Plasmodium falciparum - growth development ?

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PLASMODIUM FALCIPARUM: CONTINUOUS CULTIVATION OF ERYTHROCYTE STAGES IN PLASMA-FREE CULTURE MEDIUM

by

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1. Introduction

Continuous in vitro culture of the erythrocytic stages of Plasmodium falciparum has provided a significant advance in malaria research capabilities (Trager & Jensen, 1976; Haynes et al., 1976). However, the requirement for supplemental plasma in the culture medium poses obvious technical limitations to some biochemical and chemotherapeutic applications of this new technology. Previous attempts to eliminate the plasma requirement have resulted in very limited success; in no case has sustained continuous growth of the parasite been achieved (Geiman et al., 1966; Ifediba & Vandenberg, 1980). This report describes the development of plasma-free culture media in which P. falciparum was maintained continuously for more than four weeks.

2. Materials and methods

In vitro cultures of Plasmodium falciparum were maintained using a modification of the methods of Trager & Jensen (1976) and Haynes et al. (1976) which is briefly described below. The 5 ml cultures were maintained in 50 ml flasks with A-positive human erythrocytes parasitized with P. falciparum under 5% oxygen, 5% carbon dioxide and 90% nitrogen. The medium was changed daily and dilutions with uninfected erythrocytes were made every 3-4 days. Thin blood films were made prior to dilution, stained with Giemsa and examined microscopically. Parasitaemia was determined by counting the number of parasites per 1000 erythrocytes. Parasite growth was calculated by the equation:

$$MI = \frac{FP}{BP} TD^{2/D}$$

where MI = multiplication index, FP = final parasite rate, BP = beginning parasite rate, TD = grand multiple of all red cell dilutions and D = total days. This expression corrects for different culture intervals and results in a numerical value representing the number of red cells infected from each parasite during 48 hours.

Standard RPMI 1640 (Gibco) medium was prepared every 2-3 weeks and consisted of 1640 medium with 5.94 g Hepes buffer (Gibco) (25 mM) in one litre of pyrogen-free sterile distilled water (Gibco). It was filtered through 0.20 mμ Nalgene (R) filters and stored at 4°C. At the time of use the pH of an aliquot (43.4 ml) was adjusted to 7.4 by adding 1.6 ml of 7.5% sodium bicarbonate solution (Gibco).

Stock experimental medium was also prepared every 2-3 weeks and consisted of RPMI 1640 medium with 5.94 g Hepes buffer (25 mM), 6 g fatty acid-free bovine albumin (Sigma), and 10 mg adenosine (Sigma) in one litre of pyrogen-free sterile distilled water. It was filtered twice through Nalgene® filters (sizes 0.45 mμ and 0.20 mμ) and 50 ml aliquots were frozen for subsequent use.

Fatty acid (Sigma) solutions were prepared by dissolving 0.014 mmol in approximately 0.5 ml of ethanol, neutralized to pH 7.4 with 0.1N NaOH and diluted to a final volume of 1 ml. Ethanolic solutions of fatty acids were stored for up to one month at -20°C.

Once a week stock experimental medium was thawed, a 47.8 ml aliquot was buffered to pH 7.4 by adding 1.8 ml of 7.5% sodium bicarbonate (Gibco) and then 0.05 ml gentamycin sulfate (50 mg/ml, Schering) was added. Ethanolic solutions of fatty acid(s) were warmed and 0.36 ml added to the medium resulting in a final concentration of 10^{-4}M. (For lower concentrations the ethanolic fatty acid solutions were diluted with additional stock experimental media which had been neutralized with sodium bicarbonate.) The final experimental media were filtered again through 0.45 mμ Nalgene® filters and used for up to four days.

A-positive blood was obtained from volunteer donors and placed in sterile 50 ml tubes containing an acid citrate dextrose solution (Gibco). The blood was stored at 4°C for two weeks and used within the subsequent two weeks. When cultures were initiated or subcultures made, the plasma was removed and discarded. The erythrocytes were washed twice in plain RPMI 1640 medium. After washing, 6% (V/V) erythrocyte suspensions were prepared in experimental RPMI 1640 media which had been formulated for the specific experiment.

For control cultures, freshly frozen human plasma from A-positive donors was obtained from the Walter Reed Blood Bank, heat inactivated and stored at -20 °C for up to three months. When used, the plasma was thawed at room temperature and diluted with plain RPMI 1640 medium to a final concentration of 10% (V/V).

The parasites used in these experiments were derived from the Viet Nam Smith (Canfield et al., 1971) and the Malayan Camp (Degowin & Powell, 1965) isolates of P. falciparum. Inocula for the studies were obtained from continuously grown stock cultures which were used for drug sensitivity experiments (Desjardins et al., 1979).

3. Results

Continuous culture of malaria parasites was repeatedly achieved with the use of a variety of fatty acids as replacement for human plasma. These Plasmodium falciparum cultures were usually terminated after one month. Microscopic examination of the parasites during and at the termination of the experiments showed them to be indistinguishable from control parasites grown in medium supplemented with human plasma. In addition, the parasites grown in fatty acid-supplemented plasma-free media were occasionally frozen in glycerol, and, when thawed after several months, rapidly resumed growing in similar media.

The rate of multiplication of parasites in media containing fatty acids was always less than in positive control cultures containing human plasma. The multiplication index in these control cultures was usually 4-8 (see Table 1). Negative control cultures containing all components, except fatty acids, failed to sustain continuous growth although some invasion of red blood cells did occur for about one week.

The results of experiments to study the ability of different C-18 fatty acids to support continuous growth in plasma-free media are shown in Table 1. Cis-vaccenic, oleic, and linoleic acids supported continuous growth for longer than one month until the experiments were terminated. Neither palmitoleic, elaidic, nor stearic acids supported continuous growth, although there was some invasion of red blood cells for 1-2 weeks, similar to that observed with albumin alone.

Oleic acid was studied through the concentration range 10^{-4} to 10^{-7} M to determine the optimum concentration to support growth in the absence of plasma. The results are shown in Fig. 1. At both extremes growth was sub-optimal and ceased spontaneously. Maximum growth occurred at 10^{-5} M where growth was continuous; the multiplication index in this experiment was 4.34.

Experiments to evaluate the possibility of improved growth with combinations of fatty acids are shown in Table 2. The first two experiments showed no apparent difference between total fatty acid concentrations of 10^{-5} M and 2 x 10^{-5} M. Therefore, the remainder of the experiments were performed at total fatty acid concentrations of 10^{-5} M (approximately 3 mg/l) with no apparent difference in growth in the mixtures.

4. Discussion

Siddiqui et al. (1967) studied the effects of fatty acids on the *in vitro* development of *Plasmodium falciparum* during 24-hour cultures and reported that stearic acid was nearly as effective as plasma in supporting maturation. Oleic, palmitoleic and linoleic acids did not support maturation. In earlier work we also studied the effect of various fatty acids bound to fatty acid-free bovine albumin in a similar short-term *P. falciparum* culture system (unpublished data). These studies used morphologic maturation of parasites to assess the ability of 21 different fatty acids to replace plasma during a single parasite cycle. The best growth in these conditions seemed to occur in the presence of single unsaturated C-18 fatty acids at concentrations of 10^{-5} M. Saturated fatty acids from C-14 to C-24 did not support growth. The short duration of the experiments made it difficult to make quantitative assessments, but palmitoleic, oleic, elaidic, cis-vaccenic and linoleic acids repeatedly supported maturation in the absence of plasma. This phenomenon was not observed with the other fatty acids studied including stearic acid. When continuous culture techniques became available these previous results led to the selection of the fatty acids for studies of potential plasma replacements to support continuous growth.

The requirement for lipids by growing malaria parasites can readily be inferred from the marked increases in lipid content of infected erythrocytes from several species (Holz, 1977). Increases are reported for phospholipids, cholesterol and fatty acids. The parasite seems incapable of generating increased fatty acids or phospholipids by fatty acid biosynthesis and apparently requires an external source (Rock et al., 1971). Labelled fatty acids are readily transported and incorporated *in vivo* into phospholipids of simian (*P. knowlesi*) (Rock, 1971), avian (*P. fallax*) (Gutierrez, 1966) and rodent (*P. berghei*) (Cenedella et al., 1969) malarias.

Plasmodium spp. also appear to be incapable of cholesterol biosynthesis (Trigg, 1968a). *In vitro* exposure to labelled cholesterol precursors did not result in incorporation of label into parasite cholesterol. However, labelled cholesterol was incorporated. Cholesterol also appeared to enhance *in vitro* growth and maturation of *P. knowlesi* (Trigg, 1968b). Our cultures of *P. falciparum* grew continuously without supplemental cholesterol. Thus, in media containing added fatty acids, sufficient cholesterol for growth of the parasite was presumably provided by the periodic addition of fresh erythrocytes.

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SUMMARY

Continuous *in vitro* cultivation of the malaria parasite, *Plasmodium falciparum*, was performed in plasma-free medium. The medium used was standard RPMI 1640 supplemented with adenosine, unsaturated C-18 fatty acids and fatty acid-free bovine serum albumin. The medium was changed daily and the cultures were diluted with washed erythrocytes twice weekly. Growth was routinely maintained for one month at which time the experiments were usually terminated. Although the overall growth rates were consistently lower than in control cultures with plasma, continuous growth occurred in the absence of plasma in cultures containing cis-vaccenic, oleic and linoleic acids.

RESUME

PLASMODIUM FALCIPARUM : CULTURE CONTINUE DES STADES ERYTHROCYTAIRES EN MILIEU EXEMPT DE PLASMA

La culture continue in vitro de Plasmodium falciparum, parasite du paludisme, a été réalisée en milieu exempt de plasma. Le milieu utilisé était constitué de RPMI 1640 standard additionné d'adénosine, d'acides gras en C-18 insaturés et de sérumalbumine bovine exempte d'acides gras. Le milieu était renouvelé tous les jours et les cultures étaient diluées deux fois par semaine avec des érythrocytes lavés. La culture a été entretenue en routine pendant un mois, délai au bout duquel les expériences étaient en général terminées. Bien que les taux de croissance observés aient été régulièrement plus faibles que dans les cultures témoins contenant du plasma, on a observé une croissance continue en l'absence de plasma dans les cultures contenant les acides cis-vaccénique, oléique et linoléique.

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TABLE 1. MALARIA PARASITE GROWTH IN MEDIA SUPPLEMENTED WITH SINGLE C-18 FATTY ACIDS

Fatty acid	Concentration (M)	Days[a]	Plasmodium falciparum isolate	
			Viet Nam Smith	Malayan Camp
Cis-vaccenic	10^{-5}	39	2.81[b]	2.93
Oleic	10^{-5}	39	2.98	3.98
Palmitoleic	10^{-5}	19	0	0
Elaidic	10^{-5}	11	0	0
Linoleic	10^{-5}	31	2.96	2.95
Stearic	10^{-5}	7	0	0
None	-	13	0	0
Plasma controls		34	8.3	8.8
		36	4.4	5.0
		29	6.1	5.1

[a] Duration of experiment until terminated or growth ceased.

[b] Multiplication index (see section 2 of text).

TABLE 2. MALARIA PARASITE GROWTH IN MEDIA SUPPLEMENTED WITH MULTIPLE C-18 FATTY ACIDS

Fatty acids	Concentration (M)	Days[a]	Plasmodium falciparum isolate	
			Viet Nam Smith	Malayan Camp
Cis-vaccenic Oleic	5×10^{-6} 5×10^{-6}	34	3.91[b]	4.20
Cis-vaccenic Oleic	10^{-5} 10^{-5}	34	4.53	3.73
Cis-vaccenic Oleic Elaidic	3.3×10^{-6} 3.3×10^{-6} 3.3×10^{-6}	34	4.14	4.44
Cis-vaccenic Oleic Linoleic	3.3×10^{-6} 3.3×10^{-6} 3.3×10^{-6}	28	4.10	4.14
Cis-vaccenic Oleic Linoleic Palmitoleic	2.5×10^{-6} 2.5×10^{-6} 2.5×10^{-6} 2.5×10^{-6}	34	4.06	3.33

[a] Duration of experiment until terminated.

[b] Multiplication index (see section 2 of text).

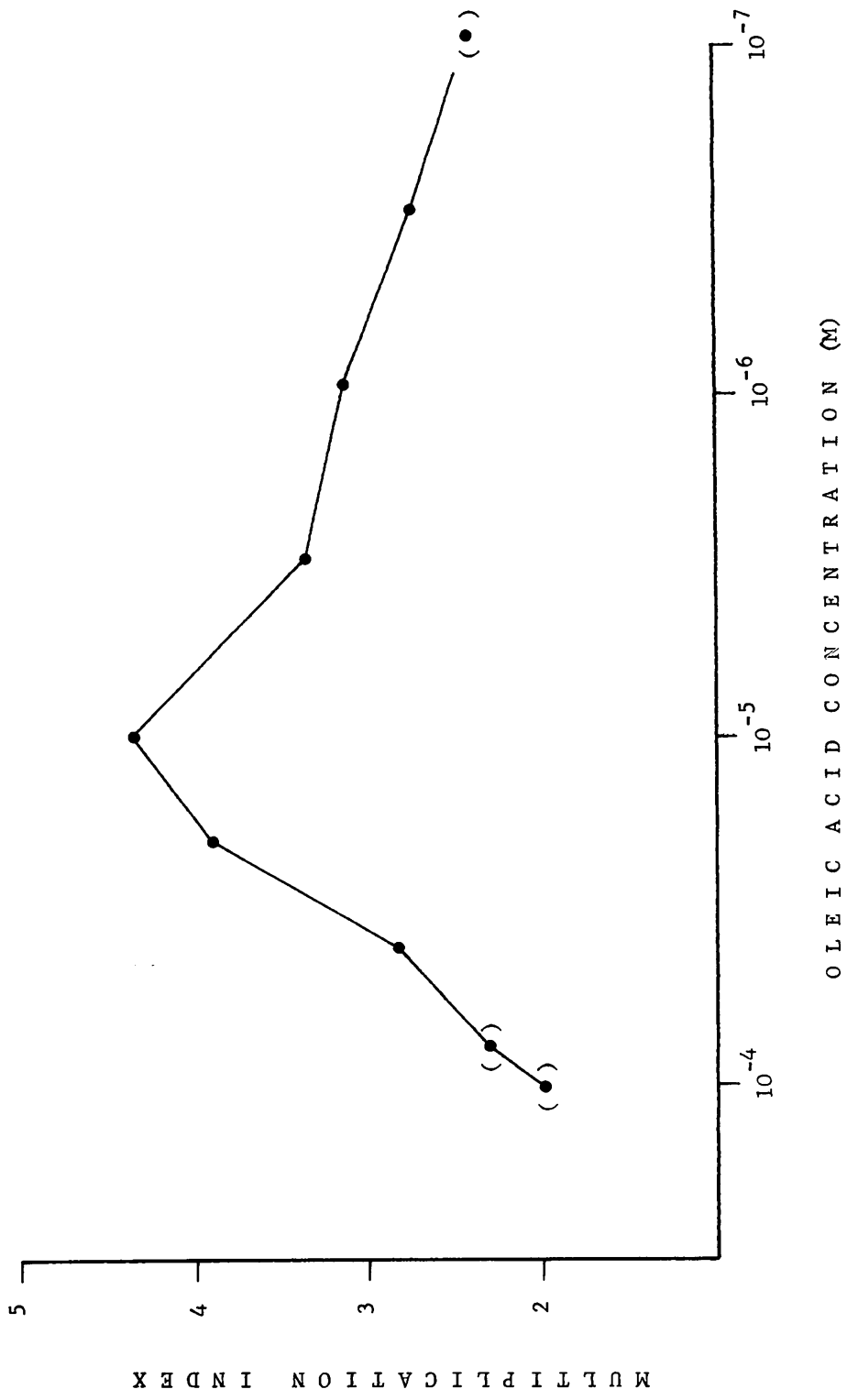

FIG. 1. THE EFFECT IN VITRO OF OLEIC ACID CONCENTRATION ON THE PLASMODIUM FALCIPARUM GROWTH RATE (EXPRESSED AS THE MULTIPLICATION INDEX - SEE SECTION 2 OF TEXT). GROWTH WAS CONTINUOUS AT ALL CONCENTRATIONS EXCEPT THOSE DESIGNATED BY( ) WHERE GROWTH CEASED SPONTANEOUSLY AFTER A FEW DAYS