EFFECTS OF ENVIRONMENTAL TEMPERATURE AND MEMBRANE FEEDING SOLUTIONS OF PURIFIED OOKINETES ON THE SPOROGENIC DEVELOPMENT OF *PLASMODIUM BERGHEI*

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Abstract: When *Anopheles stephensi* infected with *Plasmodium berghei* by bite were kept at 21°C in darkness, degenerative changes occurred in 33.8% of oocysts, as contrasted to 1.5% of such changes in mosquitoes kept at 21°C with 12-hr light period. Temperature shift of infected mosquitoes from 21°C for 24 hr to 25°C and 28°C increased the degeneration rate of oocysts to 87.8% and 85.8%, respectively, and inhibited oocysts maturation. *P. berghei* ookinetes were cultured from gametocytes of infected hamster blood and purified by discontinuous Percoll gradient, using the modified procedure described by Munderloh and Kurtti (1987). Suspensions of purified ookinetes ($1 \times 10^6/\text{ml}$) fed through membrane feeders permitted stable infection of *A. stephensi*. Mosquitoes fed purified ookinetes in phosphate buffered saline (PBS) and then maintained only with sugar, were able to produce infective sporozoites. Fetal bovine serum, mammalian cell medium F-12, and hamster red blood cell, added to PBS, improved infectivity of ookinetes to mosquitoes and decreased degenerative changes during oocyst development. The system of feeding purified ookinetes in simple chemical solution, PBS, would help to gain more information about the transformation of ookinetes to oocysts.

INTRODUCTION

Most of the life cycle of the rodent malaria parasite, *Plasmodium berghei* can be studied in *in vitro* culture systems: exoerythrocytic cycle in liver (Hollindale *et al.*, 1983), erythrocytic cycle in blood (Janse *et al.*, 1984), gametocytogenesis (Mons *et al.*, 1985) and ookinete formation (Weiss and Vanderberg, 1977; Janse *et al.*, 1985a, b; Sinden *et al.*, 1985).

The transformation of ookinetes to oocysts and the development of oocysts have not yet been studied in an *in vitro* system. Progress in cultivation of ookinetes and their development to mature oocysts has been hampered by the lack of information on the biophysiological conditions of sporogony in anopheline mosquitoes. We report the effect of environmental temperature and darkness on sporogonic development of infected *Anopheles stephensi* and the nourishing effect of the feeding solution of purified *P. berghei* ookinetes as determined by examination of the number and degenerative changes of oocysts in mosquito midguts.

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MATERIALS AND METHODS

Parasites and hosts:

The *Plasmodium berghei* (strain ANKA) — *Anopheles stephensi*—golden hamster (*Mesocricetus auratus*) complex was maintained as described by Vanderberg *et al.* (1968), and used for experiments.

Usually hamsters, 17 to 24 days old, were inoculated with *P. berghei*. Older hamsters than these were injected with phenylhydrazine HCl (100 mg/kg) four days before infection (Janse *et al.*, 1985b).

For infection, 40–50 female mosquitoes 3–7 days old, were collected from a stock colony into small mosquito cages, using disposable 350 ml plastic caps. Infected mosquitoes were maintained at 21°C with a daily cycle of 12 hr of light and darkness on 5% sucrose provided on dental cotton roll.

Media:

The ookinete culture medium consisted of Ham's F-12 medium (GIBCO) without NaHCO₃, supplemented with 10% heat-inactivated fetal bovine serum (FBS: GIBCO), 25 mM BICINE. The pH of the medium was adjusted to 8.0 with 1N NaOH. The culture medium F-12, supplemented with 10% FBS, 25 mM HEPES and 0.2% NaHCO₃, and adjusted to pH 7.3 with 1N NaOH (washing medium), was used for washing ookinetes, for dissecting mosquito midguts and for diluting blood and 90% Percoll.

Culture and harvest of ookinetes:

Ookinetes were cultured and harvested according to the modified procedure described by Munderloh and Kurtti (1987). One ml of the parasitized blood containing approximately 15 units of heparine/ml was inoculated into petri dishes (100 mm diameter) (Falcon) containing 10 ml of ookinete culture medium and incubated at 21°C for 20–22 hr to develop ookinetes.

Purification of ookinetes:

Isotonic 90% Percoll was prepared by adding 1 volume of 10× concentrated medium 199 (GIBCO) to 9 volumes Percoll (Sigma). This was further diluted to 45% and 36% with washing medium. The above cultures were collected into centrifuge tubes in an ice bath and centrifuged at 150×g for 8 min at 4°C. Both supernatant and upper greyish layer on the sediment were used to collect ookinetes. The supernatants were centrifuged at 175×g for 10 min and the sediment were resuspended in 5 ml of 36% Percoll. The upper greyish layer of the sediment were collected into centrifuge tubes containing 10 ml of washing medium and also centrifuged at 175×g for 10 min. Then again, the upper greyish layers were carefully collected and resuspended in 36% Percoll. Both 36% Percoll suspensions were separately overlayed on 5 ml of 45% Percoll and then 3 ml of washing medium was overlayed on each suspension. The preparations were spun at 150×g for 5 min and then for another 12 min at 700×g. The interfaces between the 45% and 36% Percoll were removed with a Pasteur pipet, and the number of ookinetes and contaminated red blood cells (RBC) were counted in a hemocytometer. Samples containing RBC fewer than 30 per ookinete were washed once and resuspended with described membrane feeding solutions, then used for feeding.

Effect of environmental temperature and darkness on sporogonic development:

Mosquitoes in 2 small cages were allowed to feed on an infected hamster for 10 min at 21°C and then mosquitoes in other 2 cages were fed on the same hamster for 20 min at 21°C.

After acquisition of the infected blood, mosquitoes in one cage was kept at 21°C with 12-hr light period as a standard control. Some cages were kept at 21°C for 24 hr and then kept at 25°C or 28°C with 12-hr light period, respectively. Other cages were kept at 28°C with 12-hr light period or at 21°C in darkness throughout the experiment.

Infected mosquitoes were dissected on day 11 post infection (PI) (experiment 1) and day 12 PI (experiment 2 and 3). The dissected midguts were examined with an inverted phase-contrast microscope at 360× magnification for the presence of oocysts.

Sporozoite infections of hamsters were carried out on day 15 PI in experiment 1 and 2, and day 17 PI in experiment 3. In the experiment 1 and 2, anesthetized hamster pups were placed on the top of the mosquito cages. The infected mosquitoes were allowed to feed through the nylon net of the cage on the underside of the hamster for 20 min. In the experiment 3, each mosquito was cut in half at the junction of the thorax and abdomen. The head-thoraxes of mosquitoes containing mature oocysts in the abdomen were kept in an Eppendorf containing the washing medium, 0.2 to 0.5 ml in an ice bath according to the number of mosquitoes dissected, and crashed with a round head of a spatula, after all mosquitoes were examined for the presence of oocysts. The suspension was centrifuged at 150×g for 2 min to remove the large mosquito debris. When the number of sporozoites in the supernatant could be counted in a hemocytometer, 1×10^3 sporozoites were used for infection of a hamster by intracardial inoculation carried out under sodium pentobarbital anesthesia.

Table 1 Effects of environmental temperature and darkness on sporogonic development of *Plasmodium berghei*

Environmental condition ^{a)}	No. of experiment	% Infected (No. examined)	No. of oocysts/infected mosquito $\pm$ SD	Degeneration rate (%) of oocysts (average)	Infectivity of sporozoites (No. of mosquitoes used)
21°C	1	100.0 (15)	30.2 $\pm$ 33.9	0	n.c. ^{b)}
	2	44.0 (18)	16.4 $\pm$ 42.3	3.1	+ (18)
	3	100.0 (8)	48.1 $\pm$ 25.2	1.3 (1.5)	+ (3)
21°C in darkness	1	91.7 (12)	52.9 $\pm$ 26.6	24.4	+ (17)
	3	100.0 (9)	43.0 $\pm$ 27.6	43.2 (33.8)	+ (7)
Temperature shift from 21°C to 25°C	1	100.0 (5)	19.6 $\pm$ 11.3	87.8	n.c.
Temperature shift from 21°C to 28°C	1	100.0 (7)	22.0 $\pm$ 22.7	81.8	— (8)
	2	66.6 (24)	19.0 $\pm$ 32.5	86.2	— (32)
	3	90.6 (11)	19.8 $\pm$ 15.9	89.4 (85.8)	— (2)
28°C	2	0 (23)			
	3	0 (15)			

a) Mosquitoes were kept with 12-hr light period except mosquitoes at 21°C in darkness.

b) Sporozoite infection was not checked.

If the number could not be counted, all the supernatant was used for infection of a hamster. To examine infectivity of sporozoites, the final examination of parasitemia was carried out on day 14 PI.

Nourishing effect of feeding solutions of purified ookinetes on the sporogonic development:

A. stephensi was infected by membrane feeding of purified ookinetes ($1 \times 10^6/\text{ml}$) to study the essential nourishment of blood on sporogony of *P. berghei*. Phosphate buffered saline (PBS: 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 per liter) was used as non nourishment. Five per cent glucose and washing medium were used for feeding ookinetes as moderate nourishment. A experiment of mosquitoes infected with ookinetes in PBS and in 5% glucose were maintained on 5% glucose in stead of 5% sucrose. Heparinized fresh hamster blood was diluted two times with washing medium and used for feeding ookinetes as blood meal (complete nourishment), because a small volume of whole blood easily evaporates during feeding and because it is too sticky for mosquitoes to engorge. RBC of heparinized hamster blood (1 volume) were washed once with cold PBS (100 volumes) and resuspended with PBS (1 volume) as RBC suspension.

To study the nourishing effect of blood component on sporogonic development of purified ookinetes, PBS was supplemented with each 10% of hamster RBC suspension, FBS as serum component and hamster whole blood. Each feeding solution was supplemented with 10 mM of NaHCO_3 , a phagostimulant for anopheline mosquitoes (Galun *et al.*, 1985; Yano *et al.*, in preparation).

Feeding of ookinetes to mosquitoes:

Purified ookinetes were resuspended in 0.5 to 1.5 ml of each feeding solution to obtain the concentration 1×10^6 ookinetes per ml.

Water-jacketed membrane feeders (2.6 cm in an inside diameter) (Rutledge *et al.*, 1964) fitted with a Baudruche membrane (Long and Long, Belleville, New Jersey) were used for

Table 2 Nourishing effect of feeding solution of purified *Plasmodium berghei* ookinetes on the sporogonic development

Feeding solution	Dissecting time (days)	% Infected (No. examined)	No. of oocysts/infected mosquito $\pm$ SD	Degeneration rate (%)	Infectivity of sporozoites
5% Glucose	12-18	12.1 (33)	1.3 ± 0.5	100.0	n.c. ^{a)}
PBS	8-10	40.8 (71)	3.2 ± 3.7	4.3	n.c.
	12-18	44.1 (183)	4.3 ± 5.6	19.0	4/5 ^{b)}
PBS+10% FBS	8-10	68.4 (19)	18.9 ± 20.8	0	n.c.
	12-18	87.0 (46)	29.5 ± 29.5	18.6	2/2
Washing medium	8-10	45.5 (11)	5.4 ± 5.9	0	n.c.
	12-18	76.3 (76)	6.9 ± 6.8	11.0	6/6
Blood meal	8-10	92.3 (26)	42.5 ± 41.1	4.8	n.c.
	12-18	87.4 (143)	31.6 ± 34.7	7.9	5/5

a) Sporozoite infection was not checked.

b) No. of infection/No. of trials.

Table 3 Nourishing effect of blood component in the feeding solution of purified *Plasmodium berghei* ookinetes on the sporogonic development

Feeding solution	% Infected (No. examined)	No. of oocysts/infected mosquito $\pm$ SD ^{a)}	Degeneration rate (%)
PBS+10% FBS	100.0 (31)	36.3 $\pm$ 29.8	17.6
PBS+10% RBC suspension	95.7 (23)	25.8 $\pm$ 35.6	11.6
PBS+10% Whole blood	100.0 (29)	34.2 $\pm$ 27.4	22.0
Blood meal	94.7 (18)	38.9 $\pm$ 33.0	12.9

a) The presence of oocysts was examined on day 14 PI.

feeding. Feeders were pre-warmed at 37°C in a 21°C-incubator. Mosquitoes in small cage were fed ookinetes in solution for 15 min. From day 8-10 PI, and 12-18 PI, the dissected midguts were examined for the presence of oocysts. Sporozoite infection of hamsters was carried out by intracardial inoculation of day 14-18 PI as described before.

Degenerative rate of oocysts:

The degenerative changes of oocysts proceeded from a few granules observed in the protoplasm of an oocyst, to coarse granules that filled the whole protoplasm of an oocyst. Finally, the protoplasm with coarse granules shrunk away from the oocysts wall and concentrated in the oocyst as an amorphous mass. Oocysts which under phase-contrast microscopic examination contained distinct coarse granules were counted as degenerative oocysts. Degeneration rate of oocysts was calculated from total degenerative oocysts per all oocysts of an experimental group.

RESULTS

Effects of environmental temperature and darkness on the development of malaria oocysts:

Table 1 summarizes the effects of environmental temperature and darkness on the development of malaria oocysts. The rate of infection and numbers of oocysts per mosquito did not differ significantly among mosquitoes kept at 21°C with 12-hr light period (21°C mosquitoes), at 21°C in darkness, and of temperature shift from 21°C for 24 hr to 25°C (21°C to 25°C mosquitoes) or to 28°C (21°C to 28°C mosquitoes). Almost all oocysts of 21°C mosquitoes matured without degenerative changes but the oocysts of 21°C mosquitoes in darkness, 21°C to 25°C mosquitoes and 21°C to 28°C mosquitoes degenerated significantly during their maturation; the average degeneration rates of oocysts were 33.8%, 87.8% and 85.8%, respectively, in contrast to the 1.5% of 21°C mosquitoes. Infective sporozoites were recognized both in mosquitoes kept at 21°C with 12-hr light period and those kept in darkness. Mosquitoes that were shifted from 21°C for 24 hr to 25°C or 28°C could not develop mature oocysts with sporozoites. In none of the surviving mosquitoes kept at 28°C with 12-hr light period could any development of oocysts be recognized in their midguts.

Nourishing effect of feeding solutions of purified ookinetes on the sporogonic development:

Mosquitoes fed purified ookinetes in PBS developed infective sporozoites but the infective rate and the number of oocysts per mosquito were lower than those fed ookinetes in PBS supplemented with 10% FBS, ookinetes in washing medium or in blood meal (Table 2). Addition of 10% FBS to PBS improved the infection rate, 40.8% in day 8-10 PI and 44.1% in day 12-18 PI to 68.4% and 87.0%, respectively, and the number of oocysts per mosquito, 3.2 ± 3.7 oocysts in day 8-10 PI and 4.3 ± 5.6 oocysts in day 12-18 PI to 18.9 ± 20.8 and 29.5 ± 29.5 , respectively.

Changes of PBS+10% FBS to washing medium (F-12+10% FBS+25 mM HEPES+0.2% NaHCO_3) decreased the degenerative rate, from 18.6% to 11.0% but the infected rate was not significantly different. Mosquitoes fed ookinetes in blood meal had the highest infection rates, 92.3% on day 8-10 PI and 87.4% on day 12-18 PI.

Young oocysts on day 8-10 PI of mosquitoes fed ookinetes in PBS with 10% FBS and washing medium did not degenerate but some later oocysts of mosquitoes fed ookinetes in all five kinds of solutions contained degenerative granules; the degenerative rates were 19.0% in PBS, 18.6% in PBS+10% FBS, 11.0% in washing medium and 7.9% in blood meal.

The infection rate of mosquitoes fed ookinetes in 5% glucose was very low, 12.1% of 33 mosquitoes in two experiments. The number of oocysts per mosquito was 1.3 ± 0.5 and all oocysts were degenerated.

The addition of 10% RBC suspension to PBS decreased the degenerative rate of oocysts as did the blood meal (Table 3).

The number of oocysts in mosquitoes fed ookinetes in PBS+10% RBC suspension was the lowest, 25.8 ± 35.6 in contrast to 36.3 ± 29.8 in PBS+10% FBS, 34.2 ± 27.4 in PBS+10% whole blood and 38.9 ± 33.0 in blood meal, respectively.

DISCUSSION

The temperature range which permits *P. berghei* ookinete formation in the natural vector *A. durenii*, is 18°C to 21°C (Yoeli, 1965). In the experimental vector, *A. stephensi*, oocysts did not develop at 28°C (Table 1). When ookinetes were allowed to form at 21°C for 24 hr after feeding parasitized blood, and then the environmental temperature was shifted to 25°C or 28°C, *A. stephensi* could develop young oocysts at 25°C and 28°C, but no mature oocysts formed at these temperatures.

Mosquitoes exposed to high temperature fail to develop sporozoites of *P. berghei* (Vanderberg and Yoeli, 1966). Thus the oocysts of 21°C to 25°C mosquitoes and 21°C to 28°C mosquitoes were highly degenerated during their later developmental stage. Oocysts without apparent degenerative changes were smaller and their development seemed to be retarded. Some had cloudy protoplasts with fine granules, indicating the start of degenerative changes. The damaging action of high temperature would begin before sporozoite budding, for there were no oocysts containing sporozoites.

In darkness, mosquitoes remained motionless in the cages. The degenerative changes of oocysts of 21°C mosquitoes in darkness were high. We assume that poor nourishment of mosquitoes, which did not feed on the 5% sugar solution in darkness was responsible for this finding. The transformation rate from ookinetes to oocysts increased in mosquitoes in darkness, compared to mosquitoes with 12-hr light period. We assume that the motionless of mosquitoes was responsible for this finding.

Mosquitoes fed ookinetes in PBS could develop mature oocysts and infective sporozoites when they were provided solely 5% sugar or 5% glucose on cotton roll like avian malaria ookinetes (Rosenberg and Koontz, 1984) (Table 2). Blood components were not essential on the development from ookinete to sporozoite.

Mosquitoes fed ookinetes in 5% glucose were seldom infected and they developed only a small number of degenerative oocysts per mosquito. The reason for this seems to be the weakly acidic glucose solution and its unstable pH.

The serum component, FBS, supplemented with PBS improved the viability of ookinetes in membrane feeders, and increased infectivity and the number of oocysts per infected mosquitoes almost the same as blood meal. The ookinetes kept in PBS in an ice bath for 30 min lost significantly their infectivity to mosquitoes compared to ookinetes kept in washing medium that proved completely infective for mosquitoes (100% infective rate). The RBC with PBS improved the viability of ookinetes to a lesser degree than FBS and whole blood (Table 3) but supplemented the nourishment and decreased the degeneration rate of developing oocysts.

The oocyst maturation in mosquitoes fed ookinetes in blood meal preceded by more than one day the maturation in mosquitoes fed ookinetes in PBS, PBS+10% FBS and washing medium. The maturation of oocysts could explain the higher degeneration rate of oocysts in day 8-10 PI of mosquitoes fed ookinetes in blood meal, because the degenerative changes became dominant in late stages of oocysts.

There was a large difference between the degeneration rate of oocysts in mosquitoes fed directly by bite on an infected hamster (1.5%) and those fed *in vitro*-formed ookinetes in blood meal (7.9% in Table 2 and 12.9% in Table 3). The gametocytes acquired by mosquitoes by bite should transform to ookinetes within 20 hr not to be trapped within the peritrophic membrane (Orihel, 1975). Thus ookinetes penetrating the peritrophic membrane to the midgut basement membrane would be biologically stronger than those transformed later and trapped within the peritrophic membrane, and could grow to mature oocysts and sporozoites without degeneration.

The ookinetes acquired by mosquitoes through membrane feeders could easily reach the midgut basement membrane before the peritrophic membrane hardened and had no selection of biological strongness. Some ookinetes could not retain the capacity for maturation due to unknown factors.

The experimental transformation of ookinetes to oocysts *in vitro* remained unsolved. The system of feeding purified ookinetes in simple chemical solution, PBS, would help to gain more information about it.

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Plasmodium berghei の sporogony について、蚊の飼育温度と
純化 ookinete の発育に対する membrane feeding 液の影響

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Plasmodium berghei (ANKA 株) —シリアンハムスター—*Anopheles stephensi* の系を使用した。*P. berghei* 感染ハムスターを吸血した *A. stephensi* での ookinete 形成には、蚊の飼育温度を 21°C に保つことが必須条件であった。吸血蚊を 21°C の暗闇に保つと oocyst 形成は増えたが、oocyst の発育中に 33.8% が変性を起こした。吸血後、*A. stephensi* を通常の飼育温度 28°C で飼育すると oocyst の形成は全く見られなかったが、吸血後 24 時間 21°C に保った後 28°C で飼育したものでは、oocyst の発育は見られたが、sporozoite への発育は阻害された。

P. berghei 感染ハムスター血を、10% FBS, 25 mM Bicine 加 Ham's F-12 培地 (pH 8.0) で約 10% に希釈し、21°C で 22—24 時間培養し、ookinete を得た。Percoll gradient (0~36%~45%) で純化した ookinete (1×10^6 /ml) を、5% グルコース, PBS, PBS-10% FBS, F-12-10% FBS, ハムスター全血に再浮遊し、membrane feeding 法で蚊に感染させた。血液成分を使用しなくても、PBS を feeding 液として ookinete を蚊に感染させた後、5% 蔗糖あるいは 5% グルコースで飼育して、感染力のある sporozoite を得た。その場合の低い感染率 (44.1%), oocyst 数 (4.3 個) は、PBS に 10% FBS を添加することにより感染率 (87.0%), oocyst 数 (29.5 個) とを、全血を feeding 液に使用した場合とほぼ同じ程度に回復させ得た。赤血球を全血の 10%, PBS に添加した場合は、FBS 添加より感染率, oocyst 数は劣ったが、oocyst の変性率は低下した。FBS 添加による感染率, oocyst 数の回復効果は、oocyst 発育過程での栄養供給よりも、membrane feeding 中の ookinete の viability の低下を防ぐ為と思われる。赤血球には、oocyst 発育過程での栄養供給効果があると思われ、oocyst の変性率を低下させた。

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