5	0.5	3.1
No Drug	3	4.4	2	4.6

(a) Percent of Vero cells with parasite association and percent of red blood cells (RBC) infected with parasites.

(b) Erythrocytic parasitemia of culture inoculum was 3.5% on day zero.

of this modification was to enhance the percent of Vero cells associated with P. falciparum by one day of treatment with ovomucoid and then observe the effect of the protein synthesis inhibitors on the associated parasites on days 2 and 3. The protein synthesis inhibitors were used at a low concentration ( $5 \times 10^{-8}M$ ) because preliminary experiments indicated they were both toxic to Vero and antimalarial at two to ten times higher concentrations.

The results presented in Table 5 indicate that none of the treatment regimens was antimalarial since the percent parasitemia in the erythrocytes was unchanged.

Puromycin by itself, or in combination with ovomucoid, reduced the percentage of Vero cells associated with P. falciparum as compared to the association in the appropriate control. Cycloheximide appeared to have a positive effect on enhancement of association when tested by itself as compared to the appropriate, non-ovomucoid treated control, but was not more effective than ovomucoid. In combination with ovomucoid, cycloheximide did not significantly alter the Vero cell association with parasites when compared to the ovomucoid-treated control. The morphologic evidence indicated maturation of the Vero cell associated parasites was not enhanced by any of these treatment regimens.

Although these drugs were not effective, or were no more effective than ovomucoid in enhancing association of Vero cells with the parasite nor increasing maturation of the associated parasite, they will be re-evaluated for their activity to stabilize and aid maturation of interiorized parasites.

Table 5

The Effect of Puromycin and Cycloheximide Alone and Combined with Ovomuroid, in Co-Cultures of Vero Cells and P. falciparum

Co-Cultures Treated With:		Percent Parasitemia <sup>(a)</sup>					
Ovomuroid on Days 0, 1, 2	Protein Synthesis Inhibitor: (Treatment Days)	Day 1		Day 2		Day 3	
		Vero	RBC	Vero	RBC	Vero	RBC
+	Puromycin (1, 2)	< 1	6.4 <sup>(b)</sup>	10	11.1	2	9.1
+	Cycloheximide (1, 2)	< 1	7.1	15	8.6	15	14.0
-	Puromycin (0, 1, 2)	< 1	7.7	< 1	9.0	< 1	9.4
-	Cycloheximide (0, 1, 2)	< 1	7.8	10	7.1	ND <sup>(c)</sup>	9.6
+	None	1	7.9	30	9.9	30	15.9
-	None	< 1	7.7	1	12.1	15	11.1

(a) Percent of Vero cells with parasite association and percent of red blood cells (RBC) infected with parasites.

(b) Erythrocytic parasitemia of culture inoculum was 4.5% on day zero.

(c) No datum.

F. The Effect of Steroids Alone, and Combined with Ovomuroid,  
on P. falciparum-Vero Cell Association

Using the method described in the Appendix, the steroids, hydrocortisone and dexamethasone, were tested alone and in combination with ovomuroid (1.5 mg/ml) in co-cultures. The concentration of steroids used in this test ( $5 \times 10^{-6}M$ ) was not toxic to the Vero cells; the results of preliminary experiments showed that higher concentrations of both steroids inhibited Vero cell growth and were therefore not used.

The percent of Vero cells associated with P. falciparum (predominantly merozoites and young ring stages) and the erythrocytic parasitemias are recorded in Table 6. These data indicate the steroids did not significantly enhance the quantity or the maturation of P. falciparum associated with the Vero cells when the co-cultures were treated with steroids or with the combination of steroids and ovomuroid. Therefore, enhancement by these steroids of P. falciparum association and maturation in Vero cell co-cultures will not be further explored by this methodology.

In this experiment, we observed that a high percent (46% on day 2) of Vero cells that were not treated with drug were associated with parasites. This has recently been a generally observed phenomenon (see Tables 3 and 5 of this report) for which we presently have no explanation. However, the enhancing effect of ovomuroid remains obvious; the ovomuroid-treated Vero cells have not only a much higher number of parasites associated per Vero cell than do those not treated with ovomuroid, but also a higher percentage of Vero cells are associated with parasites.

Table 6

The Effect of Hydrocortisone and Dexamethasone Alone, and in Combination with Ovomuroid, on Vero Cells and P. falciparum Co-Cultures

<u>Co-Cultures Treated With:</u>	% Parasitemia (a)			
	Day 1		Day 2	
	<u>Vero</u>	<u>RBC</u>	<u>Vero</u>	<u>RBC</u>
Hydrocortisone	6	6.7 (b)	47	12.6
Hydrocortisone and ovomuroid	18	6.4	69	13.0
Dexamethasone	22	6.6	61	13.2
Dexamethasone and ovomuroid	7	6.6	56	13.8
Ovomuroid	29	7.2	80	15.5
No Drug	6	5.6	46	12.3

(a) Percent of Vero cells with parasite association and percent of red blood cells (RBC) infected with parasites.

(b) Erythrocytic parasitemia of culture inoculum was 3.4% on day zero.

G. Research Design

The research design which we plan to use in future work evolved from results of experiments described in this report. Originally we tested drugs for their effects in P. falciparum-Vero cell co-cultures at close to the maximum level tolerated by the Vero cells (if the drug was available in sufficient quantity) which was determined by a preliminary toxicity test titration in Vero cell cultures. Early attempts to use the drugs at the maximum non-toxic concentrations were successful regarding Vero toxicity, but a significant number of the drugs were antimalarial at these dosages. We therefore had to determine the antimalarial concentration prior to using them at a maximum concentration that was neither toxic to the Vero cells nor antimalarial.

Throughout this evaluation and application of the research design, all aspects of the test system had to be carefully controlled to ensure that the design worked. Stated another way, quality control had to be built into each study. This, at times, proved to be a formidable task because of the complexity of the technologies we combined in each study. Thus, sterility was mandatory for the components that went into each drug evaluation: the P. falciparum culture from the Flow Flasks; the individual units of human and fetal calf serum; the two tissue culture media; the individual lots of human red blood cells; and the drugs. In addition, the parasite culture, both at the source, the continuous Flow Flask system, and during the test, had to be at high parasitemia, of excellent quality and asynchronous in order to supply an adequate number of segmenting parasites to

the Vero cells during all days of the test. Similarly, only top quality Vero cell cultures were acceptable for the research design to work.

Ovomucoid was found to be significantly active in enhancing P. falciparum-Vero cell association early in these studies.

It was one of the protease inhibitors we chose to study first and was neither toxic to Vero cells nor antimalarial at the maximum soluble concentration.

We proceeded to study some critical aspects of the ovomucoid induced enhancement of P. falciparum-Vero cell association, even while we were studying the effects of other drugs on the co-cultures. These studies indicated that the associated parasites appeared to be primarily exterior to the Vero cell membrane and no drug, either alone or in combination with ovomucoid, was more effective than ovomucoid in enhancing the association of P. falciparum with Vero cells or in increasing the maturation and multiplication of the associated parasites.

Therefore, in the evolved research design, Vero cell-P. falciparum co-cultures will be treated with ovomucoid and then attempts will be made to induce interiorization of the associated parasites as described in the Work Plan for the coming two and one-half months.

Before and after induction of enhanced association and interiorization of the parasite, the Vero cells will be modified in order

to stabilize the parasite and to aid its maturation and multiplication. The rationale and methods we plan to use to achieve these objectives were described in the December 15, 1978 request for extension of this contract.

V. DISSEMINATION AND UTILIZATION OF RESEARCH RESULTS

Dr. R. G. Brackett participated in the AID Contractors Meeting in Albuquerque, New Mexico in June, 1978 where he reported on the current status of the project.

Dr. Brackett and Dr. G. C. Cole participated as temporary advisors to the NMRI/USAID/WHO Workshop on the Immunology of Malaria at NMRI, Bethesda, Maryland, October 2 to 5, 1978.

Dr. Brackett gave a poster presentation on the "Growth of Plasmodium falciparum in Human Red Cell Culture for Merozoite Vaccine Production" at a meeting sponsored by the W. Alton Jones Cell Science Center, Tissue Culture Association, Inc., Lake Placid, N.Y. on October 23-26, 1978.

VI. STATEMENT OF EXPENDITURES AND OBLIGATIONS AND CONTRACTOR RESOURCES

A. Budget Statement

A budget statement showing the expenditures for the period January 1, 1978 to December 31, 1978 is presented as a detachable section of this report.

B. Involvement of Minority Personnel and Women

A total of six women were involved in project activities during the reporting period; four 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 TWO AND ONE-HALF MONTHS

The work plan for the coming two and one-half months includes the following targets.

1. To obtain more definitive information on the interrelation of the Vero cell and its associated parasites in the presence of ovomucoid and whether the associated parasites are morphologically normal, by performing electron microscopy on in situ fixed co-cultures prepared in Petri Perm plates. Even if only a small percentage of the many parasites we see associated with the Vero cells are interior to the Vero cell membrane, efforts to manipulate the cell culture to increase maturation and multiplication of the interiorized parasites will be made.
2. To attempt to fuse the two eukaryotic cells, the associated parasite and its host Vero cell. Virus fusion with Sendai and chemical fusion with lecithin or polyethylene glycol will be attempted.

3. To induce endocytosis of the Vero membrane-associated parasites with Concanavilin A and latex beads since each has been shown to induce endocytosis in other cells.

A budget statement showing planned expenditures for each of the major inputs for the period January 1, 1979 to March 15, 1979 is presented as a detachable section of this report.

## APPENDIX

### TEST METHOD FOR THE EVALUATION OF DRUG ON VERO CELL-P.

#### FALCIPARUM CO-CULTURE

Drugs were solubilized in RPMI-1640, sterilized by Millipore filtration, supplemented with 10% (V/V) human type O or AB serum that was homologous with the human erythrocytes used in the co-culture, and used within two hours. The drugs were tested at concentrations that had been found to be minimally toxic or non-toxic to the Vero cells and were not antimalarial, or at concentrations determined by the availability or solubility of the drug.

#### Test Procedure

##### Day 0

1. Vero cell cultures, grown on coverslips in 35 mm plastic tissue culture Petri dishes for one day, were treated with drug in GM-5 medium\* for two to four hours at 37° C in an atmosphere of 5% CO<sub>2</sub> in air.
2. P. falciparum (FCR-3) infected erythrocytes from in vitro culture were inoculated into the Vero cell cultures at a final concentration of 3% hematocrit.

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\*The formula for GM-5 medium is: RPMI 1640\*\* with L-Glutamine

(300 mg/liter)

- + 25 mM HEPES or 5.95 grams per liter
- + 0.2% NaHCO<sub>3</sub> or 2.00 grams per liter
- + 10% Human Serum
- + 50 mcg/ml Gentamicin

\*\*Powdered medium purchased from Grand Island Biological Co.

3. The co-culture was incubated in a candle jar at 37° C.

Days 1 and 2

1. The erythrocytes were aspirated from the co-culture into a centrifuge tube.

2. The Petri dish was inoculated with fresh drug in GM-5 medium to the original volume.

3. A coverslip was removed from the culture for determination of Vero cell-parasite interactions after rinsing, fixing with absolute methanol and staining with May-Grunwald-Giemsa.

4. The culture was then incubated at 37° C in a candle jar atmosphere.

5. The erythrocytes (step 1) were centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and a thin smear, made from the cell pellet, was air dried, fixed with absolute methanol, stained with Giemsa and examined by bright field microscopy to determine the condition of the parasites and erythrocytes as well as the percent parasitemia.

6. The erythrocyte pellet (Step 5) was resuspended in the drugged medium from the Petri dish (Step 2) and inoculated back into the Petri dish.

7. The co-culture was re-incubated in a candle jar atmosphere at 37° C.

8. The stained coverslips (Step 3) were examined by bright field microscopy for evaluation of the Vero cell-parasite inter-relationships and the condition of the associated parasites and Vero cells.

Day 3

Tests were ordinarily terminated on day 3 after determination of erythrocytic parasitemia and Vero cell-P. falciparum interactions as described for days 1 and 2.

Vero cell-P. falciparum interactions and erythrocyte parasitemias in unmedicated cultures were determined at the same times as in the drug-treated cultures. These controls were treated with GM-5 only.