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In vitro Cultivation of Sporogonic Stages of Human Malaria

Annual Progress Report

(Covering Contract Period Jan. 15, 1978 - Jan. 14, 1980)

ANNUAL REPORT SUMMARY SHEET

In Vitro Cultivation of Sporogonic Stages of Human Malaria (AID/ta-C-1486)

Project Title and Contract Number

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Contractor's Address

1/15/78 - 1/14/81 Contract Period (as amended) From - To	1/15/78 - 1/14/80 Reporting Period From - To
Total Expenditures and Obligations Through Previous Contract Year	$190,068
Total Expenditures and Obligations For Current Contract Year	$ 95,820

Narrative Summary of Accomplishments and Utilization:

The chief aim of this project is to develop methods for *in vitro* cultivation of gametocytes of human malaria, and subsequent infection of mosquitoes with these gametocytes. A series of different culture approaches have been tried in an attempt to obtain complete maturation of *Plasmodium falciparum* gametocytes *in vitro*. These include testing of new media, new gassing procedures, better maintenance of culture homeostasis, and testing different strains of the parasite. None of these procedures has yet succeeded in permitting better gametocyte maturation. Success has been reported in two areas. (1) Attempts were made to find an inexpensive, readily available substitute for the human serum requirement for the continuous culture of parasites. We found that commercially available peptones added together with calf serum gave parasite growth rates comparable to or surpassing those obtained with human serum. (2) A method to establish synchronous development of the erythrocytic stages of the parasite has been described. This involves the selective lysis of later stages of the culture by suspension of parasitized erythrocytes in 5% aqueous sorbitol solution.

B. Project Objectives

The chief aim of this project, as stated in our contract, is "to develop methods for *in vitro* cultivation of gametocytes of human malaria, and subsequent infection of mosquitoes with these gametocytes."

C. Accomplishments to Date

a) Testing of different media

Attempts were made to obtain gametocyte maturation with the use of Medium 199 (as a total replacement for RPMI-1640, and by mixing the two media in various ratios). No significant effects on gametocyte maturation were observed. (For this and all following studies, the parameters that we used to assay maturation were: ability of microgametocytes to exflagellate, ability of macrogametocytes to emerge from host erythrocytes, and infectivity of gametocytes to mosquitoes fed through a membrane).

b) Additives to medium

A number of different materials (in various concentrations) have been added to our culture medium in attempts to enhance support of gametocyte maturation. These have included peptones (Biosate, Gelysate, Trypticase, Phytone, Polypeptone, Neopeptone, and PRoteose-Peptone #3), red blood cell extract, higher concentrations of human serum (20% - 50%) pyruvate (at concentrations from 0.025 - 0.2%), adenine (at concentrations from 1 - 100 μM), and a variety of hormones, including cortisone, progesterone, thyroxine, testosterone, and prostaglandin E. The hormones was all chosen because of their known abilities to interact with the erythrocyte membrane. They were added to the culture medium at concentrations ranging from 100 nano M to

100 μM, except for prostaglandin E which was added at 100 nanograms to 100 μgrams/100 ml. Thus far, no significant effects on gametocyte maturation have been observed.

c) Gassing changes

Gametocytes in culture have been subjected to a number of different gas atmospheres, including the normal candle-jar atmosphere, ambient air (in T-culture flasks), and a series of different ratios of O_2/CO_2 as described by Scheibel et al. (1979) that produce optimum parasite growth. No significant effects on gametocyte maturation have been observed.

d) Use of freshly collected erythrocytes and sera

Several studies were done in which gametocyte cultures were initiated with human serum and erythrocytes within a day of collection. Results thus obtained were no more satisfactory than when stored serum and erythrocytes were used.

e) Better maintenance of pH and culture homeostasis

(1) Gametocyte purification procedures

Attempts were made to separate developing gametocytes from asexual parasites in order to reduce "competition" for nutrients, and excessive build-up of toxic waste products. Separating techniques tested included: a Sepharose column (Kass et al.), centrifugation on a discontinuous gradient (Lymphoprep), centrifugation on a continuous gradient (Renografin - BSA), centrifugation in physiogel or gelatin-saline solutions, and preliminary studies to determine whether the plasma membrane of gametocyte-infected erythrocytes had unusual lectin-binding characteristics. None of these attempts resulted in a satisfactory procedure for concentration of gametocytes.

(2) Control of asexual parasites with antimalarial drugs

Chloroquine and quinine were tested at final dilution levels of 0.25 - 2.0 nano M. Drugs were added on day 7 of gametocyte culture.

(When added at this time, they do not have an effect on already differentiated gametocytes.) The results showed effective control of asexual parasites, with most of them vacuolated and deteriorated. Much of the debris from dead parasites could be removed by washing. Gametocytes continued to develop normally in the presence of these drugs. However, they did not reach full maturity, as indicated by any of the previously cited parameters.

(3) Reduction of hematocrit

Attempts to obtain better culture homeostasis by increasing the ratio of medium to cells were made. No significant effects on gametocyte maturation were noted.

f) Co-cultivation of gametocytes with fibroblasts

This project has not yet been initiated. In view of recent advances in culture of a number of different human cell lines, we are planning to begin work in this area shortly.

g) Testing various strains of *P. falciparum*

A number of different established isolates have been compared with one another for their ability to consistently produce gametocytes. These include FCR-1, FCR-2, FCR-3, and "Mr. M" (from Rockefeller University), FCN-1 (isolated by us), FCC-2 strain (from China) Z strain (from N.I.H.), and Panamanian, Haitian, and African strains (from C.D.C.). We have found "Mr. M" and FCN-1 to be the most dependable gametocyte producers under our conditions, and virtually all of our work is now restricted to these strains.

h) *P. vivax* in culture

In view of repeated reports of failure in the culture of these parasites by others, we felt that initiation of gametocyte studies was not justified until further basic progress was made.

i) Induction of new gametocytes

In view of the fact that P. falciparum cultures already produce adequate numbers of gametocytes under normal circumstances, we did not consider attempts at purposeful induction to be an area of high research priority. However, our inability to separate gametocytes from asexual parasites (see item e, 1 above) now makes this area more attractive. Inducing all or most of the parasites in a culture to become gametocytes might solve the problem of "competition" from asexual parasite growth. Preliminary studies with cyclic AMP and with phosphodiesterase inhibitors (caffeine and theophylline) have been initiated.

j) Mass production

(1) Parasite growth in tissue culture flasks

Based on information from Dr. W. Trager obtained during his visit to China, we attempted to grow parasites in tissue culture flasks exposed to ambient air. Our studies have confirmed this simple procedure. We have used two sizes of plastic T-flasks: 25 cm^2 (in which we place a total culture volume of 5 ml) and 75 cm^2 (in which we place a total culture volume of 25 ml). Parasites are grown at 10% hematocrit. After the daily changing of medium, the flask cap is closed tight. Our results indicate good parasite growth and excellent gametocyte production under these circumstances (better gametocyte production is obtained at lower hematocrits of about 4%). This is perhaps the simplest procedure yet described for production of large numbers of parasites.

(2) Substitutes for human serum in medium

Attempts were made to find an inexpensive, readily available substitute for the human serum requirement for the continuous culture of

parasites, in view of the fact that the requirement for human serum in the culture medium often poses technological problems. Human serum may be difficult to obtain in large quantities on a regular basis; it may be expensive to purchase; and its efficacy may vary from donor to donor. In a region endemic for malaria further problems may result from a high incidence of antimalarial antibodies among blood donors, and a relatively high incidence of Hepatitis B virus, which could pose hazards for experimenters. A satisfactory and inexpensive substitute for human serum in these cultures would be helpful.

An approach that has been useful in the replacement of serum for culture of some mammalian cells has been the employment of peptones (review in Taylor and Parshad, 1977). Often, cell lines exhibiting a serum dependence may not survive an abrupt transfer to a medium in which the serum has been replaced by peptone. However, it may be possible to gradually adapt the cell line to a serum-free medium by progressive, stepwise reduction of the serum concentration and concomitant addition of peptone over a period of time (Evans et al., 1964). We attempted to utilize this approach in the culture of P. falciparum. We found that we could thereby eliminate the requirement for human serum in the culture medium, but that one of several animal sera were needed, presumably to maintain the integrity of the host red blood cells.

MATERIALS AND METHODS

Studies were conducted with the FCR-3 strain of P. falciparum (Trager and Jensen, 1978) cultured in petri dishes and maintained within candle jars (Jensen and Trager, 1977). We routinely used 60 x 15 mm dishes containing total culture volumes of 5 ml. Red blood cells (type A^+ collected in citrate-phosphate-dextrose solution) were obtained from the New York Blood Center, and were cultured at either a 10% or 12% hematocrit. Control cultures throughout the study were maintained in RPMI 1640 (Gibco) with 10% human serum, prepared as described by Jensen and Trager (1977). To compare the growth of parasites maintained in different media, all cultures were initiated with parasitemias of approximately 0.2%, and cultured for 96 hr with daily changes of the media. Blood films were prepared from each culture at the time of initiation and after 48 and 96 hr of growth, and were stained with Giemsa's stain. Percent parasitemia was expressed as the number of parasites per 100 erythrocytes, and was arrived at by counting 10,000 erythrocytes per slide. Growth rates of the parasites in the various media were compared by comparing their rates of multiplication over the 96 hr experimental period.

Peptones tested as media supplements included: Gelysate, Phytone, Polypeptone, Thiotone, and Trypticase (all from BBL), and Bacto-Peptone, Bacto-Casamino acids, Neopeptone, and Proteose-Peptone No. 3 (all from DIFCO). After testing various concentrations of these peptones, we found that the most satisfactory results were obtained when the last 2 peptones were prepared as 15% (w/v) aqueous solutions, and all other peptones were prepared as 10%

(w/v) solutions. The peptone preparations generally required heating to dissolve the peptones. They were subsequently dispensed in 100 ml bottles, autoclaved for 30 min (at 15 psi, 121C), and stored at 4C. These stock solutions could be stored satisfactorily for at least 3 months under these conditions. The slight precipitate often observed in Neopeptone solutions after autoclaving had no deleterious effect on parasite growth. For some of our preparations we added 1% glucose (w/v) to the peptone solution prior to autoclaving (as suggested by Pumper et al., 1965; Lasfargues et al., 1973).

Other additives tested were Bacto-Yeast Extract (DIFCO), and Polyvinylpyrrolidine (GAF). Sera and serum components tested were human type A+ serum (New York Blood Center), human gamma globulins [fraction II], human alpha globulins [fraction IV], human albumin [fraction V] (all from Miles), fetal bovine, calf, sheep, and horse sera (all from GIBCO), and swine serum (Irvine Scientific). Human serum was obtained from blood collected without anticoagulants, and was stored frozen in our laboratory; non-human sera were obtained and stored frozen. Human serum fractions were obtained in lyophilized form and added to a final concentration of 120 mg/100 ml of complete culture medium.

EXPERIMENTS AND RESULTS

Adaptation of parasites to media with low levels of human serum

In our initial series of studies, we attempted a progressive stepwise reduction in the amount of human serum added to the culture medium, at the same time reciprocally increasing the amount of peptone stock solution added. The peptone stock solutions used were Gelysate, Phytone, Polypeptone, Thiotone, Trypticase, Bacto-Peptone, and Bacto-Casamino acids (each at 10% w/v). The progressive changes in the ratio of human serum/peptone stock solution added

to the culture media were: 7.5% serum/2.5% peptone; 5% serum/5% peptone; 2.5% serum/7.5% peptone; 1% serum/9% peptone; 0.5% serum/9.5% peptone; 0.1% serum/9.9% peptone; 0% serum/10% peptone. Parasites were grown continuously in media containing each of these ratios for approximately 6 weeks before they were introduced to the next stepwise reduction in human serum.

We found that parasite adaptation to levels below 1% human serum was possible only with Gelysate and Bacto-Peptone. In each case there tended to be a progressive reduction in parasite multiplication rate as the percentage of human serum decreased (Table I). By the time that human serum had been entirely eliminated from the culture medium, parasite growth was consistent, but at a level of only 8-16% of normal growth in control media with 10% human serum. Erythrocytes maintained in media with human serum levels of 1% or less had various degrees of hemolysis and hemagglutination. Additives such as yeastolate, polyvinylpyrrolidone, additional glucose, or human blood fractions II, IV, and V did not significantly improve parasite growth rates. Parasites transferred abruptly from normal cultures containing 10% human serum directly into media with these low levels of serum failed to grow. The remaining peptones tested (Phytone, Polypeptone, Thiotone, Trypticase and Bacto-Casamino acids) failed to support parasite growth at various times during attempted adaptation of the parasite, and were consequently discontinued.

Continuous culture of parasites in media without human serum

In view of the poor in vitro survival of erythrocytes in media with low levels of human serum, we attempted to determine whether the addition of commercially available animal sera or human serum fractions could stabilize the erythrocytes and enhance parasite growth in media without human serum. At the

same time, 2 additional peptones, Neopeptone and Proteose-Peptone No. 3, were tested. Parasite lines already pre-adapted to growth in 0.5% human serum plus 9.5% Gelysate or Bacto-Peptone solution were used in these studies.

The results showed that excellent parasite growth could be obtained with Neopeptone and Proteose-Peptone No. 3 in the presence of 10% calf, fetal bovine, or swine serum (Table II). Growth under these conditions was equal to or better than that observed in the 10% human serum controls. Parasite growth began to decrease at levels of about 5% or less of the added animal sera. Continuous cultivation of parasites in these peptones with 10% calf serum and without any human serum has now been carried out for over a year with no signs of diminution of parasite growth.

Though swine serum supported excellent parasite growth (Table II), agglutination of some erythrocytes was noted after about 96 hr in culture. Thus attempts at continuous culture of parasites with this serum were terminated. Parasite growth was relatively unsatisfactory with horse or sheep serum, or with human serum fractions IV and V in lieu of whole serum. Accordingly, these cultures were also abandoned.

Our original adaptation of parasites to growth in media without human serum was done over a 9 month period. We have since found that this adaptation can be accomplished within a 1 month period by gradually reducing the concentration of human serum from 10 to 0% and correspondingly increasing the calf serum concentration from 0 to 10%. The entire transition, and subsequent continuous culture of the adapted parasite line is done in the presence of Neopeptone or Proteose-Peptone No. 3 at the levels indicated (1 ml of 15% peptone stock solution per 100 ml RPMI medium). Our attempts to abruptly transfer non-adapted parasites from a medium containing 10% human serum directly

to the peptone-calf serum medium have met with repeated failure. However, parasites once adapted to growth in media without human serum can be transferred back abruptly to media with 10% human serum.

DISCUSSION

Our ability to culture P. falciparum continuously without any requirement for human serum should be helpful in future research with this parasite. Peptone additives are inexpensive, relatively consistent in their composition, and have an indefinite shelf life. That some continuous parasite growth occurs in the presence of these peptones, and without any serum at all in the medium suggests that RPMI-1640 plus the peptones may contain all the nutrients required by the parasite for development. However, parasite growth can be enhanced considerably by the addition of any of several non-human sera. Blood serum may contain hormones or other components necessary for increased stabilization of the erythrocyte plasma membrane (Valeri, 1975). Conceivably, future progress may permit us to use a serum fraction or a specifically defined serum component in place of the whole serum that is now required for optimal parasite growth. Still, at the present time calf serum remains an easily obtainable, relatively inexpensive additive. It may be used in lyophilized form for more practical storage under adverse tropical conditions.

Jensen (1979) reported that several different species of non-human sera including bovine, horse, sheep and swine were incapable of sustaining parasite growth rates comparable to those obtained with human serum. He also obtained unsatisfactory results with a serum substitute containing Bacto-

peptone as one of its components. However, no attempts were made to gradually adapt the parasite to media containing this serum substitute. More recently, Jensen (personal communication) was able to confirm our own results by gradually adapting parasites to the peptone-calf serum media as described in the present paper.

Several other substitutes for human serum in the continuous culture of P. falciparum have been reported. These include fatty acid-free bovine or human albumin (Siddiqui and Richmond-Crum, 1977), horse serum (Butcher, personal communication), rabbit serum (Rieckmann et al., personal communication), and bovine serum fortified with an extract of red blood cells (Siddiqui, personal communication). The relative usefulness of each of these proposed substitutes remains to be confirmed and assessed in comparative laboratory trials.

ACKNOWLEDGEMENTS

We thank Drs. J. Jensen and W. Trager for training the first author of this paper in culture techniques, and for supplying the FCR-3 strain to us. We also thank Dr. Jensen for repeating and confirming our procedure in his own laboratory. The studies reported here were supported by a contract from the United States Agency for Independent Development.

TABLE I. Adaptation of P. falciparum (FCR-3 strain) to continuous culture in media without human serum by stepwise reduction in human serum and concomitant increases in peptone solutions.

Components added to media* (Amounts added per 100 ml RPMI medium)						Increases in parasitemia after 96 hr growth+
Human serum			Peptone (as 10% w/v solution)			
10	ml		0	ml		51 X
7.5	ml	+	2.5	ml	Gelysate	47 X
5.0	ml	+	5.0	ml	"	39 X
2.5	ml	+	7.5	ml	"	26 X
1.0	ml	+	9.0	ml	"	23 X
0.5	ml	+	9.5	ml	"	19 X
0.1	ml	+	9.9	ml	"	19 X
0	ml	+	10	ml	"	4 X
7.5	ml	+	2.5	ml	Bacto-Peptone	48 X
5.0	ml	+	5.0	ml	"	34 X
2.5	ml	+	7.5	ml	"	24 X
1.0	ml	+	9.0	ml	"	17 X
0.5	ml	+	9.5	ml	"	10 X
0.1	ml	+	9.9	ml	"	18 X
0	ml		10	ml	"	8 X

* Parasites were continuously cultured in each serum/peptone combination for about 6 weeks before progressing to the next step down.

+ Cultures were initiated at approximately 0.2% parasitemia. Each 96 hr multiplication rate is the mean of 24 replicate cultures.

TABLE II. Continuous culture of adapted P. falciparum (FCR-3 strain) in media without human serum*

Components added to media* (Amounts added per 100 ml RPMI medium)							Increase in parasitemia after 96 hr growth+
Serum			Peptone (as 15% w/v solution)				
10	ml	Human (Control)	+	0	ml		60 X
10	ml	Calf	+	1	ml	Neopeptone	65 X
7	ml	"	+	1	ml	"	54 X
5	ml	"	+	1	ml	"	49 X
2.5	ml	"	+	1	ml	"	30 X
10	ml	Fetal Bovine	+	1	ml	Neopeptone	52 X
7	ml	"	+	1	ml	"	51 X
5	ml	"	+	1	ml		49 X
2.5	ml	"	+	1	ml	"	29 X
10	ml	Swine	+	1	ml	Neopeptone	65 X
7	ml	"	+	1	ml	"	48 X
5	ml	"	+	1	ml	"	47 X
2.5	ml	"	+	1	ml	"	23 X
10	ml	Calf	+	1	ml	Proteose-Peptone #3	65 X
7	ml	"	+	1	ml	" "	56 X
5	ml	"	+	1	ml	" "	52 X
2.5	ml	"	+	1	ml	" "	28 X
10	ml	Fetal Bovine	+	1	ml	Proteose Peptone #3	50 X
7	ml	"	+	1	ml	" "	52 X
5	ml	"	+	1	ml	" "	42 X
2.5	ml	"	+	1	ml	" "	36 X
10	ml	Swine	+	1	ml	Proteose-Peptone #3	66 X
7	ml	"	+	1	ml	" "	52 X
5	ml	"	+	1	ml	" "	50 X
2.5	ml	"	+	1	ml	" "	33 X

* Cultures were initiated with parasite lines that had been previously adapted to grow in media with 0.5 ml human serum/100 ml RPMI medium (See Table I).

+ Cultures were initiated at approximately 0.2% parasitemia. Each 96 hr multiplication rate is the mean of 24 replicate cultures.

k). Synchronization of cultures

Studies were also done on synchronization of parasite stages in culture. The rationale for this as stated in our original contract proposal was: "This may be an especially important problem to solve in gametocyte culture, in view of the fact that mature gametocytes may be produced synchronously, and may be short-lived in their maturity when produced. Thus, if all of the gametocytes within a culture could be produced in synchrony, a high percentage of them might be shown to be mature at the same time after an appropriate period of development. Attempts will be made to impose such synchrony by fluctuations of levels of oxygen, CO_2, hormones, temperature, cyclic AMP, and various regulators of cell division and the cell cycle."

None of these procedures was helpful in the production of synchrony. However, a useful procedure resulted from our accidental finding that sorbitol solution selectively lysed red cells containing mature parasites, and that this approach could be used to select for a synchronous population of ring forms.

MATERIALS AND METHODS

The FCR-3 strain of *P. falciparum* (Trager and Jensen, 1978) was cultured in petri dishes as described by Jensen and Trager (1977). We used 60 x 15-mm dishes containing total culture volumes of 5 ml.

To establish synchrony, each culture was centrifuged at 200 g for 5 min, the supernatant discarded, and the pellet (approx. 0.5 ml) resuspended in 2.5 ml of aqueous 5% D-sorbitol (0.274 M) for 5 min at room temperature. After an additional centrifugation, an equal volume of RPMI-1640 containing 10% human serum was added to the pellet. Cultures were re-established by addition of uninfected erythrocytes and medium to give a 12.5% hematocrit with

an appropriate starting parasitemia (generally 0.1%). The medium then was changed daily, as previously described (Jensen and Trager, 1977).

The resulting cultures were followed by examination of Giemsa-stained smears taken at various intervals to determine parasitemia and percent distribution of the different stages of development (rings, trophozoites, and schizonts). Staining generally was done with 8% Giemsa (pH 7.2) for 10 min. Parasitemia was determined by counting 10,000 erythrocytes per sample and is given as number of parasites per 100 erythrocytes. Osmolality values were obtained with a Wescor 5100B vapor pressure osmometer.

RESULTS

Cultures examined immediately after sorbitol treatment consisted almost entirely of single and multiple ring-form infections, and uninfected erythrocytes. No significant lysis of uninfected erythrocytes was observed. Erythrocytes infected with the more mature stages of the parasite were in varying degrees of lysis. This lytic effect could be enhanced by an additional wash and incubation in sorbitol.

Observations on culture samples taken at 24-hr intervals after sorbitol treatment are shown in Figure 1A. At 48 and 96 hr posttreatment, approximately 95% of the parasites were found to be in the ring stage of development. At 24, 72, and 120 hr, more than 95% of the parasites were in the trophozoite and schizont stages. These shifts in frequency distribution of the stages were correlated with discrete and discontinuous increases in parasitemia (Fig. 1B) occurring on alternate days in association with the formation of new ring forms.

To determine the synchrony of parasite transition from trophozoites to mature schizonts, culture samples were taken at frequent intervals during this transition period (Fig. 2). The relative frequency of schizonts increased gradually, reaching a peak at 80 hr. Thereafter, as the schizont population decreased, there was a concomitant increase in the frequency of ring forms, with a peak by 96 hr. The time at which schizonts reach their maximum frequency may vary depending upon culture conditions, and the strain of parasite used. An even greater degree of synchrony can be produced by a second treatment with sorbitol approximately 34 hr after the first. Because this is a time of transition between schizonts and new ring formation, this second treatment ("fine tuning") selects for a relatively narrow age distribution of newly formed rings. The subsequent schizont populations that develop from these rings approaches 100% at a given time.

The 5% sorbitol solution used has an osmolality of 287 mOs/kg. Sorbitol solutions with osmolalities ranging between 141 and 855 mOs/kg also were effective in inducing selective lysis of erythrocytes infected with later stages of the parasite. Another alcohol sugar, D-mannitol, had a similar lytic effect; sucrose and α-lactose were ineffective.

DISCUSSION

Synchronization of our cultures was induced by a selective destruction of the later stages of parasite development during sorbitol treatment. The osmotic fragility of malaria-infected erythrocytes is known to increase during the development of the parasite (Shen et al., 1946; Fogel et al., 1966). However, the selective lysis that occurred in our system was not caused by simple osmotic shock. The osmolality of the 5% sorbitol solution we used

(287 mOs/kg) is not significantly different from that of the culture medium in which the parasites are grown (276 mOs/kg), and sorbitol solutions having a wide range of osmolalities were similarly effective. The mechanism of this effect is not clear. Sorbitol is known to have a "stabilizing" effect on the erythrocytic plasma membrane (Meryman and Hornblower, 1968), and has been used as a protective agent during cryopreservation of mammalian cells (Meryman, 1966; Karow, 1969). It also has been used during cryopreservation of erythrocytes infected with malaria parasites (Diggs et al., 1975; Wilson et al., 1977). Indeed, our original observations on synchronization of P. falciparum cultures were made on culture material that had been thawed after cyropreservation.

The ability to synchronize cultures of P. falciparum should permit future quantitative studies on the immunological, biochemical, and physiological differences among the various stages of development.

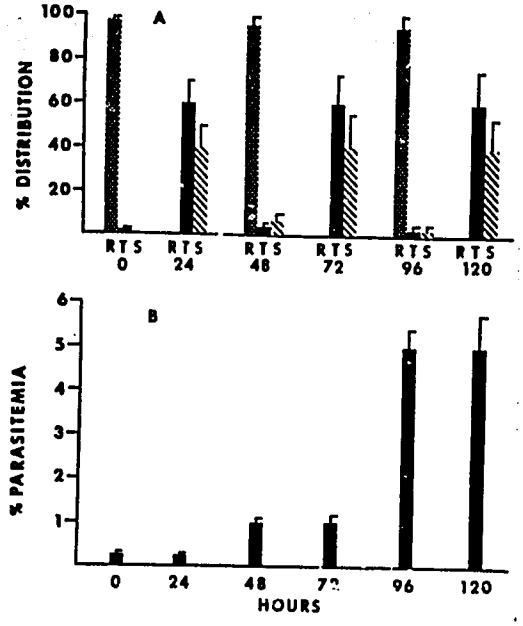

FIGURE 1. Synchronous development of *P. falciparum* in culture after sorbitol treatment. Each value is the mean of 12 samples (+1 standard deviation). Time is given in hours after sorbitol treatment (0 time was immediately after treatment). A. Relative percent distribution of parasite stages at 24-hr intervals. R = rings, T = trophozoites, S = schizonts. B. Parasitemias of cultures at 24-hr intervals.

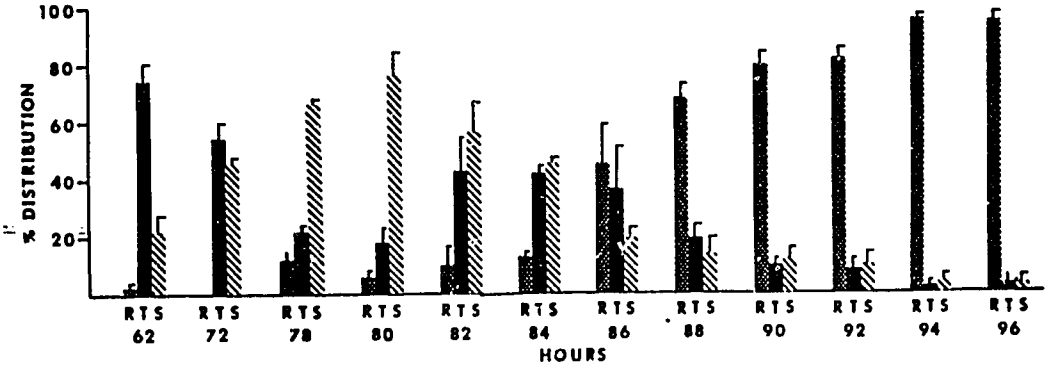

FIGURE 2. Relative percent distribution of *P. falciparum* stages during one cycle of synchronous development in culture. Each value is the mean of 4–12 samples (+1 standard deviation). Time is given in hours after sorbitol treatment. R = rings, T = trophozoites, S = schizonts.

D. Dissemination and Utilization of Research Results

Publications developed under the contract:

1. Lambros, C. and Vanderberg, J. P. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *Journ. Parasitol.* 65: 418-420.

2. Vanderberg, J. P. 1979. Isolation and purification of sporozoites of the malaria parasites: A review. Bull. W.H.O. (In Press).

3. Ifediba, T., and Vanderberg, J. P. 1980. Peptones and calf serum as a replacement for human serum in the cultivation of *Plasmodium falciparum*. *Journ. Parasitol.* (In Press).

4. Schneider, I., and Vanderberg, J. P. 1980. Culture of the invertebrate stages of plasmodia and the culture of mosquito tissues. *In* "Research in Malaria", vol. II. (J. Kreier, ed.) Academic Press, N.Y. (In Press).

5. Vanderberg, J. P., and Gwadz, R. 1980. The transmission by mosquitoes of plasmodia in the laboratory. *In* "Research in Malaria", vol. II. (J. Kreier, ed.) Academic Press, N.Y. (In Press).

Utilization by Other Laboratories

We have been informed that our procedure for synchronization of *P. falciparum* has become established as a standard procedure in many laboratories using this parasite. The procedure permits more efficient harvesting of specific parasite stages for immunization and other purposes.

E. Work Plan for the coming year

(a) The chief approach for the final year of this contract will be the continued testing of media additives and replacements with the aim of getting

complete development of P. falciparum gametocytes. Most of these additives are as listed in our contract proposal.

(b) Co-cultivation of our parasites with various human cell lines will also be attempted. These include fibroblasts and endothelial cells.

(c) Recent reports from other laboratories have suggested that maintenance of P. falciparum gametocytes at constant temperatures of around 37°C will permit their maturation. (Normally the temperature is lowered during the daily feeding of the culture.) We will be testing this procedure.

(d) As also stated in our contract proposal, "Attempts will be made to get further in vitro development of gametocytes, eventually leading to mature, immunogenic sporozoites." Previous studies were done by us on in vitro transformation of rodent malaria gametocytes to ookinetes. We will continue these studies with in vitro-formed ookinetes. Studies will first be done to determine the most satisfactory procedure for separation of ookinetes from uninfected and infected erythrocytes, prior to returning the ookinetes to culture for further development. We will then initiate studies on attempts to get further development of these ookinetes in culture. It is hoped that any culture procedures we develop may be transferable to human malaria.

(e) We also hope to complete our analytical studies on components of Anopheles mosquito hemolymph. This could help us in our attempts to design a rational medium for the cultivation of the mosquito stages of the parasite.

(f) We also plan to continue pilot studies on the protein components of P. falciparum, and on the changing patterns of protein synthesis during the life cycle of the parasite. Though this was not specifically proposed as part of our contract, someof our contract-related results (i.e., the synchronization results) have logically led us to this study. We hope to propose this study as a portion of our new contract renewal proposal, and therefore we plan to

continue our pilot studies in order to test the feasibility of our projected

F. Involvement of Minority Personnel and Women

One woman was involved in project activities as a research technician during the reporting period. Four members of minorities were involved in project research activities; a Black as a research technician, a Black as a graduate research assistant, a Black as an animal room technician, and a Hispanic as an insectary technician (the services of the latter 2 are shared on a cooperative basis with other members of the Department.)

FINANCIAL REPORT

The financial reports required by this contract are submitted directly by the New York University Medical Center business office according to our institutional policy.

SMITHSONIAN
SCIENCE INFORMATION EXCHANGE, INC.
1730 M STREET, N.W. PHONE 202-381-6511
WASHINGTON, D.C. 20036

SIE NO.

NOTICE OF RESEARCH PROJECT

SUPPORTING AGENCY:

Agency for International Development

AGENCY'S NUMBER(S):

Contract No: AID/ta-C-1486
and/or
Control No:

TITLE OF PROJECT:

In Vitro Cultivation of Sporogonic Stages of Human Malaria

PRINCIPAL INVESTIGATOR, ASSOCIATES

	School or Division	Department
Dr. Jerome Vanderberg	Division of Parasitology	Dept. of Microbiology

RECIPIENT INSTITUTION:

Name and Address: Including Zip Code.
New York University School of Medicine
550 First Avenue
New York, New York 10016

PERIOD FOR THIS NRP:

Start Date: 1/15/79
End Date: 1/14/80
Annual Funding: $95,201

SUMMARY OF PROJECT: Be brief-200 word maximum: (Include Objective, Approach, Current Plans and/or Progress)

The chief aim of this project is to develop methods for in vitro cultivation of gametocytes of human malaria, and subsequent infection of mosquitoes with these gametocytes. A series of different culture approaches have been tried in an attempt to obtain complete maturation of Plasmodium falciparum gametocytes in vitro. These include testing of new media, new gassing procedures, better maintenance of culture homeostasis, and testing different strains of the parasite. None of these procedures has yet succeeded in permitting better gametocyte maturation. Success has been reported in two areas. (1) Attempts were made to find an inexpensive, readily available substitute for the human serum requirement for the continuous culture of parasites. We found that commercially available peptones added together with calf serum gave parasite growth rates comparable to or surpassing those obtained with human serum. (2) A method to establish synchronous development of the erythrocytic stages of the parasite has been described. This involves the selective lysis of later stages of the culture by suspension of parasitized erythrocytes in 5% aqueous sorbitol solution.