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Effect of Temperature to *Plasmodium berghei* and *P. yoelii* on Mosquito Stage in *Anopheles stephensi*

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Abstract

The effect of exposure to high temperature on the fertilization stage and the sporogonic stage of rodent malaria, *Plasmodium berghei* and *P. yoelii*, was studied *in vivo* and *in vitro*. It has been recommended that *P. berghei*-infected mosquitoes need to be maintained at a temperature of about 21°C, but *P. yoelii*-infected mosquitoes can be reared at normal insectary temperature (24–27°C). We found that exposure of mosquitoes to high temperature (26°C) after an infective blood meal remarkably impeded the development of both *P. berghei* and *P. yoelii* in the mosquitoes. The damaging effect occurred by the exposure to 26°C from 0–30 min in *P. berghei* and from 0–180 min in *P. yoelii* after the blood meal. Exflagellation and resulting ookinete formation of *P. berghei*, *in vitro*, were also impaired by exposure to 26°C for the first 30 min of incubation. Exflagellation in *P. yoelii* was not impaired at 26°C but was impaired at 31°C. These results indicate that exposure to low temperature is essential for transformation of malaria parasites in mosquito stage, and each species of *Plasmodium* has an appropriate low temperature as a trigger for the transformation.

Key words: *Anopheles stephensi*; *Plasmodium berghei*; *Plasmodium yoelii*; cell cycle; exflagellation; temperature.

Introduction

P. berghei, *P. yoelii* and *Anopheles stephensi* are widely used as experimental malaria systems in laboratories all over the world. It has been recommended that the mosquitoes fed on *P. berghei* infected mice or rats should be maintained at temperature 20–22°C, but mosquitoes infected with the other malaria species can be reared at normal insectary temperature (24–27°C) (Vanderberg and Gwadz, 1980). *P. berghei*, which was isolated in a highland region of Zaire, is a unique parasite because no oocysts are developed when mosquitoes fed on *P. berghei* infected animals are reared at 24–27°C. Vanderberg and Yoeli (1966) observed that complete sporogonic development of *P. berghei* took place at 16 to 24°C, and mosquitoes exposed to 28°C for as short a time as 2 hr following their infective blood meal failed to develop oocysts and sporozoites. *P. berghei* is thought to complete its mosquito stage in relatively low temperatures (below 22°C) in natural conditions in Africa (Yoeli *et al.*, 1964). These recommendations on the suitable temperature for rearing mosquitoes have persisted as a requirement for the necessary experimental conditions to complete the malaria life cycle in laboratories.

We have raised and investigated the following questions of *P. berghei*: why does *P. berghei* need such a low temperature in mosquito stage development, which step in the mosquito stage is most critical, and how different from other malaria species. In this report we used two species of rodent malaria, *P. berghei* and *P. yoelii*, and have counted oocysts on mosquito midgut in addition to observing exflagellation of microgametes and ookinete formation *in vitro*.

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Materials and Methods

Mosquito feeding and treatment at various temperatures

The *P. berghei* ANKA strain, clone 234, and the *P. yoelii* 17X lethal strain were both transmitted to BALB/c mice by *An. stephensi* mosquitoes. Infected blood containing 20% glycerin was stored at –80°C (passage 0). Stored blood was thawed and injected intra-peritoneally (1×10^7 parasites) into BALB/c mice (passage 1). When the parasitemia in the passage 1 mice reached to 5 to 10%, passage 2 mice were prepared by injection of 1×10^6 parasites from the passage 1 mice blood. *An. stephensi* mosquitoes were reared at 25–27°C and 70–75% relative humidity under long day conditions (14 L: 10 D). Adult females, 4–7 days old were held at 21°C for 2 hr and allowed to feed for 30 min on a mouse, which was in the 3rd day of patent infection with 0.5–5% of parasitemia. Each engorged mosquito was immediately caught, put into a plastic tube individually and transferred into incubators at various temperatures (e.g. 21°C, 26°C, 31°C) for various times (e.g. 30 min, 1 hr, 3 hr, 24 hr). After the incubation, mosquitoes were reared in pots at 21°C with 5% fructose.

Oocyst count and sporozoite count

Mosquitoes were dissected 12–14 days after their infective blood meals. Oocysts were counted on the midgut under a microscope at 10×40 magnification. Salivary glands were taken, fixed and stained with Giemsa, and examined for sporozites. To evaluate the degree of salivary gland infection, we used Positive Gland Index (PGI, Collins *et al.*, 1977), which was divided into 5 classes: PGI 0 (0 sporozoite in a pair of salivary glands), PGI 1 (1–10 sporozoites), PGI 2 (11–100 sporozoites), PGI 3 (101–1000 sporozoites) and PGI 4 (more than 1001 sporozoites).

Observation of exflagellation and ookinete formation

About 0.5 μl of infected mouse blood was taken from the tail vein and mixed with 25 μl of RPMI 1640 medium supplemented with hypoxanthine at 50 μg/ml, 25mM HEPES, 20% heat inactivated fetal calf serum, penicillin (5 μg/ml) and streptomycin (5 μg/ml) at pH 8.4 (Winger *et al.*, 1988). All medium components were obtained from Sigma Co. (St. Louis, USA). The mixture was covered with a Vaseline-sealed cover-slip (18×18 mm) and incubated for 30 min at various experimental temperatures (e.g. 16°C, 21°C, 26°C, 31°C). All glassware, pipettes and medium had been kept at the experimental temperatures. The number of exflagellation was counted against 10^5 erythrocytes. Then all samples were moved to an incubator at 21°C. The number of ookinetes against 10^5 erythrocytes was counted 24 hr later. Statistical analyses were carried out by the χ-square test and the Mann-Whitney U-test.

Results

Effect of high temperature on development of P. berghei *and* P. yoelii in vivo

Early exposure

When mosquitoes were exposed to 26°C for the first 30 min after feeding and subsequently transferred to 21°C, the number of oocyst was significantly smaller than that of the control group in *P. berghei* (Table 1-1, 2), but was not significantly different in *P. yoelii* (Table 2-1). When the exposure time at 26°C was extended to 180 min, no oocysts were found in *P. berghei* infected mosquitoes (Table 1-1), and a significant reduction of oocyst number appeared in *P. yoelii* (Table 2-3). When mosquitoes were allowed to stay at 21°C for the first 30 min after a *P. berghei* blood meal, exposed to 26°C in the next 30 min, and transferred to 21°C again, a normal number of oocysts was observed (Table 1-2).

Early to late exposure

When mosquitoes were kept at 21°C for the first 30 min after feeding and transferred to 26°C, the oocyst infection rate was lower and the number of oocysts was significantly smaller than those of control group in *P. berghei* (Table 1-3). No fully developed oocysts and sporozoites were found. But when exposure time at 21°C was extended to 60 min, the oocyst infection rate increased (80.0%) and a few sporozoites were found in the salivary glands (Table 1-3). On the other hand, when mosquitoes infected with *P. yoelii* were kept at 21°C for the first 60 min and transferred to 26°C thereafter, no significant

Table 1 Effects of high temperature on development of *P. berghei* oocysts in *An. stephensi*

Experiment no.	Exposure time to high temperature* after blood meal	Oocyst infected positive/total (%)	No. of oocysts mean±SD (range)	% oocyst No. to control	Sporozoites in saliv. glands positive/total (%)	mean PGI†
1	control	7/7 (100)	107.1±75.7 (36–232)	100	ND	ND
	0 min.–30 min.	3/7 (42.9)	4.3±5.7 (0–13)	4.0¶	ND	ND
	0 min.–180 min.	0/6 (0)§	0±0 (0–0)	0.0¶	ND	ND
2	control	8/13 (61.5)	13.0±19.1 (0–66)	100	ND	ND
	0 min.–30 min.	4/17 (23.5)	0.6±1.2 (0–4)	4.6¶	ND	ND
	30 min.–60 min.	10/13 (76.9)	34.1±33.2 (0–99)	262.3	ND	ND
3	control	6/7 (85.7)	69.3±92.6 (0–230)	100	6/7 (85.7)	2.9
	30 min.–18 days	1/7 (14.3)‡	0.1± 0.4 (0–1)	0.1¶	0/7 (0)§	0¶
	60 min.–18 days	4/5 (80.0)	5.8± 8.2 (0–20)	8.3¶	1/5 (20.0)	0.2‖
4	control	13/14 (92.9)	60.5±84.4 (0–289)	100	12/13 (92.3)	3.4
	10 days–13 days	14/15 (93.3)	58.1±67.8 (0–218)	96.7	12/15 (80.0)	1.2¶
	10 days–16 days	13/14 (92.9)	87.5±76.1 (0–241)	145.6	8/14 (57.1)	0.9¶
	13 days–16 days	12/14 (85.7)	47.6±81.6 (0–313)	79.2	10/13 (76.9)	2.5

*Mosquitoes were kept at 26–27°C for exposure to high temperature. The control mosquitoes were kept at 21°C.
†Mosquitoes were scored PGI individually. The mean PGI of the group was calculated arithmetically.
‡Difference significant from the control (χ^2-test, $p<0.05$). §Difference significant from the control (χ^2-test, $p<0.01$).
‖Difference significant from the control (U-test, $p<0.05$). ¶Difference significant from the control (U-test, $p<0.01$).

difference in the oocyst infection rate occurred but the mean oocyst number was significantly smaller than that of the control group (Table 2-2). Even when exposure time to 21°C was extended to 24 hr, the numbers of oocyst were significantly smaller than control (Table 2-4). This suggests that *P. yoelii* needs a low temperature (21°C), even after ookinete penetration of midgut.

Late exposure

When mosquitoes fed on *P. berghei*-infected mice were kept at 21°C for 10 days after feeding, exposed to 26°C for 3 days or 6 days with subsequent transfer to 21°C, normal oocyst development was observed but the mean PGI was reduced (Table 1-4). When the exposure time at 21°C was extended to 13 days, no apparent reduction of PGI was noted. At 21°C sporozoites of *P. berghei* start their migration to the salivary glands from day 12. We consider that most of sporozoites in the salivary gland in the mosquito group which were exposed at 27°C from day 13 to 16 had migrated from oocysts in the midgut by day 13.

Effect of various temperatures on fertilization stage in vitro

In the mixture of *P. berghei*-infected blood and RPMI1640 medium, which was exposed to 26°C for the first 30 min, followed by incubation at 21°C for 24 hr, a few exflagellations and no ookinete formation were observed (Table 3). When the sample were exposed to 16°C for 30 min, exflagellation occurred at similar rate to the control sample exposed to 21°C, but the number of ookinetes was lower than the control sample.

In *P. yoelii*, when the samples were exposed to 26°C for 30 min with subsequent incubation at 21°C for 24 hr, the numbers of exflagellation and numbers of ookinetes were lower than those of control (21°C) groups. This indicates that an incubation temperature of 26°C is not suitable even for *P. yoelii*.

In the sample exposed to 31°C for 30 min, no exflagellation and ookinete formation were observed in *P. berghei*, and a few exflagellation and no ookinete formation were observed in *P. yoelii*.

In three conditions of temperature (21, 24 and 27°C), the number of exflagellations was highest at

Table 2 Effects of high temperature on development of *P. yoelii* oocyst in *An. stephensi*

Experiment no.	Exposure to high temperature* after blood meal	Oocyst infected positive/total (%)	No. of oocysts mean±SD (range)	% oocyst No. to control
1	control	11/11 (100)	105.3±101.1 (10–376)	100
	0 min–30 min	8/8 (100)	72.9± 59.9 (1–126)	69.2
	0 min–9 days	9/13 (69.2)	6.3± 9.8 (0–35)‡	6.0
2	control	30/30 (100)	176.3±176.3 (9–356)	100
	0 min–60 min	24/24 (100)	131.0± 96.0 (1–288)	74.3
	60 min–10 days	14/17 (82.4)	36.0± 43.7 (0–133)‡	20.4
	0 min–10 days	14/18 (77.8)	8.7± 11.3 (0–43)‡	4.9
3	control	11/11 (100)	115.8± 67.4 (41–250)	100
	0 min–180 min	9/10 (90.0)	20.4± 37.3 (0–125)‡	17.6
	180 min–11 days	9/10 (90.0)	5.2± 3.5 (0–10)‡	4.5
	0 min–11 days	4/10 (40.0)†	0.7± 1.1 (0–3)‡	0.1
4	control	15/15 (100)	135.5± 74.0 (13–255)	100
	0 min–24 hr	14/14 (100)	29.8± 41.8 (1–136)‡	22.0
	24 hr–11 days	15/16 (93.8)	12.7± 12.9 (0–50)‡	9.4
	0 min–11 days	4/15 (26.6)†	1.5± 4.6 (0–18)‡	1.1

*Mosquitoes were kept at 26°C for exposure to high temperature. The control mosquitoes were kept at 21°C.
†Difference significant from control (χ^2-test, $p<0.01$). ‡Difference significant from control (U-test, $p<0.01$).

Table 3 Effects of various maintenance temperature on fertilization of *P. berghei* and *P. yoelii*

Experimental No.	Parasitemia (%)	Gametocytemia (%)	No. of exflagellation*				No. of ookinetes†			
			16°C	21°C	26°C	31°C	16°C	21°C	26°C	31°C
P. berghei										
1	8.0	0.16	ND	350	15	ND	ND	25	0	ND
2	30.0	0.6	ND	800	80	ND	ND	20	0	ND
3	21.6	2.1	920	1,340	ND	ND	28	108	ND	ND
4	37.7	1.6	ND	1,710	ND	0	ND	102	ND	0
P. yoelii										
1	14.0	0.20	ND	146	6	ND	ND	3	0	ND
2	15.0	0.20	ND	98	5	ND	ND	2	0	ND
3	20.0	0.40	ND	53	38	ND	ND	4	2	ND
4	13.0	0.10	ND	ND	30	2	ND	ND	4	0

*Infected blood was taken, mixed with medium and incubated in various temperatures for 30 min. Then samples were moved to a room of 21°C and the number of exflagellation was counted against 10^5 erythrocytes.
†Samples were further incubated in 21°C for 24 hr, and the number of ookinates was counted against 10^5 erythrocytes.

21°C for *P. berghei*, and at 24°C for *P. yoelii* (Table 4).

Effect of high temperature for various times on fertilization in vitro

Table 4 Effects of various maintenance temperatures on exflagellation of *p. berghei* and *P. yoelii*

Parasite	Praasitemia (%)	Gametocytemia (%)	No. of exflagellation* 21°C	24°C	27°C
P. berghei	20.0	0.20	236	114	2
P. yoelii	47.0	0.40	14	30	1

*Infected blood was taken, mixed with medium and incubated in various temperatures for 30 min. Then the number of exflagellation was counted against 10^5 erythrocytes.

When infected blood was kept at 21°C for the first 30 min and then transferred to 26°C for the next 30 min or 24 hr, the numbers of exflagellations and ookinete formation in the experimental samples were not different from those of the control samples in both *P. berghei* and *P. yoelii* (Table 5). When infected blood was kept at 26°C for 24 hr or even for the first 30 min, exflagellation and ookinete formation were decreased compared to control samples in both *P. berghei* and *P. yoelii*.

Discussion

Vanderberg *et al.* (1966) noted a significant effect on *P. berghei* in mosquitoes that were moved to 28°C a few minutes after feeding, and were exposed to that temperature for 2 hr before being transferred to 21°C for the reminder of sporogonic

Table 5 Effects of high temperatures on fertilization of *P. berghei* and *P. yoelii*

Experimental No.	Exposure time to high temperature*	No. of exflagellation†	(% of control)	No. of ookinetes‡	(% of control)
P. berghei					
1	0	360	(100)	15	(10-0)
	30 min–60 min	370	(102.8)	12	(80.0)
	30 min–24 hr	310	(86.1)	11	(73.3)
	0–24 hr	38	(10.6)	1	(6.7)
	0–30 min	60	(16.7)	3	(20.0)
2	0	2,560	(100)	140	(100)
	30 min–60 min	ND		129	(92.1)
	30 min–24 hr	ND		84	(60.0)
	0–24 hr	1,500	(58.6)	18	(12.9)
	0–30 min	ND		18	(12.9)
P. yoelii					
1	0	53	(100)	4	(100)
	30 min–60 min	67	(126.4)	2	(50.0)
	30 min–24 hr	57	(107.5)	1	(25.0)
	0–24 hr	38	(71.7)	2	(50.0)
	0-30 min	40	(75.5)	1	(25.0)
2	0	146	(100)	3	(100)
	30 min–24 hr	159	(108.9)	2	(66.6)
	0–24 hr	6	(4.1)	0	(0.0)
	0–30 min	5	(3.4)	0	(0.0)

*Slides were exposed to 26°C for various time.
†Number of exflagellation was counted after 30 min incubation against 10^5 erythrocytes.
‡Number of ookinetes was counted after 24 hr incubation against 10^5 erythrocytes.

development. They mentioned that the mode of action of high temperature damage was unknown. We consider that this phenomenon occurred not by the exposure of high temperature but by lucking of exposure to low temperature. In our present study using *An. stephensi* and *P. berghei*, we reduced the exposure time of high temperature to 30 min and found a significant reduction of oocyst development on the midgut. Moreover, when the fed mosquitoes were placed at 21°C for the first 30 min and transferred at 26°C for the next 30 min, oocysts on the midgut developed sufficiently. DNA synthesis and exflagellation occur during the first 30 min in microgametocytes of *P. berghei*, thus, we speculate that exposure to low temperature is essential for malaria parasites to transform into mosquito-stage form. Our speculation was confirmed by *in vitro* observation. The number of exflagellation at 21°C was higher than that at 26°C or 31°C. When samples were placed at 26°C for the first 30 min, exflagellation and ookinete development were impaired. As Kawamoto *et al.* (1991) has pointed out, DNA synthesis of malaria during microgametogenesis is regulated by a temperature-dependent mechanism.

Stimulation of low temperature at the beginning of mosquito stage is also essential in the other *Plasmodium* species. As Sinden and Croll (1975) observed that exflagellation of *P. yoelii nigeriensis* occurred at the temperature 25°C but did not at 30°C, *P. yoelii* also needed to be kept in a low temperature condition for starting exflagellation. From our results, the appropriate temperature for this was 24°C, which was 3°C higher than that of *P. berghei*. We recognized that 26°C was not appropriate for *P. yoelii* to develop ookinetes *in vitro* and oocysts in mosquitoes, indeed, the degree was not as strict as *P. berghei*. The life cycle of *P. yoelii* through the mosquito stage can be maintained at 26°C as former researchers recognize, however, we recommend that the temperature condition of 21–24°C is more appropriate for *P. yoelii*.

In most cells, the major cell-cycle checkpoint is said to be in G1 phase, and some regulatory proteins (e.g. G1 cyclin, cyclin-dependent protein kinese) regulate at the end of G1 phase (Hartwell, 1991). Mature microgametocytes stay at G1 phase (Kawamoto *et al.*, 1991). When the microgametocytes are placed in cold conditions, the cells would pass the G1 checkpoint and begin DNA synthesis. In the other organisms such as bacteria, yeast, plants etc., cold shock proteins have been identified (Jones *et al.*, 1987; Kondo *et al.*, 1991; Willimsky *et al.*, 1992). When cells are placed in cold conditions, mRNAs of several cold shock proteins are synthesized, and the cold shock proteins are produced. In these cases some regulatory proteins will direct synthesis of mRNA, although the mechanism is unclear. Similar mechanisms of DNA synthesis and exflagellation in *Plasmodium* would work because the cold shock will stimulate regulatory proteins. Budding yeast (*Saccharomyces cerevisiae*) has a normal G1 and S phase. However, microtuble-based spindle formation begins to form very early in the cycle, during S phase. Thus there dose not appear to be a normal G2 phase (Hartwell and Weinert, 1989). This is similar to the microgametocytes of malaria, which does not have G2 phase; DNA synthesis (S phase) and microtuble formation for flagella (M phase) occurs almost at the same time.

We next consider the epidemiological fact that human malaria cases generally increase in the rainy season and decrease in the dry season. The reason has been explained by mosquito density: breeding sites increase in rainy season, the number of mosquitoes increases accordingly, and the chance of malaria transmission increases. We do not deny this explanation, however, we propose another possibility on the basis of our experimental results: temperature generally increases in dry season, e.g. more than 30°C, the malaria parasites cannot start transformation in mosquitoes in such a high temperature, and the chance of malaria transmission decreases. Although our hypothesis needs more experimental and epidemiological data, we suppose that malaria parasites, mosquitoes and the mammalian host have kept their relationships in such natural conditions.

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