LARGE SCALE CULTURE TECHNIQUE FOR PURE *PLASMODIUM FALCIPARUM* GAMETOCYTES

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Abstract. A large scale technique for pure gametocyte cultures of *Plasmodium falciparum* was established in culture flasks by using treated erythrocytes and RPMI medium supplemented with 15% human plasma instead of serum. Pure gametocyte cultures were successfully obtained following treatment with 5% sorbitol on day 8 and 9 of cultivation. This method resulted in approximately 97% reduction of asexual parasites and provided pure gametocytes in culture. The highest numbers of gametocytes were obtained from cultures starting with 2% parasitemia and 2% erythrocyte suspension. On day 12 of cultivation, approximately 35×10^6 gametocytes per 100 ml of cell suspension could be harvested.

INTRODUCTION

Studies of the asexual stages of *Plasmodium falciparum* were greatly facilitated with the establishment of an *in vitro* continuous cultivation system (Trager and Jensen, 1976). On the other hand, understanding of the properties of the sexual stage of *P. falciparum* has been delayed due to a lack of adequate amounts of pure gametocytes. The candle jar method provided a limited amount of imperfect gametocytes after two months of continuous cultivation (Trager and Jensen, 1976) and this approach is not convenient because of the requirement for daily changes of culture medium.

The first significant success in gametocyte culture was achieved by Smalley (1976) who observed the *in vitro* development of mature *P. falciparum* gametocytes. Since then, not only other manual methods have been described for small scale of cultivation of gametocytes (Campbell *et al*, 1980; Ifediba and Vanderberg, 1981; Ponnudurai *et al*, 1982), but an automated culture system providing large numbers of gametocytes has also been developed employing a flow system for medium change (Ponnudurai *et al*, 1983, 1986). However, both the manual and automated methods do not provide pure gametocytes in culture but produce a mixture of both sexual and asexual forms of *P. falciparum*.

In this report, a simple, convenient and inexpensive large scale technique for cultivation of pure *P. falciparum* gametocytes is described.

MATERIALS AND METHODS

Parasites

Gametocyte-producing *Plasmodium falciparum* isolate KT3, collected from Kanchanaburi province in Thailand, was used in this study (Petmitr *et al*, 1995).

Maintenance of culture

Parasites were grown in 162 cm^2 sterile screw-capped tissue culture flasks (Costar). Each flask contained 100 ml of RPMI 1640 medium supplemented with 15% human plasma and 1% of treated red cells. The latter were prepared by incubating ten volumes of red blood cells, group O, with one volume of PIGPA solution (50 mM sodium pyruvate, 50 mM inosine, 100 mM glucose, 500 mM disodium hydrogen phosphate, pH 7.2, and 5 mM adenine in 0.9% (w/v) NaCl) for one hour at 37°C. Erythrocytes were then washed with RPMI 1640 medium two times and the buffy coat was removed. Duplicate cultures were started with parasitemias varying from 0.1% to 2%, at the ring stage obtained from synchronization of parasite growth using sorbitol treatment (Lambros and Vanderberg, 1979). Flasks were then flushed for one minute with a gas mixture containing 5% CO_2, 5% O_2 and 90% N_2, immediately tightly capped and placed horizontally in a 37°C incubator. Medium was changed on day 4 and 6. On day 8 and 9, cultures were treated with 2.5 volumes of 5% sorbitol according to the method

of Saul *et al* (1990). Thereafter, the medium was changed every 4 days until day 16 of initiation of culture.

Determination of parasitemia

One ml of cell culture suspension was taken from each flask and centrifuged at 700*g* for five minutes. A smear was taken of the packed erythrocytes and stained with Giemsa. Parasitemia was determined by counting the number of parasites per 10,000 erythrocytes.

RESULTS AND DISCUSSION

Large scale cultures of *P. falciparum* gametocytes were conducted using 1% erythrocyte suspension and 2% initial parasitemia. Sorbitol treatment of cultures on day 8 and 9 after initiation of parasite cultivation resulted in elimination of the sexual stages of the parasites; the numbers of asexual parasites dropped 97% whereas those of gametocytes increased from 228 to 322 per 10,000 erythrocytes (Table 1).

The effects of initial parasitemias on gametocyte production were also investigated. At low parasitemias, there was a concomitant increase in the numbers of gametocytes with duration of cultivation, but at higher initial parasitemias, maximum numbers were observed on day 12 (Table 2). At the highest parasitemia used (2%), yield of gametocytes on day 12 (306 gametocytes per 10,000 erythrocytes) was less than double expected using an initial parasitemia of 1% (256 per 10,000 erythrocytes).

Gametocytes were able to develop from stage I to stage V (Table 3). On day 8 and 9, over 97% of the gametocytes were of stage I-III, but by day 16, 86% were of stage IV. However on day 19, stages IV and V constituted only 52% owing to a drop in the numbers of stage IV gametocytes developing into stage V.

A comparison was also made between cultures maintained in 1% and 2% erythrocyte suspension (using an initial parasitemia of 2%). At day 12, $23 \pm 1 \times 10^6$ (mean ± range) gametocytes per flask were obtained in 1% cell suspension whereas $36 \pm 4 \times 10^6$ gametocytes per flask were seen in the 2% cell suspension.

The large scale cultivation technique described herein provides a simple, convenient and inexpensive means for producing pure gametocytes. Using treated erythrocytes (Chavalitshewinkoon and Wilairat, 1991) wasteful daily medium change was eliminated during the long cultivation period required to obtain gametocytes. Furthermore this method did not require any special equipment such as an automated culture system (Ponnudurai *et al*, 1983, 1986) so it should find application in many laboratories working on malaria cultures.

There have been two previous approaches used in the preparation of gametocytes *in vitro*: application of differential centrifiguation to separate the sexual from asexual forms (Williamson and Cover, 1975; Hommel *et al*, 1979; Knight and Sinden, 1982) and use of drugs, such as mitomycin C (Sinden *et al*, 1984) or pyrimethamine (Chutmongkonkul *et al*, 1992) to eliminate the asexual forms. However, mitomycin C is also toxic to gametocytes, and appearance of pyrimethamine resistance limits this

Table 1

Effect of sorbitol treatment on gametocyte production of *P. falciparum* KT3.

	Parasites/10,000 RBC* Sorbitol treatment		
	Before	After	
Parasite stage	day 8	day 8	day 9
Asexual stage	948 ± 5	359 ± 5	31 ± 1
Gametocyte stage	228 ± 8	289 ± 10	322 ± 15

*Mean ± range

Table 2

Effect of initial parasitemia on gametocyte production of *P. falciparum* KT3.

Percent initial parasitemia (asexual stage)	No. of gametocytes/10,000 RBC*			
	Day 4	Day 8	Day 12	Day 16
0.10 ± 0.01	-	1 ± 1	11 ± 1	44 ± 1
0.20 ± 0.08	-	6 ± 0	44 ± 4	47 ± 6
0.50 ± 0.06	2 ± 0	38.5 ± 5.5	153.5 ± 7.5	96.5 ± 6.5
1.00 ± 0.04	6 ± 0	118 ± 4	256 ± 11	137.5 ± 5.5
2.00 ± 0.02	10 ± 0	228 ± 8	306 ± 7	229 ± 10

*Mean ± range

Table 3

Distribution of gametocyte stages during *in vitro* culture of *P. falciparum* KT3 isolate.

Gametocyte stage	The No. of gametocytes/10,000 RBC* Sorbitol treatment					
	Before		After			
	day 4	day 8	day 9	day 12	day 16	day 19
I-II	1 ± 0	197.5 ± 10.5	221 ± 9	52 ± 12	9.5 ± 3.5	5 ± 2
III	1 ± 1	24 ± 3	97.5 ± 7.5	146 ± 12	22 ± 6	11.5 ± 4.5
IV	7.5 ± 1.5	5.5 ± 0.5	3 ± 1	105 ± 8	162.5 ± 9.5	69 ± 6
V	0.5 ± 0.5	1 ± 0	1 ± 1	3 ± 0	35 ± 1	94.5 ± 3.5

*Mean ± range

method to places where the parasites are still sensitive to the antifolates. On the other hand, sorbitol used in the lysis of infected cells containing the asexual stages has no effect on the morphology nor maturation of gametocytes (Petmitr *et al*, 1995), and following lysis of the uninfected erythrocytes with 0.2% (v/v) saponin the sexual forms can be easily collected by sedimentation.

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