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JANUARY 1, 1979 TO JUNE 30, 1979

SEMI-ANNUAL

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

SEMI-ANNUAL REPORT SUMMARY SHEET

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

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Contract Period: January 1, 1976 to June 30, 1979

Reporting Period: January 1, 1979 to June 30, 1979

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

Total Expenditures and Obligations through previous contract year: $638,343

Total Expenditures and Obligations for current six months: $30,176

SUMMARY OF ACCOMPLISHMENTS AND UTILIZATION

Scanning and transmission electron microscopy of Vero cell-associated parasites obtained from ovomucoid treated co-cultures indicated that the associated parasites were normal appearing merozoites, free of erythrocytes and intimately associated with the outside of the Vero cell membrane.

Treatment of the Vero cell-*P. falciparum* co-culture with erythrocyte extract did not stabilize or induce maturation of Vero cell associated parasites.

Centrifugation of Vero cell-*P. falciparum* co-cultures resulted in a marked increase in the number of merozoites that were associated with the tissue culture cells. This procedure did not result in detectable interiorization of the parasites or their maturation, as determined by bright field microscopy of stained specimens.

Vero cells did not undergo chemical- or virus- induced fusion nor did they interiorize latex particles. Therefore, these approaches for facilitating interiorization of Vero cell- associated merozoites do not appear to be feasible. Because of this, we investigated ovomucoid-enhanced association of other cell types with *P. falciparum*. Ovomucoid did not abet association of Cynomolgus monkey kidney cells with *P. falciparum*, but did facilitate association of merozoites with Rhesus monkey embryonic diploid cells that was almost as efficient as the association seen with Vero cells and, to a lesser extent, with African Green monkey embryonic diploid cells. A remarkable association of infected erythrocytes with a human umbilical vein endothelial cell line was observed. The parasites were late trophozoites and schizonts and about 40% of the endothelial cells were associated with infected erythrocytes. Bright field microscopy revealed that some of these infected red blood cells were interiorized by the tissue culture cells. This association was not ovomucoid dependent.

We plan to take advantage of the enhanced association of merozoites with tissue culture cells caused by centrifugation of co-cultures and the efficient association of parasites with Rhesus monkey diploid cells and human endothelial cells to attempt to enhance interiorization, maturation and growth of *P. falciparum* in these tissue cell cultures.

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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 increase.

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 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-*P. falciparum* co-cultures we attempted to improve the association of the parasite with Vero cells and the maturation and multiplication of the cell-associated parasites by treatment of the Vero cell-*P. falciparum* co-culture with protease inhibitors, protein synthesis inhibitors and steroids. These treatments did not enhance maturation or multiplication of the cell-associated parasite, but a protein protease inhibitor, ovomucoid, markedly enhanced the number of Vero cells associated with the merozoite or young ring states and increased the number of parasites per Vero cell. The degree of association induced by ovomucoid was dose related. The association of parasites with the Vero cells was tenuous in that a significant decline in associated parasites occurred within two hours after the infected erythrocytes were washed out of the co-culture. Interference, phase and transmission electron microscopy indicated that the parasites were predominantly located on the outside of the Vero cell membrane.

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, 1979 to June 30, 1979

A. Evaluation of the Ovomucoid-Enhanced Vero Cell-*P. falciparum* Association by Scanning and Transmission Electron Microscopy

We are indebted to Dr. Thomas Seed of Argonne National Laboratories for performing the sectioning and evaluation by electron microscopy of Vero tissue culture cells and their associated *P. falciparum*.

Co-cultures of Vero cells and *P. falciparum* were prepared in Petri culture dishes that have a gas-permeable membrane as the culture surface (Petriperm®, W.C. Heraeus, Hanau, West Germany), as described in our 1978 Annual Report. The scanning electron microscopy was performed on the Vero cells and their associated parasites directly on the Petriperm® membrane after fixation *in situ* with glutaraldehyde.

The transmission electron microscopy was performed on suspended tissue culture cells and their associated parasites that were fixed on the Petriperm® membrane and then scraped into suspension and on suspended cells and associated parasites that were scraped off the membrane and then fixed. Flow diagrams of the methods used to produce the transmission electron microscope preparations and their appearance as determined by light microscopy, are presented in Appendix A.

A representative scanning electron photomicrograph of the merozoites on the Vero cell membrane is presented in Figure 1. This view correlates closely with what we observe by bright field microscopy of stained preparations (see the photomicrograph, Figure 1, June 30, 1978 Six-Month Report),supporting our original interpretation.

Definitive information on the Vero cell-associated parasites was obtained by transmission electron microscopy, examplified in the photomicrograph (Figure 2) of a representative associated merozoite with its apical end indenting the Vero cell membrane. This apposition of the merozoite to the cell is very similar in appearance to that depicted by Miller *et al* for *P. knowlesi* at the time of infection of red blood cells.

Miller, L.H. *et al*. 1979 Interaction Between Cytochalasin B-Treated Malarial Parasites and Erythrocytes. Attachment and Junction Formation. J. Exptl. Med. 149: 172-184.

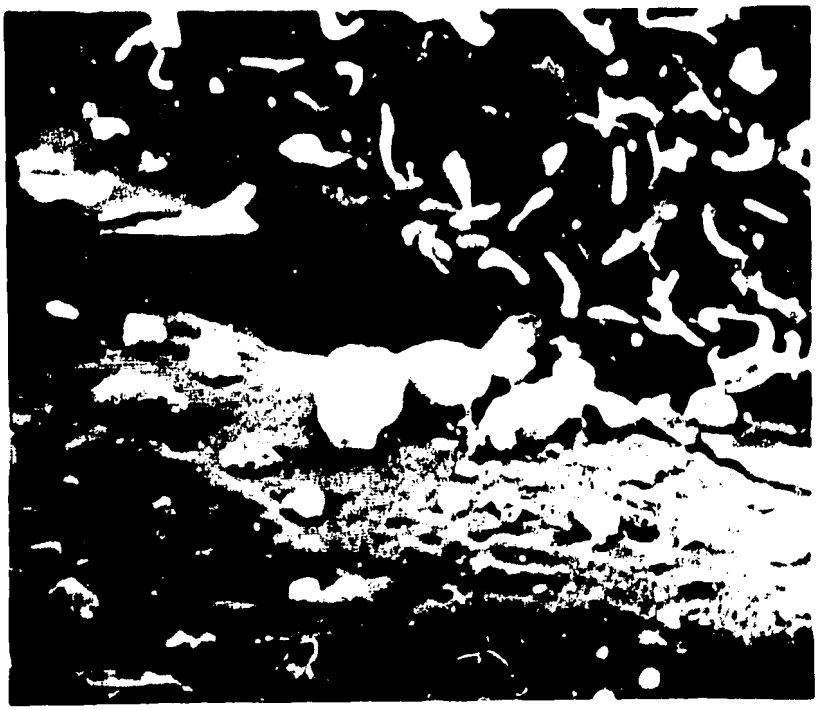

Figure 1. Scanning electron photomicrograph of a Vero cell and associated *P. falciparum* merozoites.

Figure 2. Transmission electron photomicrograph of the edge of a Vero cell and an associated *P. falciparum* merozoite.

The merozoites were normal in appearance and Dr. Seed reported that he did not see any parasites that were interior to the Vero cell membrane.

He did not see any Vero cell-associated merozoites in the preparations that were fixed in glutraldehyde and then scraped off the membrane; the associated parasites were found only in the preparations in which the cells were first scraped off the Petriperm® membrane and then fixed. The control preparations (normal red blood cells in the co-culture) revealed only red blood cells associated with the Vero cells; no evidence was obtained that either normal or infected red blood cells were interiorized by the Vero cells.

These electron microscopic results indicate that the Vero cell-associated parasites that we see by light microscopy in stained coverslips preparations are intimately associated with the Vero cells in a manner similar to that observed between susceptible red blood cells and plasmodia. Furthermore, the associated parasites are morphologically normal and, therefore, presumably infective; these parasites are in fact merozoites; and the merozoites are unable to penetrate through the Vero cell membrane either actively or passively, although the first stage of attachment and penetration may be occurring.

These results validate our past efforts and justify continued efforts to stabilize and enhance maturation of the associated parasites and to facilitate their interiorization. The proposition that Vero cells may not be the ideal tissue culture cells for these efforts will be developed later in this report.

B. The Effect of Red Blood Cell Extract on P. falciparum-Vero Cell Association

In the December 31, 1978 AID/ta/C-1258 Annual Report we presented the results of several different approaches designed to enhance Vero cell parasite association, stabilize the Vero cell associated parasites, enhance their maturation and encourage their interiorization. The single significantly positive result from these efforts was to markedly increase the number of parasites associated with each Vero cell and increase the percentage of Vero cells that became associated with parasites by treating the Vero cell-*P. falciparum* co-culture with the protein protease inhibitor, ovomucoid.

We also reported that the association of *P. falciparum* merozoites with Vero cells was transitory, in that within two hours after infected erythrocytes were washed out of ovomucoid treated co-cultures in which association of parasite with tissue culture cells had occurred, a significantly diminished percentage of Vero cells was associated with parasites. The number of parasites per Vero cell were also diminished and the remaining Vero cell-associated parasites were morphologically more degenerate.

We attempted to stabilize the parasites that were associated with Vero cells in ovomucoid treated co-cultures and enhance their maturation by treating the co-culture with red cell extract that was made according to Siddiqui*. The method was that described in Appendix B.

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*Siddiqui, W.A. et al Effects of Red Cell Extract on *in vitro* Growth and Multiplication of Malarial Parasites. The J. of Parasitology 61(2): 189-194 (1975)

TABLE 1 - THE EFFECT OF RED BLOOD CELL EXTRACT(RCE) ON *P. FALCIPARUM* ASSOCIATION AND STABILITY WITH VERO CELLS IN CO-CULTURES.

Co-culture (1) Treatments			Percent Vero cells associated with parasites on day/time:					
OM	RCE	RBC removed on day 2 (1320)	1	2			3	
			0815	0815	1300	1500	0830	1330
+	+	+	12	85 (2)	86	75	<0.3-<1 (2)	<1
+	+	-	NT (3)	NT	70	30-76 (2)	39-76 (2)	25
+	-	+	28	83	81-88 (2)	55	<1	<1
+	-	-	NT	NT	91-86 (2)	9	62 (2)	33

(1) % parasitemia of erythrocyte cultures were:

day 0 = 6.9
day 1 = 6.8 to 11.8
day 2 = 13.1 to 17.5
day 3 = 21.1 to 25.4

(2) Duplicate coverslips from different Petri dishes were evaluated.

(3) NT = Not Tested

The red cell extract was incorporated into the GM-5 medium at 0.1 ml per 2 ml per 35mm Petri dish and ovomucoid was incorporated into the medium at a concentration of 3.0 mg/ml. On days 1 and 2 the alternate method for preparating blood smears from the co-cultures was used. On day 2, after a high degree of association of parasites with cells had occurred, the infected erythrocytes were removed from the Petri dishes, the dishes were thoroughly rinsed with GM-5, the erythrocytes were pelleted, resuspended in fresh medium and, as indicated in Table 1, returned to some of the Petri dishes.

Evaluation of the coverslips subsequently removed from Petri dishes revealed that the thorough rinsing had reduced the percentage of Vero cells associated with parasites in the co-cultures that were not treated with red cell extract. This evidence that red cell extract helped stabilize the parasite's association with Vero cells appeared to be a temporary phenomenon, since by the next day evaluation of coverslips from those co-cultures which did not have the erythrocytic cultures returned to them on day 2 revealed no parasite association with the Vero cells irrespective of the presence or absence of red cell extract in the medium.

The data presented in Table 1 indicate the red cell extract did not stabilize Vero cell associated parasites for nineteen hours. Also, maturation of the associated parasites did not occur in the presence or absence of red cell extract during the three days of the test.

It was also observed from the evaluation of the erythrocyte smears that the red blood cell extract modified two other aspects of the co-culture: although the parasitemia was not affected, the parasites on day 2 and 3 were in poorer condition when the extract was present; secondly, the extract precipitated in the medium, forming what looked like mini-red blood cells about one-tenth the size of erythrocytes and fibrillar debris.

Since there was no apparent advantage in incorporating red cell extract in our culture medium, this approach will be abandoned.

C. The Effect of Centrifugation of the Co-culture on P. falciparum -Vero cell association

During an on-site visit, Dr. R. Beaudoin suggested that we test the effect of centrifugation on the interiorization and maturation of Vero cell-associated *P-falciparum* merozoites. This suggestion was made because of his remarkable success in tissue culture propagation of *P. berghei* sporozoites that was achieved by centrifuging co-cultures of the sporozoites and tissue culture cells.

Ovomucoid treated co-cultures of P. falciparum (FCR-3) and Vero cells were set up as described in Appendix B. On day 1, when the erythrocyte parasitemia was 12.4% to 15.8%, one Petri dish co-culture was centrifuged at 1500 RPM (450 x g) for two hours and another for 16.5 hours. Centrifugation was performed in air at 37°C and the medium was changed from GM-5 to GM-5 modified by deletion of the $NaHCO_3$ and pH adjustment to 7.2-7.4 with NaOH. After centrifugation, the medium was changed to GM-5. Co-cultures that were not centrifuged (control cultures) received the modified medium and were incubated at 37°C in air during the c trifugation period.

Evaluation of coverslips removed from the Petri dishes before centrifugation showed that 26% of the Vero cells were associated with merozoites, mostly 1 to 3 parasites per cell. After the two-hour centrifugation the merozoite association had increased so that 75% of the Vero cells contained associated parasites and some of these cells were associated with a hundred or more merozoites. Many of the Vero cell-associated merozoites appeared to be in good condition. On the next day many of the associated parasites had disappeared from the Vero cells centrifuged for two hours (5% Vero cells associated with P. falciparum) but some of the Vero cells were associated with more than the expected number of parasites, compared to the uncentrifuged Vero cell-P. falciparum control which contained 37% associated Vero cells, with 1 to about 10 normal merozoites per cell.

After the 16.5 hour centrifugation only about 1% of the Vero cells were associated with merozoites. The coverslip from the control Petri dish (incubated in air at 37°C during the 16.5 hours) also showed minimal (1%) Vero cell-*P. falciparum* association. The erythrocyte parasitemia was also reduced in these Petri dishes, especially in the centrifuged Petri dish, and the parasites were in fair to poor condition, as compared to parasites in good to excellent condition in the erythrocyte cultures exposed to air and modified GM-5 medium for only two hours.

In none of these preparations was there any maturation of associated parasites; nor was there evidence for interiorization as determined by bright field microscopy of stained coverslips.

Since centrifugation of co-cultures of Vero and *P. falciparum* for two hours resulted in "instant" association of enormous numbers of normal merozoites with the Vero cells, the technique will prove especially useful during experiments in which culture conditions are modified so as to enhance interiorization of associated parasites.

D. Attempts to Induce Fusion and Interiorization of Latex Beads by Vero cells.

Because all data indicates Vero cell associated *P. falciparum* merozoites are located on and external to the Vero cell membrane

and under the culture conditions we have tested so far these external merozoites are not maturating, we believe interiorization of the merozoites into the Vero cells may abet maturation.

Our approach to achieve this has been to study the effects on the Vero cell of a variety of agents which are known to pertubate the integrity of tissue culture cell membranes.

Attempts were made to induce cell to cell fusion with two molecular weights of polyethylene glycol, alone, and in combination with Triton X-100 and dimethylsulfoxide in monolayers of Vero cells. Polyethylene glycol induced fusion was also attempted on Vero cells in suspension. Fusion did not occur between Vero cells during nine experiments even though polyethylene glycol repeatedly induced cell to cell fusion of a mouse skin fibroblast cell line, L cells.

Attempts to induce fusion between Vero cells by Sendai and Newcastle Disease viruses were unsuccessful.

It is reported that during interiorization of latex particles by tissue culture cells that part of the cell membrane is endocytosed with the latex beads. If Vero cells could be shown to take up these particles it was thought that merozoites attached to the membrane may also be interiorized with the latex particles. However, in one attempt, Vero did not ingest 0.81μ polystyrene latex beads and, therefore, further evaluation of this approach was not made.

E. The Effect of Co-culturing *P. falciparum* with Tissue Culture Cells other than Vero.

Because the electron microscopy of Vero cells and associated merozoites indicated the parasites were located on the outside of the cell membrane and because fusion and ingestion of latex particles by Vero cells as methods for interiorizing the associated merozoites into the Vero cells appeared to be unpromising approaches for interiorizing associated parasites, we tested other cell types which we had previously shown to be associated with *P. falciparum* after co-culturing. Although these other cell types had shown inefficient association with parasites, this was also true of Vero before we started using ovomucoid to enhance the association. Therefore, the other cell types were re-tested in co-culture with and without ovomucoid.

1. Co-culture with primary Cynomolgus monkey kidney cells

The first of these cell types was primary frozen Cynomolgus monkey kidney cells. The test method was that described in Appendix B; the co-culture was treated with ovomucoid at 3 mg/ml.

As previously observed, and the reason why more extensive work had not previously been done with primary Cynomolgus monkey cells, the GM-5 medium was toxic to these cells, destroying all but a few cells that then proliferated. Interestingly, ovomucoid protected the cells from this toxicity.

In the Cynomolgus co-cultures that were treated with ovomucoid P. falciparum associated with one percent of the tissue culture cells. We previously presented pictures of this association obtained from the original experiments with P. falciparum and Cynomolgus monkey kidney co-cultures in the June 30, 1977 Six-Month AID/ta/C-1258 Report. The association was very similar to that observed using Vero cell-P. falciparum co-cultures (as depicted in the June 30, 1978 AID/ta/C-1258 Six-Month Report) but was much less extensive.

2. Co-culture with embryonic diploid monkey cells

Since the above results did not indicate that the primary Cynomolgus monkey kidney cell was an attractive candidate cell for further studies, other cells derived from monkeys were tested in co-culture.

Vero is an African Green monkey cell line derived from the kidney of a normal adult. In recent years diploid fibroblast lung cell cultures have been derived from Rhesus monkey and African Green monkey embryos and, reasoning somewhat teleologically, we tested these cells in co-culture with P. falciparum in the presence and absence of ovomucoid. Ovomucoid also protected these cells from the toxicity of GM-5 medium. More important, fair to good association of P. falciparum merozoites with both of these cell types has been repeatedly observed. These data are summarized in Table 2. The erythrocytic parasitemia in these cultures

TABLE 2 - THE EFFECT OF CO-CULTURING *P. FALCIPARUM* AND MONKEY EMBRYONIC DIPLOID TISSUE CULTURE CELLS

Cell Line	Expt #	Day	% monkey cells associated with parasites	
			c̄OM	c̄/o OM
F-Rh-L-2 (Rhesus Lung, Diploid, Embryonic)	1	1	<1	<1
		2	75	13
		3	<1	Toxic
	2	1	5-7*	NT**
		2	67-80*	NT
		3	42	NT
F-C-L-1 (African Green Lung, Diploid, Embryonic)	1	1	<1	Toxic
		2	8	Toxic
		3	Toxic	Toxic
	2	1	<1	NT
		2	27	NT
		3	Toxic	NT
Vero (African Green Kidney, Adult)	1	1	5	<1
		2	83	75
		3	ND	ND
	2	1	19	32
		2	82	75
		3	ND	ND

* Coverslips from duplicate Petri dishes were evaluated.

** NT: Not tested

*** ND: No datum because tissue culture cells overgrew and could not be evaluated.

ranged from 8.4% to 22.0% on days 1 and 2 of these experiments and, after being split on day 2, it ranged from 4.0 to 11.1 on day 3.

The African Green monkey diploid cells, F-C-L-1, demonstrated only fair association with the P. falciparum merozoites and, therefore, we do not plan to use them in future experiments.

Because the Rhesus monkey diploid cells, F-Rh-L-2, demonstrated association with merozoites that was almost equal to that of Vero cells, we plan to use the Rhesus monkey cells in future experiments designed to increase the associated parasite's maturation, interiorization and growth in tissue culture.

3. Co-culture with human umbilical vein endothelial cells.

We previously reported (December 31, 1977 Annual Report AID/ta/C-1258) that human umbilical vein endothelial tissue culture cells associated with P. falciparum in co-culture. Since then we have had significant difficulty culturing these cells and only recently have appropriate cell growth enhancing factors become available which make routine culturing of these cells feasible. We are indebted to Dr. T. Maciag of Collaborative Research Inc., Waltham, MA, for the cell line we are currently using.

The endothelial cell cultures were co-cultured with P. falciparum (FCR-3) in the presence and absence of ovomucoid, as described in Appendix B.

Erythrocytic parasitemia was about 8% on day 1, 13% to 14% on day 2 and, after a 1:3 split on day 2, was about 5% on day 3.

A remarkable association of infected red blood cells with the endothelial cells was observed on days one and two after the co-cultures were set up. The association occurred equally in the presence or absence of ovomucoid. One or more red blood cells were associated with about 75% of the endothelial cells and 40% to 50% of these red blood cells were infected with P. falciparum. The parasites were predominantly late trophozoites and schizonts; ring stages were not observed in the associated red blood cells. In some instances the infected red blood cells appear to be in vacuoles within the cells and degenerating red blood cells and their intracellular parasites were observed. Merozoites, free of red blood cells, were infrequently associated with endothelial cells.

The endothelial cell from the umbilical venule lining is an unusual nucleated cell; it is reported to contain blood clotting Factor VIII antigens and its surface contains the red blood cell group antigens of the donor. From our results it appears that the surface of red blood cells that are infected with late P. falciparum stages is modified so it becomes preferentially associated with endothelial cells in culture. A few of these associated red blood cells appear to become interiorized by the endothelial cells. It will be of great interest to see if the associated parasites mature and multiply, either in their red blood cells or in interiorized endothelial cell vacuoles after the endogenous infected red blood cells have been removed from the co-culture.

F. Research Design

During this reporting period we used the Research Design described in the AID/ta/C-1258 December 31, 1979 annual report and in the December 15, 1978 request for extension of the contract in order to study some critical aspects of the ovomucoid induced enhancement of P. falciparum-Vero cell association.

The results of these experiments indicated that the associated parasites, the merozoite, were morphologically normal by electron microscopy and, therefore, presumably are infective. The parasites were external to the Vero cell membrane and in intimate contact with it, even indenting the membrane in a manner similar to that observed when the merozoites infect erythrocytes. Parasites were not seen in the interior of the cell. Therefore, the reseaarch design will in part continue to be devoted to efforts to interiorize the parasite.

When we found that fusion by chemical and virological methods could not be induced in Vero cells, enhanced induction of parasite associateion with other cell types was investigated.

Ovomucoid treated fetal Rhesus monkey diploid cells were shown to efficiently associate with P. falciparum merozoites in a manner that appears to be similar to that observed with Vero cells. We therefore plan to substitute the Rhesus monkey diploid cells for Vero cells in the original research design with the hope that these cells can be modified so as to interiorize the associated parasites, which can then be induced to maturate and multiply.

Because centrifugation of the Vero cell-P. falciparum co-culture resulted in a marked increase in the number of merozoites associated with Vero cells, this technique will be incorporated into the research design. Using centrifugation we will induce parasite-tissue culture cell association "on cue" immediately before and after drug- or virus-induced modification of the tissue culture cells rather than waiting for spontaneous association to occur, as we have done in previous studies.

A fourth major discovery that has occurred in the project in the last six months, which requires a quite different modification of the research design is the remarkable observation that erythrocytes infected with late stages, but not early trophozoites, of P. falciparum are preferentially associated with human umbilical vein endothelial cells. This appears to occur primarily at the cell surface, but there is some evidence these infected erythrocytes are also interiorized by the endothelial cell. We plan to use this cell in experiments designed to modify culture conditions so as to enhance maturation and growth of the parasite.

Another, somewhat minor, change in the research design involves hardware. In order to conserve on the number of tissue culture cells and parasites used in these experiments, we will perform some of them in Tek Lab microscope culture slides, which have been reintroduced by the manufacturer after modification to correct leakage problems. These were used by us for attempted

propagation of P. berghei in tissue cultures and, therefore, are not a new technology to us. They should facilitate our procedures since they obviate the need for flying coverslips in Petri dishes.

Although never a part of our Research Design, pertinent information on the anatomic location of parasites in/on tissue culture cells and the parasites condition obtained by electron microscopy of thin sections has proved to be of great value to us. We do not have thin-sectioning technology or a competent malaria electron microscopist to perform the evaluation of these specimens. We therefore would like to suggest that a sub-contract for such technology by a competent individual be let at this time. During a twelve-month period we estimate we would make use of this technology about four times.

V. DISSEMINATION AND UTILIZATION OF RESEARCH RESULTS

The program was reviewed in-depth with Mr. E. Smith and Dr. R.P. Beaudoin when they visited us on March 8-9, 1979. Drs. J. Erickson and P. Contacos also paid us an on-site visit June 20, 1979, at which time we reviewed the project with them and presented much of the data presented in this report. On June 19 Messrs. C. Miller and A. Ryder met with Drs. Erickson and Contacos to inform them of the techniques we use at Warner-Lambert to apply Critical Path Planning to Research and Development and how it may be adapted to fit AID research programs. They plan to continue to work with AID, as requested, to develop a suitable Critical Path Plan.

VI. STATEMENT OF EXPENDITURES AND OBLIGATIONS AND CONTRACTOR RESOURCES

A. Budget Statement

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

B. Involvement of Minority Personnel and Women

A total of four women were involved in project activities during the reporting period; three as professional laboratory investigators and one as a secretary. One black professional laboratory investigator was 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 determine if interiorization and maturation of *P. falciparum* merozoites can be induced in Rhesus monkey diploid tissue culture cells by modification of the culture conditions, e.g. by fusion of the parasites and tissue culture cells; by perturbation of the cell membrane and induction of endocytosis of parasites on the cell membrane by treating the cultures with Concanavallin A or latex particles.
2. To determine if the parasites in erythrocytes that are associated with human umbilical vein endothelial cells will mature and multiply after association occurs.

3. To attempt to stabilize and induce maturation of parasites associated with Rhesus monkey diploid cells and human endothelial cells by modifying the culture conditions.

A budget statement showing planned expenditures for each of the major inputs for the period March 16, 1979 to September 15, 1979 is presented as a detachable section of this report.

APPENDIX-A

METHOD OF PREPARATION AND BRIGHT FIELD MICROSCOPICAL RESULTS ON VERO CELL-ASSOCIATED *P. FALCIPARUM* SPECIMENS FOR TRANSMISSION ELECTRON MICROSCOPY

PROCESS I. A suspension of Vero cells with associated *P. falciparum* (P.f.) = Cells-P.F. fixed *in situ* and then harvested.

Vero cell monolayer in Petriperm dishes (PP) treated with ovomucoid and inoculated with *P. falciparum* (P.f.) infected red blood cells (RBC) and incubated for three days.

↓

1. RBC rinsed off
2. Vero cells-P.f. on PP membrane are fixed for one hr. *in situ*
3. PP membrane rinsed and washed for one hour in PBS-sucrose at room temperature

↓

4. The Vero cells-P.f. were gently scraped from the membrane into PBS-sucrose.

Specimen A: A piece of the membrane stained with MGG.* Staining is blue but parasites are easily discernible. 41% of the Vero cells are associated with P.f. which are free of RBC. Several P.f. are associated with each Vero cell.

↓

Specimen B: The cell suspension in 2.5 ml volume contains 360,000 Vero cells, many in small cell sheets.

↓

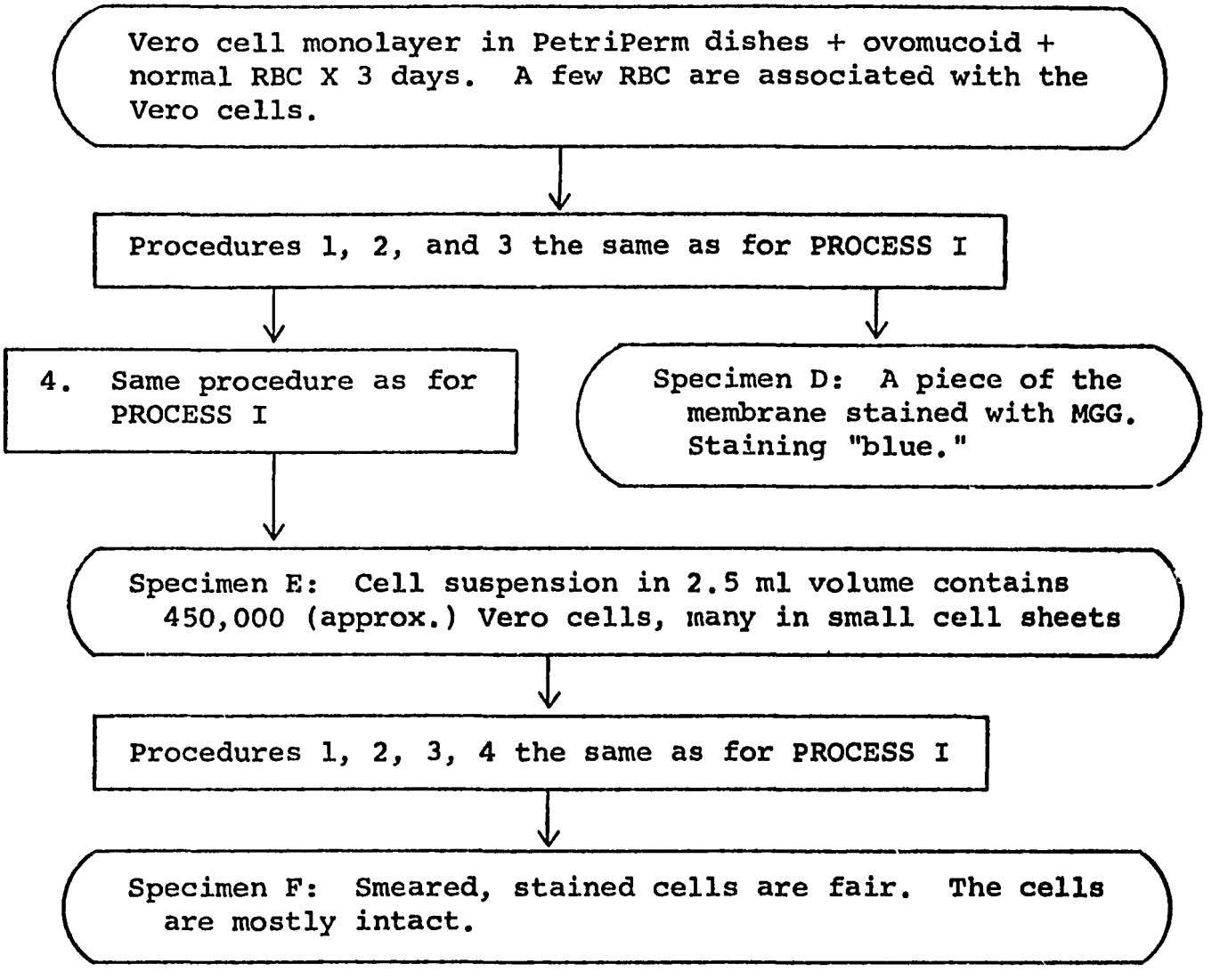
1. A "thick drop" smear was made from pelleted cells from a small aliquot of the cell suspension
2. Air dry
3. Fix with methanol
4. Stain with MGG*

↓

Specimen C: Smeared, stained cells are fair. The cells are mostly intact. Parasites are easily discernible but differentiation is poor.

* MGG: May Grunwald Giemsa

PROCESS II. Cell Control for Specimens A, B, and C. A suspension of Vero cells without associated P. falciparum. Cells fixed in situ and then harvested.



PROCESS III. A suspension of Vero cells with associated P. falciparum (P.f.). Cells = P.f. harvested from PetriPerm membrane and then fixed.

Vero cell monolayer in PetriPerm dishes (PP) treated with ovomucoid and inoculated with P. falciparum infected red blood cells (RBC) and incubated for three days.

↓

1. RBC rinsed off
2. Vero cells-P.f. on PP membrane were scraped off membrane into PBS
3. An aliquot of this cell suspension in PBS was pelleted, smeared (thick drop) on a slide, fixed with MeOH and stained with MGG

↓

4. The remainder of the cell suspension in PBS was added dropwise to cold 1.25% glutaraldehyde in PBS-sucrose. Fixation for one hour. Pelleted. Resuspended in PBS-sucrose for one hour. Stored at 4°C.

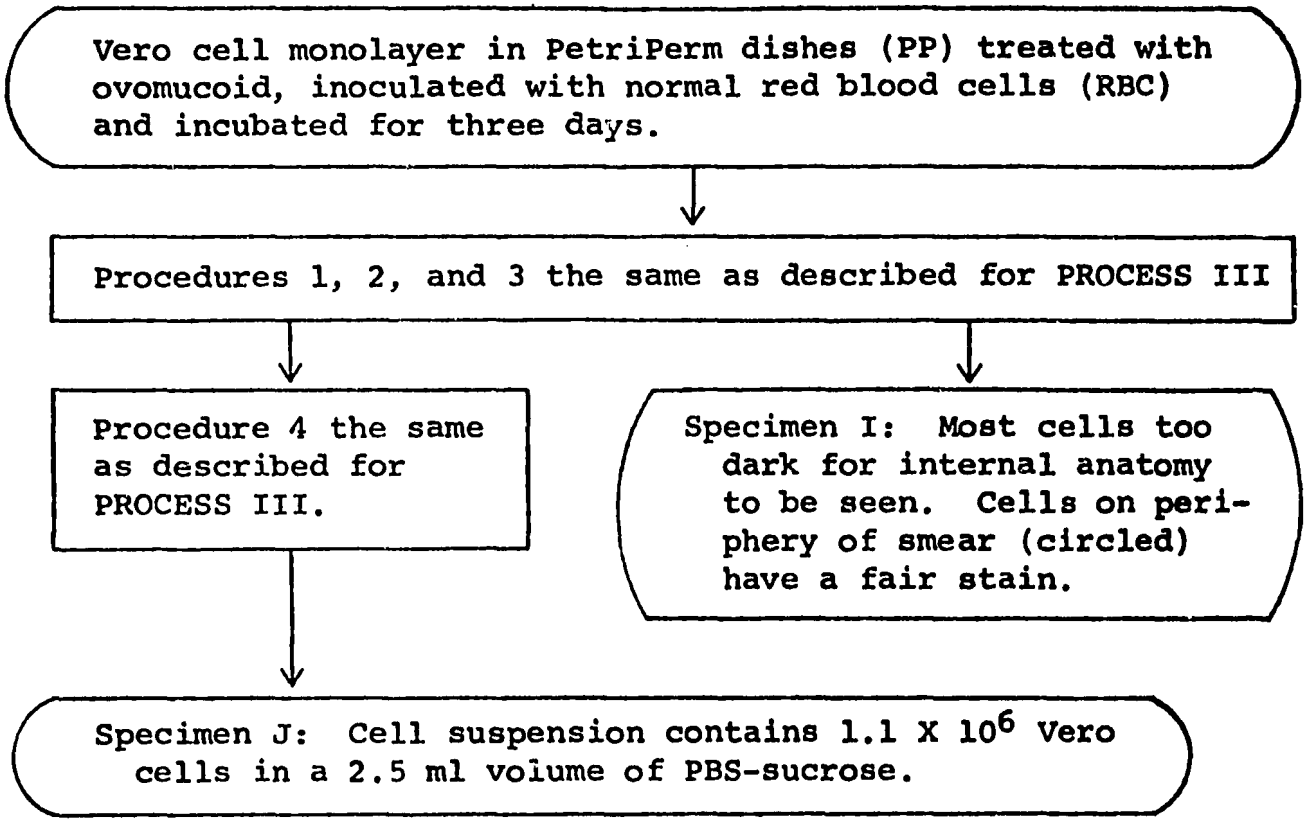
↓

Specimen G: Most cells too dark for internal anatomy and parasites to be seen. Cells on periphery of smear (circled) have a fair stain. Parasites can not be seen. Cell viability = 83% (trypan blue).

↓

Specimen H: Cell suspension contains 990,000 Vero cells in a 2.5 ml volume of PBS-sucrose.

PROCESS IV. Cell Control for Specimens G and H. A suspension of Vero cells <u>without</u> associated <u>P. falciparum</u> (P.f.). Cells harvested and then fixed.



APPENDIX-B

TEST METHOD FOR THE EVALUATION OF DRUG ON VERO CELL-P. FALCIPARUM CO-CULTURE

A drug was 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 inoculated into the co-culture within two hours. A drug was used 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_2 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% $NaHCO_3$ 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 resuspended and aspirated from the co-culture into a centrifuge tube.
   Alternately, the erythrocytes were left in situ and only the medium was removed by aspiration (Step 5 was not done). A thin smear was made directly from the erythrocytes on the bottom of the Petri dish.
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 was made from the cell pellet.
6. The erythrocyte smear 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.
7. The erythrocyte pellet (Step 5) was resuspended in the drugged medium from the Petri dish (Step 2) and inoculated back into the Petri dish.

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8. The co-cultrue was re-incubated in a candle jar atmosphere at 37°C.
9. 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.