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Continuous in-vitro cultivation of *Plasmodium falciparum* in Ibadan: solutions to scientific and logistical problems

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Summary

The technique of continuous in-vitro cultivation of *Plasmodium falciparum* has not been widely applied in malaria-endemic areas, due to scientific as well as purely logistical problems. Methods for solving or coping with these problems are described. They have already proved effective for over 4 years. The parasites harvested have been satisfactory, as judged by reproducible logarithmic growth curves and normal morphology in Leishman-stained smears as well as phase-contrast microscopy of wet-preparations. They have also been successfully used as antigen sources in the malarial fluorescent-antibody test and to investigate enhanced platelet aggregation in experimental malaria.

Résumé

La cultivation in-vitro sans cesse de *Plasmodium falciparum* n'a pas été largement appliqué dans les zones paludisme-endémiques à cause de raisons scientifiques aussi bien que les raisons logistiques. Les méthodes de résoudre ces problèmes sont décrites. Elles ont déjà prové effectives pour plus de quatre ans. Les parasites cultivés ont été satisfaisants comme on a montré par la courbe de croissance logarithmique et la morphologie normale de Leishman-frottis-colorés aussi bien que les préparations humides de microscope à contraste-de-phase. Ils ont été successivement bien utilisés comme les sources d'antigène dans l'expérience du paludisme fluorescent des anticorps et pour démontrer l'aise de l'aggrégation de plaquette dans l'expérience du paludisme.

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Introduction

Malaria research has been greatly enhanced since the successful development of a method for the continuous cultivation of *Plasmodium falciparum in vitro* [1]. In areas highly endemic for malaria, however, this very useful research tool has not been widely applied, apparently because of certain problems. For instance, the need for human serum in the culture medium poses a scientific problem because malaria antibody present in locally derived sera would tend to inhibit parasite growth [2]. However, it is well known that adults who have always lived in malaria-endemic areas suffer from attacks of malaria, implying that at least some sera support parasite growth. Purely logistical problems, such as irregular water and electricity supplies and lack of suitable, long-term cryopreservation facilities, also abound in these areas. Yet, since the majority of the ultimate beneficiaries live in these malaria-endemic areas, it is desirable that scientists who work there contribute to the research effort. To this end we have successfully used the candle-jar method [3] to maintain *P. falciparum* in continuous in-vitro culture under the conditions prevalent at the University College Hospital, Ibadan, Nigeria.

Materials and methods

To prevent air-borne contamination, all manipulations of the cultures, sera, media and other solutions were carried out inside a laminar

flow hood (Bioflow model, Flow Laboratories) which was left running for at least 15 min before use. To prevent manual contamination, the operator's hands and forearms (up to the elbows) were scrubbed for at least 10 min with 4% (w/v) chlorhexidine gluconate (Hibiscrub, ICI). To cope with interruptions in water supply, enough clean tap water to last several days was permanently stored in the laboratory in aspirator bottles and other capped plastic containers.

All centrifugations were done in a bench-top centrifuge (MSE) at 1000 *g* for 2 min at room temperature, except for haematocrit determinations which were carried out in a Hawxley microhaematocrit centrifuge (11,000 *g*) for 10 min.

Glass, rubber and plasticware

These were cleaned by soaking overnight in 5% 7X detergent (non-toxic for tissue culture, Flow Laboratories) and washed three times in tap water followed by three times in distilled water and then dried in a warm-air oven. Glassware including cotton wool-plugged graduated and Pasteur pipettes were sterilized in a hot-air oven at 200°C for 2 h. Rubber and plastic items, including membrane filters (Millipore) were sterilized by autoclaving at 100 kPa for 1 h. Plastic Petri dishes were obtained already sterilized (Flow Laboratories).

Deionized water

For all solutions which would come into contact with the parasites, water was prepared by trapping steam and then running the water through a deionizer (Elgastat). Water resistivity was greater than 2000 ohm cm^{-1}.

Culture medium

RPMI 1640 culture medium (Flow Laboratories) was used. Exactly 10.4 g powdered medium (with glutamine but without sodium bicarbonate) and 5.96 g HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) buffer were dissolved in 960 ml of water. This solution was sterilized immediately by filtration through a membrane filter (0.22 μm; Millipore) under positive pressure applied with a 50 ml syringe via a two-way tap. Five, 200 ml aliquots were made and stored at −20°C until use. Just before use, an aliquot was thawed and to each 100 ml was added 4.2 ml of 5% $NaHCO_3$ solution (sterilized by membrane filtration) as well as 5 mg gentamycin. This was called incomplete medium and comprised RPMI 1640 supplemented with 25 mM HEPES, 2 g/l $NaHCO_3$ and 50 μg/ml gentamycin, all being final concentrations.

Sera. Type AB Rh+ve fresh frozen plasma was obtained from the University College Hospital blood bank. It was used only if it was negative for hepatitis B surface antigen. To each 100 ml of this plasma was added 12 ml 0.24 M $CaCl_2$ solution previously sterilized by autoclaving. After clotting and clot retraction at room temperature, the serum was divided into aliquots and stored at −20°C. For use, serum was added to the incomplete medium at a final concentration of 10%. This was called complete medium.

Initially, the ability of blood bank-derived serum to support parasite growth was tested by comparison with the serum of one of us (O.S.). While working abroad, serum from O.S. had been shown to support parasite growth as satisfactorily as sera from donors in Europe. Subsequently, every new batch of AB+ serum was tested against the previous one, i.e. the batch just about to be used up.

Parasite strain. The Wellcome–Liverpool strain of *P. falciparum* (a gift from Prof. L. Luzzatto) was used throughout. Strain purity was maintained as described below.

Red cells. For maintaining the stock culture, only red cells from one of us (O.S.) was used. This was done because blood from this source could be most easily guaranteed free of antimalarial drugs and wild-type malaria parasites. Each time blood was withdrawn (by venepuncture), Leishman-stained films were made. The blood was only used for culturing parasites if no malaria parasites were found on microscopic examination of such stained films. One volume of acid phosphate dextrose (ACD, anticoagulant) was mixed with four volumes of venous blood and stored in screw-capped glass tubes at 4°C. Aliquots of this solution were washed three times in incomplete medium by centrifugation, followed by removal of the supernatant and buffy coat. Final resuspension was in complete medium.

Culture technique. This was by the candle-jar method [3], except that culture haematocrit was usually kept between 3% and 5% and Leishman stain was used. Culture parasitaemia was diluted by the addition of unparasitized red cells on Mondays and Fridays. However, fresh venepuncture was carried out whenever haemolysis was noticed in the supernatant in the Petri dish, the washed red cells, the blood stored in ACD, or if the stained smears showed markedly distorted red cells or pyknosis in the parasites. Leishman-stained smears of the culture were examined daily or on alternate days as necessary. Occasionally, a wet preparation was examined by phase-contrast microscopy.

Results

As judged by several criteria, the parasites grown under the foregoing conditions proved satisfactory.

Parasite multiplication. This occurred in a logarithmic fashion as shown in Fig. 1. In one series of experiments represented by the continuous line, the red cells used to set up the Petri dishes on day 0 were freshly obtained by venepuncture. In a second series 10 months later, represented by the broken line, the red cells used on day 0 had already been stored in ACD at 4°C for 10 days. This may explain why its gradient is less than the continuous line. However, it still represents a fourfold multiplication over the 84 h (less than two parasite life cycles) of the experiment, with sampling at 12-h intervals.

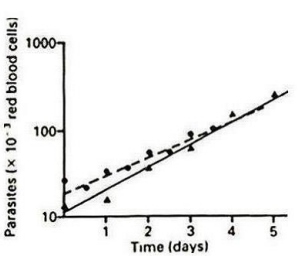

Fig. 1. Reproducible logarithmic growth of *P. falciparum in vitro*. Each point represents the mean of duplicate Petri dishes. (▲——▲) Red cells used on day zero from fresh venepuncture; stained smears made daily for 5 days. (●- - - -●) Data generated 10 months later; red cells used on day zero had been stored in ACD at 4°C for 10 days; smears were made twice daily for 4 days.

Parasite morphology. Leishman-stained smears revealed normal morphology, with good nucleo-cytoplasmic differentiation and well-dispersed brown malaria pigment in trophozoites and schizonts (Fig. 2). Occasional 'crisis forms' were seen, i.e. parasites in various stages of pyknosis (poor nucleo-cytoplasmic differentiation) with clumping of the malaria pigment. In general, crisis forms became prominent as culture parasitaemia approached or exceeded 100 per 1000, or if red cells stored for more than 16 days were used for culture.

Phase-contrast microscopy of wet preparations showed normal nuclear and cytoplasmic bi-refringence. In trophozoites only, vigorous motility of granules of malaria pigment was seen in the parasite which itself lay within the parasitophorous vacuole.

Other indices of satisfactory parasite culture. These parasites were used as antigen sources for the malarial fluorescent-antibody test many times with good results. They have also been used to demonstrate enhanced platelet reactivity in experimental malaria [4].

Red cell storage in vitro. Blood which had been stored for 16 days or more at 4°C in ACD and in its own plasma frequently showed evidence of haemolysis in the supernatant plasma. Also, parasites grown in such red cells were pyknotic, with little or no increase in parasitaemia. Under these conditions, haemolysis was very often evident in the supernatant culture medium in the Petri dish.

Interruptions in electricity supply. This usually meant some delay in changing the medium, since the use of the laminar flow hood is crucial for maintaining sterility. A delay of up to 12 h (i.e. 36 h after the last change) was well tolerated by the cultures even at high culture haematocrit (5%–10%) and parasitaemia (up to 100 per 1000 red cells). A 24-h delay was usually lethal. However, whenever such a delay was anticipated or deliberately planned, the cultures continued to thrive if culture haematocrit and parasitaemia had been reduced to 2% and 2/1000 red cells respectively.

The use of a water-jacketed incubator coupled with generally high ambient temperature (28–35°C depending on time of day and year) produced very gradual cooling during electrical power cuts. Incubator temperature dropped about 5°C over a 12-h period.

Laboratory accidents. Three earlier attempts

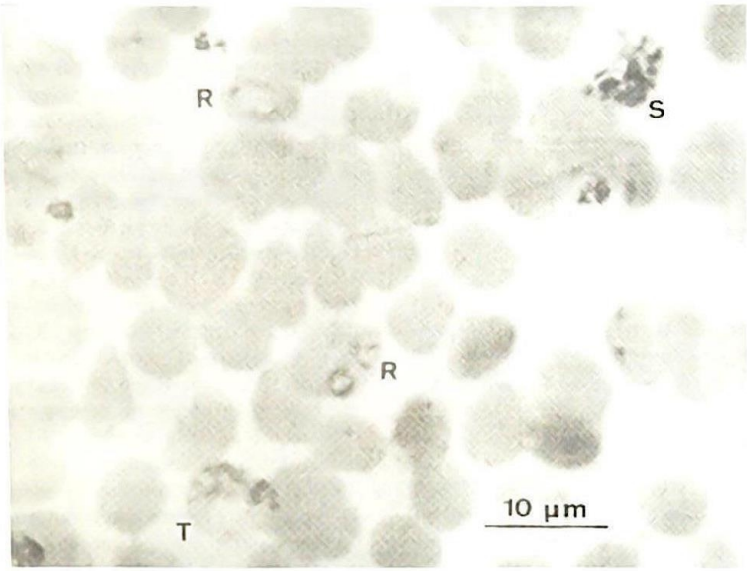

Fig. 2. *P. falciparum* in continuous culture *in vitro*. Leishman-stained smears showing ring forms (R), trophozoites (T) and schizonts (S).

(in 1982) to grow *P. falciparum* continuously *in vitro* were frustrated by laboratory accidents. One was traced to an improperly sterilized bijou bottle into which donor blood had been collected. The cultures became contaminated with Gram-negative bacilli, resistant to several antibiotics including gentamycin. Species identification was not attempted because the urgent priority was antibiotic sensitivity which would enhance the effort to rescue the parasites from the contaminants. The attempt was unsuccessful. On another occasion, contamination by *Candida* species was traced to a membrane filter used for 'sterilizing' the RPMI 1640 culture medium. It had been autoclaved at 100 kPa for 15 min holding time. After this, the holding time for malaria culture work was increased to 1 h. On a third occasion, the culture was lost, evidently because of the combined effects of high parasitaemia and delayed medium change. Owing to a prolonged interruption in electricity supply, the culture medium could not be changed until about 40 h after the last one at which time the parasitaemias in the Petri dishes were already approximately 8%, at a culture haematocrit of 5%.

No cases of laboratory-acquired malaria or other diseases have occurred in our hands so far.

Discussion

As far as we are aware, this is the first successful application of the continuous culture technique for *P. falciparum* in Nigeria. The results obtained are similar in many respects to data published earlier [1,3,5]. Logarithmic patterns of multiplication are consistent with thriving parasite cultures. Outdated red cells (i.e. red cells stored for 3–4 weeks under blood-banking conditions) are known to support parasite growth [3] but their competence as hosts declines gradually over the period [6]. This probably explains why the slope of the growth curve for 10-day-old red cells *in vitro* is less than that for absolutely fresh red cells (Fig. 1). However, since the sera used were also different, the difference in gradients could be due to differences in their ability to support parasite growth.

In our hands, red cells remain competent hosts for malaria parasites for only 2 weeks. This rapid ageing of blood stored *in vitro* is

probably due to a combination of factors. First, the ordinary household refrigerator used is not as efficient as blood-bank equipment. Secondly, frequent interruptions in electricity supply impair its function further.

The system described here differs from previously published work in one important respect, namely, that locally derived sera which probably contain high titres of malarial antibodies have been used for routine maintenance of continuous cultures. There is evidence that such sera impair parasite growth *in vitro* [2]. *In vivo*, globulin fractions prepared from adults have been successfully used to treat malaria in children [7]. However, growth inhibition *in vivo* was not total and γ-globulin therapy was not uniformly effective. In addition to these, a 10-fold dilution factor is present in the culture system described here, since serum is added to culture medium at a final concentration of 10%.

This system has the advantage that it resembles closely what happens *in vivo* in malaria-endemic areas. Inhabitants of such areas nearly always have some malarial antibodies in their sera, whether maternally acquired or from de-novo synthesis. Thus, when they contract malaria, the parasites have to grow in the presence of varying titres of malarial antibodies. Investigation of the malarial antibody titres of the sera used over the last 18 months is the subject of a separate communication. Caution must be exercised in extrapolating *in-vitro* data to *in-vivo* situations because of differences inherent in the two systems. The closer the *in-vitro* system simulates the *in-vivo* situation, the more valid such extrapolations are likely to be. The culture method described here reflects more accurately what happens *in vivo* in endemic areas than methods using non-immune sera.

In the area of laboratory safety, the risk of hepatitis B transmission constitutes one major problem. The prevalence of the hepatitis-B virus varies from 10 to 40% in Nigeria [8,9]. Thus, it is important to screen every unit of serum to be used for malaria cultures for hepatitis B surface antigen using a sensitive method, e.g. reversed passive haemagglutination or radioimmunoassay. More recently, we have also had to screen sera for the human immunodeficiency virus.

One problem which we have not been able to solve so far concerns cryopreservation. Working elsewhere, one of us had demonstrated that parasites stored in −70°C freezers (Revco), using a modification of the method of Wilson *et al.* [10], remain viable for a least 3 months. We attribute our failure to obtain viable parasites even after storage for just 1 week here in Ibadan, to the malfunctioning of the freezers. Their temperatures often rose to −50°C or higher due, among other things, to interruptions in the electricity supply. Although available locally, the supply of liquid nitrogen is not reliable.

The lack of cryopreservation has meant that the parasites have been kept growing round the year, implying an obligatory constant consumption of culture medium and other materials. It has also meant that personnel who look after these cultures have had to live with some restriction to their movements, e.g. it has been impossible to be away for more than 48 hours at a time. But most importantly, it has meant that contamination of the cultures, which occurs occasionally in most laboratories, implies in our case a simultaneous loss of the parasite strain. For these reasons, the solution to the problem of cryopreservation is receiving priority attention.

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