The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of *Plasmodium berghei in vitro*

O. BILLKER, M. K. SHAW, G. MARGOS *and* R. E. SINDEN*

Molecular and Cellular Parasitology Research Group, Infection and Immunity Section, Department of Biology, Imperial College of Science, Technology and Medicine, London SW7 2BB, UK

(*Received 9 July 1996 ; revised 23 December 1996 ; accepted 31 December 1996*)

SUMMARY

Developmentally arrested malarial gametocytes undergo gamete formation in the mosquito midgut immediately after ingestion of the infected bloodmeal. In the rodent malaria parasite *Plasmodium berghei* male gametogenesis (exflagellation) can be induced *in vitro* by a temperature decrease (from 39 °C in the vertebrate host to 20 °C) and a concomitant pH increase (from 7·3 in mouse blood to 8·0). We report the presence of additional Gametocyte Activating Factor(s) (GAF) present in *Anopheles stephensi* tissue extracts, which induce both male and female gametogenesis at the otherwise non-permissive pH of 7·3 *in vitro* but are unable to overcome the low temperature requirement. All constituent cellular events of microgametogenesis studied here are induced by the same triggers *in vitro*. A temperature decrease is also required for exflagellation in the mosquito midgut. The possible role of GAF as a second obligatory natural trigger of gametogenesis is discussed.

Key words: *Plasmodium*, malaria, gametogenesis, induction, exflagellation, regulation.

INTRODUCTION

In all plasmodia studied to date exflagellation can be triggered *in vitro* by the combination of a decrease in temperature of at least 5 °C and a concomitant increase in extracellular pH (reviewed by Sinden *et al*. 1996). A role for different, mosquito-derived factors regulating exflagellation *in vivo* has long been suggested (Micks, de Caires & Franco, 1948). Nijhout (1979) reported a factor (Mosquito Exflagellation Factor or MEF), present in midguts and heads of *Aedes aegypti* and in anal excretions of *Anopheles stephensi*, that was able to replace the obligatory pH increase required for *Plasmodium gallinaceum* exflagellation *in vitro*. A role for *Anopheles* gut factors in the induction of exflagellation in *P. falciparum* has also been suggested (Mendis, Noden & Beier, 1994). Here we present evidence that in the rodent malaria parasite *P. berghei* male and female gametogenesis can also be induced by mosquito extracts at an otherwise non-permissive pH *in vitro* and have examined whether mosquito extracts can also overcome the temperature restriction.

MATERIALS AND METHODS

Parasites

Plasmodium berghei ANKA clone 2.34 was maintained by cyclic passage in Theiler's Original mice and *Anopheles stephensi* and used between the 1st and 4th passage after mosquito transmission. Mice were checked daily for exflagellation and were used for experiments between day 7 and day 11 of infection when exflagellation, under optimal conditions (pH 8·0, 20 °C), exceeded 5 centres of exflagellation per microscopic field at ×400 magnification.

Mosquito extracts

Batches of 100 heads from adult female *An. stephensi* mosquitoes were homogenized on ice and sonicated in 100 μl phosphate-buffered saline (PBS), pH 7·3, centrifuged for 5 min at 10000 ***g***, 4 °C, and the pellet discarded. Supernatants were stored at −20 °C for up to 3 weeks.

To exclude bicarbonate as the active factor (Butcher, Sinden & Billker, 1996), *An. stephensi* head extracts were 'de-bicarbonated' following the procedure of Nijhout (1979). The extracts were acidified to pH 4·5 by addition of 1 M HCl, evacuated for 5 min and the pH was restored to 7·3 using freshly made (bicarbonate-free) 1M NaOH.

Standard culture medium, test solutions and test conditions

RPMI 1640 containing 25 mM HEPES (Sigma Chemicals, UK) was supplemented with an additional 40 mM glucose and used as the standard culture medium in all experiments. Test solutions consisted of 1 part RPMI and 2 parts of either

* Corresponding author. Tel: 0171 594 5425. Fax: 0171 594 5424. E-mail: r.sinden@ic.ac.uk.

https://doi.org/10.1017/S0031182097008895 Published online by Cambridge University Press

Table 1. Exflagellation responses of *Plasmodium berghei* microgametocytes to PBS extracts of female *Anopheles stephensi* heads under different conditions of temperature and pH *in vitro*

(The exflagellation responses were quantified as exflagellation centres in 20 microscopic fields and are expressed as a percentage of a PBS control at pH 8·0/20 °C.)

	Percentage exflagellation response (±S.E.)	*n**
20 °C		
PBS, pH 8·0	100·0	23
PBS, pH 7·3	1·5 (±0·8)**	21
Head extract, pH 8·0	115·7 (±9·4)	6
Head extract, pH 7·3	91·8 (±8·3)	6
Head extract, pH 7·3, de-bicarbonated	95·9 (±4·7)	15
37 °C		
PBS, pH 8·0	0·0 (±0·0)**	12
PBS, pH 7·3	0·0 (±0·0)**	6
Head extract, pH 8·0	0·0 (±0·0)**	6
Head extract, pH 7·3	0·0 (±0·0)**	6

* *n*, Number of replicates.
** Significantly different from pH 8·0/20 °C condition (Wilcoxon's signed rank test, $P < 0·05$).

mosquito extract or PBS. In all experiments heat-inactivated foetal calf serum (FCS) was present at a final concentration of 5%. Test solutions were adjusted to the required pH (±0·02 pH units) immediately before each experiment and, in preliminary studies, the pH of all test solutions was found to be stable over the time of the experiments. To investigate the effect of temperature, experiments were alternately carried out in 20 °C and 37 °C constant temperature rooms using pre-warmed equilibrated equipment and solutions and fresh tail blood from the same infected mouse.

Exflagellation assay

Exflagellation was quantified in a mini assay developed from that of Kawamoto *et al*. (1990). Samples of 2·5 μl of tail blood were taken into heparinized pipette tips, immediately mixed with 7·5 μl of test solution, placed on a microscope slide and covered with a Vaseline-rimmed cover-slip to give a contiguous layer of red blood cells (RBC), from which contact with the air was excluded. Slides were observed between 15 and 30 min later, when exflagellation under optimum conditions was within 80% of the maximum (see Fig. 1). Exflagellation was scored in each preparation by counting centres of movement in 10 microscopic fields (×40 phase objective, ×20 ocular lens) and the average recorded. All exflagellation results are expressed as a percentage of a positive control (medium at pH 8·0/20 °C) examined in parallel with the test conditions.

Ookinete cultures

To examine induction of macrogametogenesis in response to mosquito head extracts, 5 μl of tail blood were immediately mixed with 15 μl of test solutions in a 0·5 ml test tube at 20 °C. Cultures were diluted after the first hour of culture with 180 μl RPMI 1640 (pH 7·3) supplemented with 5% FCS, penicillin (50 U/ml), streptomycin (0·1 mg/ml) and neomycin (0·05 mg/ml), transferred to 24-well plates and further cultured overnight to permit ookinete development. After 24 h, macrogametocyte-derived parasite stages in these cultures were detected and counted by indirect fluorescent antibody labelling (Ponnudurai *et al*. 1989). The mAb 12.1, which specifically recognizes the macrogamete/zygote/ookinete surface protein Pbs21 (Sinden *et al*. 1987), was conjugated to fluorescein isothiocyanate (FITC, Sigma) according to manufacturer's recommendations. Ookinete cultures were centrifuged (5 min, 200 ***g***) and pellets incubated for 30 min at room temperature in 30 μl PBS, pH 7·2, containing the FITC-conjugated mAb (1:100 dilution). Cultures were then washed twice, resuspended in 50 μl of PBS, then the labelled ookinetes and round parasites (macrogametocytes and zygotes) were counted by fluorescence microscopy in a modified Neubauer haemocytometer.

Examination of constituent events of gametogenesis

To investigate the effect of different combinations of triggers on the constituent events of gametogenesis, 5 μl of tail blood were cultured with 15 μl of test solutions with or without *An. stephensi* head extract; at pH 7·3 or pH 8·0; at 20 °C or 37 °C. After 8 and 20 min, samples were taken for blood smears and electron microscopy.

For the quantification of nuclear stages and DNA synthesis in these cultures, methanol-fixed blood smears were rehydrated in an ethanol series and DNA was stained with propidium iodide (PI) in the presence of ribonuclease A as described by Kawamoto *et al*. (1991). Slides were mounted in Vector-Shield and observed by fluorescence microscopy in a Bio-Rad MRC 600 confocal microscope. For quantification of microgametocyte activation in response to different culture conditions, 100 microgametocytes on each blood film were assigned to 1 of 3 categories: (1) *non-activated* microgametocytes with a large nucleus which, on smears, frequently appears in a lateral position and always displays weak and disperse fluorescence (Fig. 2A); (2) *replicated* microgametocytes, which have not released gametes but which have rounded up and possess a large, centrally located nucleus that confines pigment granules in the cytoplasm to a narrow ring and displays strong fluorescence as reported previously (Janse *et al*.

https://doi.org/10.1017/S0031182097008895 Published online by Cambridge University Press

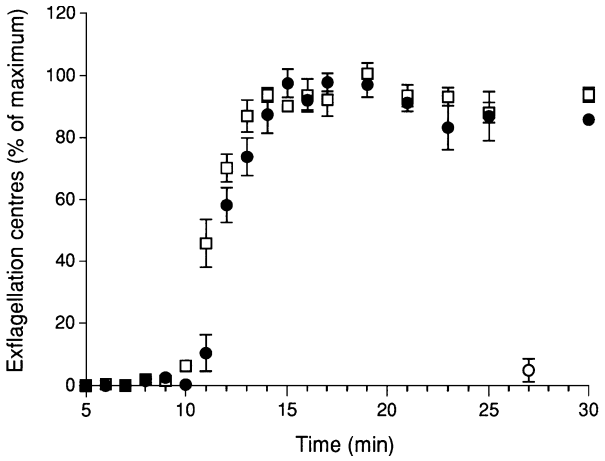

Fig. 1. Time-course of *in vitro* exflagellation of *Plasmodium berghei* at 20 °C induced by pH 8·0 (□) or by a mosquito head extract at pH 7·3 (●). For each condition exflagellation on the same slide was scored every 1 min and expressed as a percentage of the average of the 3 highest scores. A negative control (medium at pH 7·3 in the absence of head extract, ○) was scored at 27 min only and the score expressed as a percentage of the pH 8·0 condition. Values represent the mean of 3 experiments ±S.E.

Table 2. Expression of Pbs21 in *Plasmodium berghei* following 24 h culture at 20 °C in response to either a pH increase or *Anopheles stephensi* head extracts during the first hour

(The data are expressed as a percentage of the total number of Pbs21-positive parasites in the pH 8·0/20 °C condition (±S.E.); average of 4 experiments.)

	Pbs21-positive parasites	
	All stages	Ookinetes
PBS, pH 8·0	100	25·7 (±3·5)
PBS, pH 7·3	1·1 (±0·8)	0·2 (±0·2)
Head extract, pH 7·3	153·8 (±26·0)	30·6 (±11·3)

1986; Kawamoto *et al*. 1991) (Fig. 2B and C); (3) microgametocytes, which are in the process of *releasing* gametes and in which the previously central mass of DNA is separated into as many as 8 smaller portions, which may be located in the forming microgametes (Fig. 2D and E).

For electron microscopical examination of gametocytes, samples from the above cultures were fixed in 200 volumes of 2% glutaraldehyde, 4% formaldehyde and 0·1% picric acid in 50 mM HEPES buffer (pH 7·2) for 1 h at 4 °C. The samples were pelleted, washed in HEPES buffer and post-fixed in 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7·2) for 2 h at 4 °C. Cells were washed several times in distilled water to remove excess phosphate and then stained *en bloc* with aqueous 0·5% uranyl acetate for 6–16 h at 4 °C. The samples were dehydrated in an acetone series and embedded in an Epon-Araldite mixture. Ultrathin sections were

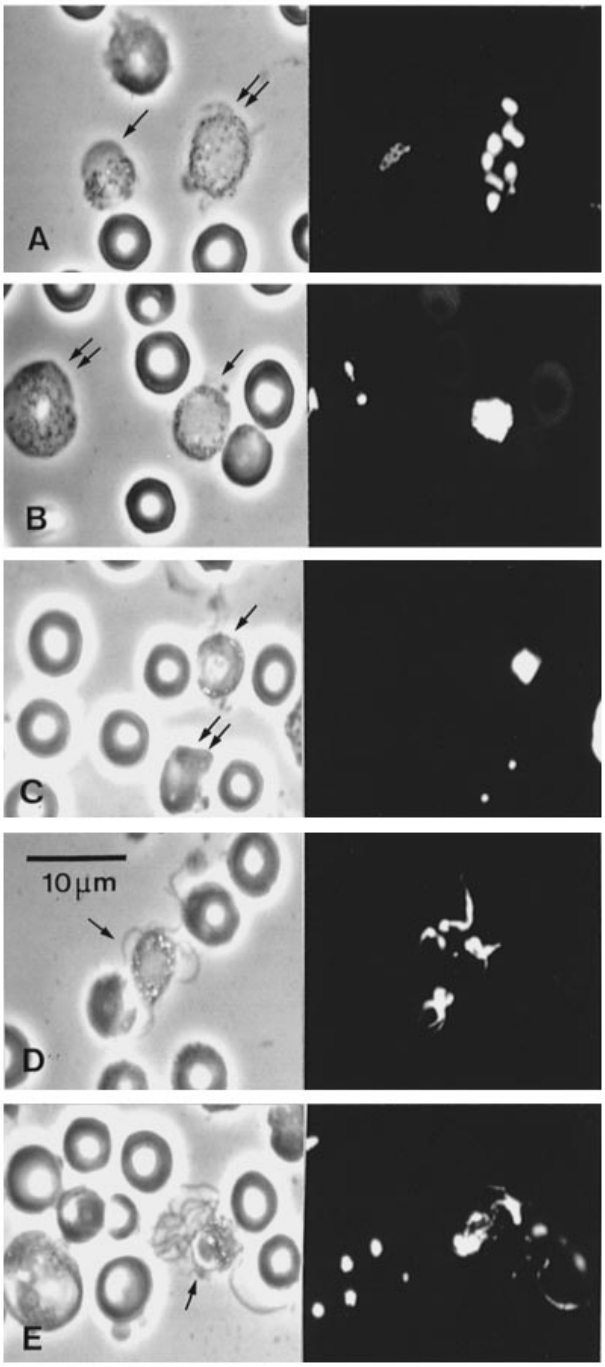

Fig. 2. Detection of DNA synthesis in *Plasmodium berghei* microgametocytes stained with propidium iodide. A phase-transmission image (left side of each panel) and a confocal fluorescence image (right) show the same field. All photographs are from a smear of a culture that was incubated for 20 min at pH 8·0/20 °C and contained microgametocytes in different stages of gametogenesis. Non-replicated microgametocytes (A, arrow) with a lateral nucleus exhibiting weak and diffuse fluorescence. Replicated microgametocytes with a large, strongly fluorescent nucleus containing either non-condensed (B, arrow) or condensed (C, arrow) DNA in the centre of a rounded gametocyte with pigment granules confined to a narrow ring (note the much smaller fluorescent nucleus from asexual stages (double arrows in B and C)). Replicated microgametocytes in the process of releasing gametes, in which the replicated DNA has partially (A, double arrow; D, arrow) or completely (E, arrow) migrated into the forming microgametes.

https://doi.org/10.1017/S0031182097008895 Published online by Cambridge University Press

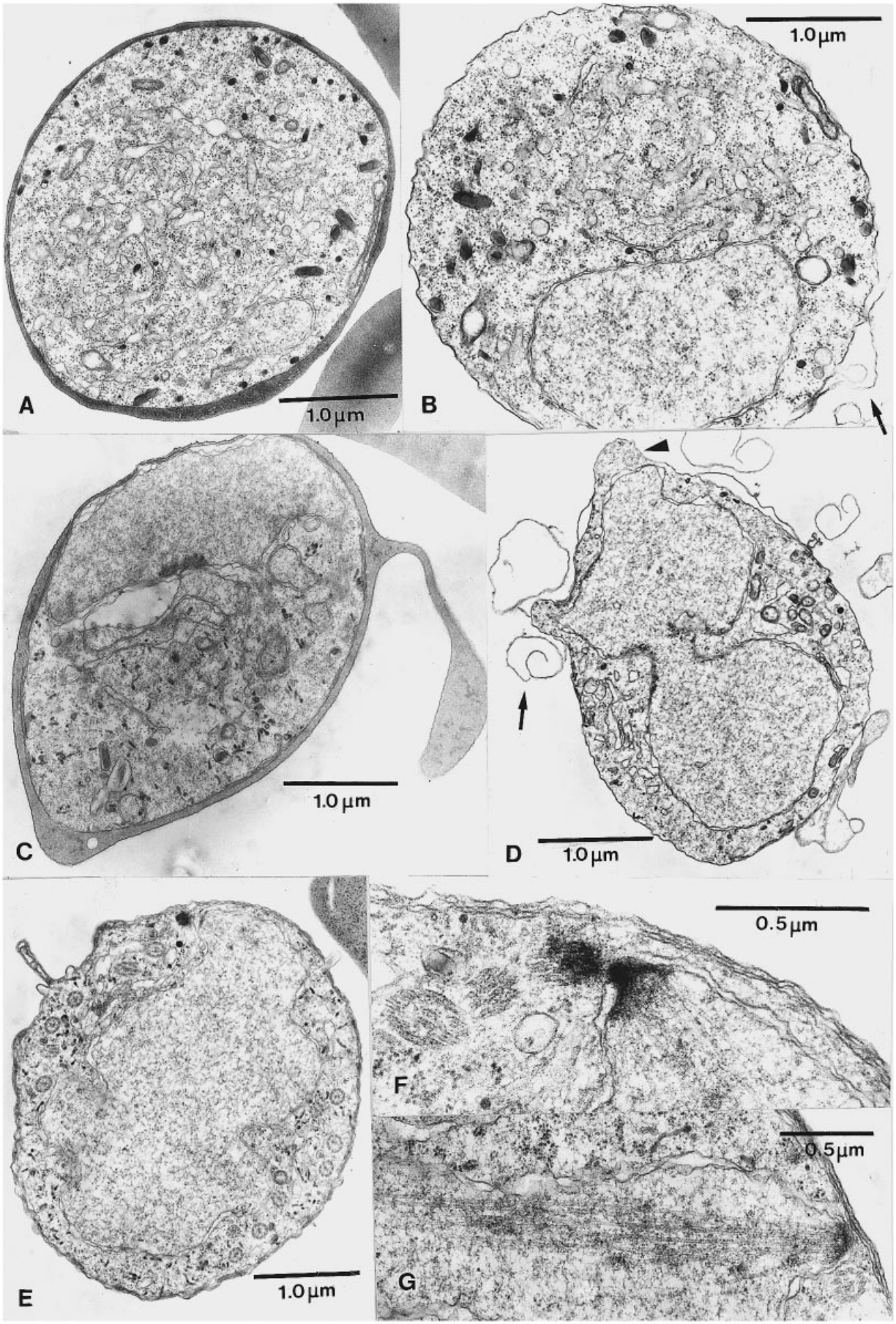

Fig. 3. For legend see opposite.

https://doi.org/10.1017/S0031182097008895 Published online by Cambridge University Press

Table 3. DNA synthesis and exflagellation in *Plasmodium berghei* microgametocytes in response to different combinations of triggers after 8 and 20 min incubation *in vitro*

(The average frequencies (±S.E.) of different developmental stages are expressed as a percentage of all microgametocytes. In each experiment 100 microgametocytes were observed per condition.)

	At 8 min			At 20 min			
		Replicated			Replicated		
	Non-replicated	Not releasing gametes	Releasing gametes	Non-replicated	Not releasing gametes	Releasing gametes	*n**
20 °C							
pH 8·0	13·2 (±4·7)	85·9 (±4·5)	0·9 (±0·6)	12·9 (±2·7)	17·3 (±2·9)	69·8 (±1·3)	5
pH 7·3	95·2 (±3·1)	4·6 (±2·9)	0·2 (±0·2)	94·2 (±2·7)	2·7 (±1·2)	3·1 (±1·5)	5
37 °C							
pH 8·0	100	0	0	100	0	0	1
Head extract, pH 7·3	100	0	0	100	0	0	1
Head extract, pH 8·0	100	0	0	100	0	0	1

* *n*, Number of experiments.

stained with aqueous uranyl acetate and lead citrate, and examined in a Philips 400T electron microscope.

RESULTS

Exflagellation responses to mosquito extracts in vitro

The standard culture medium induced exflagellation at 20 °C when adjusted to pH 8·0 but exflagellation was rarely observed at pH 7·3 (the pH of mouse blood) and, on average, did not exceed 1·5% of the pH 8·0 control. This pH restriction of exflagellation was completely overcome *in vitro* by *An. stephensi* head extracts, which at 20 °C induced the process at either pH (Table 1). Removal of bicarbonate from head extracts did not abolish the effect. Neither pH 8·0 nor mosquito extract triggered exflagellation at 37 °C (Table 1). Whilst the overall time-courses of exflagellation induced at 20 °C by either the pH stimulus or *An. stephensi* head extract were very similar, the onset of exflagellation was invariably delayed by approximately 1 min when mosquito extract was used (Fig. 1). The exflagellation response to *An. stephensi* head extracts was concentration dependent. A 1:8 dilution in pH 7·3 medium reduced the exflagellation response to 50% of the pH 8·0 control and the effect was completely abolished at a dilution of 1:16 (data not shown).

Exflagellation at different temperatures in the mosquito midgut

Groups of *An. stephensi* mosquitoes were allowed to feed for 10 min on the same *P. berghei*-infected mice (day 3 p.i.) at 20 °C or 37 °C. Mosquitoes were dissected on slides between 15 and 35 min after the onset of feeding and gut contents immediately observed for exflagellation. In mosquitoes fed and examined at 20 °C exflagellation was observed in 12 out of 15 individuals with exflagellation levels reaching up to 3 centres of exflagellation per microscopic field. No exflagellation was seen in any of 15 mosquitoes fed and dissected at 37 °C.

Effects of pH and head extracts on macrogametogenesis

In the absence of mosquito extract expression of the surface protein Pbs21 by macrogamete-derived parasite stages in ookinete cultures (20 °C) was dependent on the pH during the first hour of culture. When the pH was 8·0 during the first hour the number of Pbs21-positive parasites in 4 separate cultures, each established from a different mouse, ranged from 58 to 193/10^5 RBC at the end of the 24 h culture period. Ookinetes represented *ca.* 25% of all Pbs21-positive parasites. If the pH was maintained at 7·3 (20 °C) during the first hour of culture, development of macrogametocytes to the Pbs21-positive stage was almost completely suppressed. This suppression was completely reversed by the prior addition of an *An. stephensi* head extract at pH 7·3 (Table 2).

Effects of individual triggers on constituent events of gametogenesis in vitro

Non-activated microgametocytes (Fig. 2A) can be

Fig. 3. Transmission electron micrographs of non-activated and activated *Plasmodium berghei* macrogametocytes (A and B respectively) and microgametocytes (C and D–G respectively) from 20 min *in vitro* cultures (20 °C) at pH 7·3 (A, C) or pH 8·0 (B, D–G). Note red blood cell lysis in B and D with fragments of the host cell membrane (arrows); sections of axonemes in D (arrowhead) to G but not in C; intranuclear spindles in E–G but not in C.

https://doi.org/10.1017/S0031182097008895 Published online by Cambridge University Press

recognized on propidium iodide-stained smears by their weak and disperse nuclear stain. These non-replicated microgametocytes can thus easily be distinguished from replicated microgametocytes which have a strong fluorescence signal from a large, central nucleus (Fig. 2B and C) and from those that have reached the stage of releasing gametes (Fig. 2D and E). Measurements of fluorescence intensities on confocal images confirmed that microgametocytes classified as 'non-replicated' had a DNA content of 1·5 C (when compared to ring stages) whereas those classified as 'replicated' or 'releasing gametes' had increased their DNA content to a maximal 8·5 C level (data not shown).

Under permissive conditions (pH 8·0/20 °C) 87% of all microgametocytes had replicated their DNA after 8 min in culture (Table 3). When cultured for 20 min under these conditions approximately 70% were releasing gametes. After 20 min in the absence of a pH increase more than 94% of the microgametocytes still had a peripheral (unreplicated) nucleus exhibiting only weak fluorescence. The small numbers of microgametocytes that replicated their DNA in the absence of a pH increase were consistent with the low level of exflagellation generally observed under these conditions (see above). No signs of DNA synthesis or changes in nuclear morphology were seen at 37 °C regardless of pH increase or the presence of *An. stephensi* head extract.

Transmission electron microscopy performed on material from the same cultures (Fig. 3) demonstrated that significant levels of axoneme assembly in microgametocytes, and host cell lysis by gametocytes of either sex were only seen under permissive conditions; i.e. at 20 °C and pH 8·0, but not at 37 °C or at pH 7·3.

DISCUSSION

Exflagellation in *P. berghei* can be induced *in vitro* by a drop in temperature and a concomitant pH increase from the physiological pH of mouse blood (pH 7·3) to an optimum range of 7·8–8·1 (reviewed by Sinden *et al.* 1996). The present study confirms these findings and demonstrates that the pH increase is not obligatory and can be fully replaced *in vitro* by a mosquito extract prepared from female *An. stephensi* heads. Exflagellation induced by either MEF or pH is additionally absolutely dependent upon a temperature decrease. Increasing the pH of *An. stephensi* head extracts to 8·0 did not significantly increase their ability to induce exflagellation, indicating that both stimuli individually and completely activated microgametocytes. The activity or activities may be identical to the mosquito exflagellation factor (MEF) detected in tissue extracts from *Ae. aegypti* and *An. stephensi* and in anal excretions of the latter species, which has previously been reported to induce exflagellation in *P. gallinaceum in vitro* (Nijhout, 1979).

The time-courses of mosquito extract and pH 8·0-induced exflagellation are very similar, although the slightly more rapid response to pH suggests it acts 'downstream' to GAF. While the mechanisms by which either pH 8·0 or a mosquito extract induce exflagellation are unknown, it is interesting to note that both are sensitive to amiloride (O. Billker, unpublished observations), an inhibitor of Na^+/H^+ exchange thought to affect exflagellation by inhibiting a rise in cytoplasmic pH (Kawamoto *et al.* 1992).

Macrogametogenesis *in vitro*, like exflagellation, requires a pH increase in addition to a temperature fall, and the pH increase can be replaced by *An. stephensi* head extracts. The effect of MEF is thus not confined to the induction of exflagellation and we suggest it is more appropriate to call this moiety Gametocyte Activating Factor (GAF). The physicochemical nature of GAF is still unknown but monitoring of pH and buffer capacities in all test solutions used in the present study suggests that it is different from either pH or bicarbonate because the activity of test solutions is not affected by removal of bicarbonate ions (Table 1 and Nijhout, 1979).

In contrast to the earlier conclusions of Kawamoto *et al.* (1991, 1993) we find that a combination of at least 2 inducers is required for DNA synthesis and axoneme assembly in the microgametocyte, and host cell lysis in gametocytes of both sexes, suggesting that these constituent events of gametogenesis are not separately induced by different triggers.

The observation that only 2 of the 3 known triggers of gametogenesis are required *in vitro* poses the question as to the natural triggers in the mosquito midgut. Our findings on the role of temperature *in vivo* show unequivocally that exflagellation in the mosquito requires a temperature decrease and highlights the critical role of temperature in overall transmission of *Plasmodium* by the mosquito vector (Vanderberg & Yoeli, 1966; Noden, Kent & Beier, 1995). The possible role of a pH increase as the second trigger in the midgut has been questioned in the past because pH measurements in mosquito bloodmeals revealed values suboptimal for exflagellation *in vitro* (Micks *et al.* 1948; Bishop & McConnachie, 1956). In contrast, GAF is detected in midgut homogenates of a number of mosquito species (Nijhout, 1979; Mendis *et al.* 1994), suggesting that such factors may indeed come into contact with gametocytes in the bloodmeal and thus be the natural secondary trigger of gametogenesis.

O. Billker thanks the German Academic Exchange Service for financial support (Doktorandenstipendium HSPII/AUFE) and the work was supported by the World Health Organization.

REFERENCES

BISHOP, A. & MCCONNACHIE, E. W. (1956). A study of the factors affecting the emergence of the gametocytes of *Plasmodium gallinaceum* from the erythrocytes and the exflagellation of the male gametocytes. *Parasitology* **46**, 192–215.

BUTCHER, G. A., SINDEN, R. E. & BILLKER, O. (1996). *Plasmodium berghei*: infectivity of mice to *Anopheles stephensi* mosquitoes. *Experimental Parasitology* **84**, 371–379.

JANSE, C. J., VAN DER KLOOSTER, P. F. J., VAN DER KAAY, H. J., VAN DER PLOEG, M. & OVERDULVE, J. P. (1986). Rapid repeated DNA replication during microgametogenesis and DNA synthesis in young zygotes of *Plasmodium berghei*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 154–157.

KAWAMOTO, F., ALEJO-BLANCO, R., FLECK, S. L., KAWAMOTO, Y. & SINDEN, R. E. (1990). Possible roles of Ca^{2+} and cGMP as mediators of the exflagellation of *Plasmodium berghei* and *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **42**, 101–108.

KAWAMOTO, F., ALEJO-BLANCO, R., FLECK, S. L. & SINDEN, R. E. (1991). *Plasmodium berghei*: ionic regulation and the induction of gametogenesis. *Experimental Parasitology* **72**, 33–42.

KAWAMOTO, F., FUJIOKA, H., MURAKAMI, R. I., SYAFRUDDIN, HAGIWARA, M., ISHIKAWA, T. & HIDAKA, H. (1993). The roles of Ca^{2+}/calmodulin- and cGMP-dependent pathways in gametogenesis of a rodent malaria parasite, *Plasmodium berghei*. *European Journal of Cell Biology* **60**, 101–107.

KAWAMOTO, F., KIDO, N., HANAICHI, T., DJAMGOZ, M. B. A. & SINDEN, R. E. (1992). Gamete development in *Plasmodium berghei* regulated by ionic exchange mechanisms. *Parasitolgy Research* **78**, 277–284.

MENDIS, C., NODEN, B. H. & BEIER, J. C. (1994). Exflagellation responses of cultured *Plasmodium falciparum* (Haemosporida: Plasmodiidae) gametocytes to human sera and midguts of anopheline mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* **31**, 767–769.

MICKS, D. W., DE CAIRES, P. F. & FRANCO, L. B. (1948). The relationship of exflagellation in avian plasmodia to pH and immunity in the mosquito. *American Journal of Hygiene* **48**, 182–190.

NIJHOUT, M. M. (1979). *Plasmodium gallinaceum*: exflagellation stimulated by a mosquito factor. *Experimental Parasitology* **48**, 75–80.

NODEN, B. H., KENT, M. D. & BEIER, J. C. (1995). The impact of variations in temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*. *Parasitology* **111**, 539–545.

PONNUDURAI, T., LENSEN, A. H. W., VAN GEMERT, G. J. A., BENSINK, M. P. E., BOLMER, M. & MEUWISSEN, J. H. E. TH. (1989). Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. *Parasitology* **98**, 165–173.

SINDEN, R. E., BUTCHER, G., BILLKER, O. & FLECK, S. (1996). Regulation of infectivity of *Plasmodium* to the mosquito vector. *Advances in Parasitology* **38**, 53–117.

SINDEN, R. E., WINGER, L., CARTER, E. H., HARTLEY, R. H., TIRAWANCHAI, N., DAVIES, C. S., MOORE, J. & SLUITERS, J. F. (1987). Ookinete antigens of *Plasmodium berghei*: a light and electron-microscope immunogold study of expression of the 21 kDa determinant recognized by a transmission-blocking antibody. *Proceedings of the Royal Society*, B **230**, 443–458.

VANDERBERG, J. P. & YOELI, M. (1966). Effects of temperature on sporogonic development of *Plasmodium berghei*. *Journal of Parasitology* **52**, 559–564.