Development of malaria parasites in mosquitoes fed with ookinetes suspended in defined media [1]

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Summary. Information concerning the specific nutritional requirements of malarial parasites developing in the mosquito host has been difficult to obtain, owing primarily to the complex nature of the blood meal that accompanies the parasites and the lack of success in culturing the complete invertebrate cycle of *Plasmodium* in vitro.
The present report describes a blood-free system for infecting mosquitoes with ookinetes of *Plasmodium berghei* and for allowing the latter to develop into infective sporozoites. Ookinetes cultured in vitro were separated from blood proteins, suspended in defined medium, and fed to *Anopheles stephensi* mosquitoes through a membrane. The mosquitoes were then maintained on the same defined medium plus 5% sucrose. Infectivity of the parasites was demonstrated 17–19 days later by intracardial inoculation of the macerated mosquitoes into hamsters.
This system makes it possible to evaluate nutritional factors that affect parasite development in the mosquito host under controlled conditions.
Key words. Anopheles stephensi; Plasmodium berghei; transmission of malaria; artificial feeding of mosquitoes; in vitro development of *Plasmodium*.

When a mosquito feeds on a malaria-infected vertebrate host, the imbibed gametocytes undergo a sequence of development which can be outlined as follows; fertilized zygotes are formed, which change into motile ookinetes; these pass through the midgut wall and form oocysts. Sporozoites break out of the oocysts and migrate to the salivary glands, from which they can infect a new host during the next blood meal.
When *Plasmodium berghei* develops at 21 °C in the vector, *Anopheles stephensi*, ookinetes appear 16–22 h after an infected blood meal. The appearance of infective sporozoites requires 16–20 days. Ookinetes can be developed in vitro by culturing infected rodent blood diluted with medium at 21–22 °C [3, 4]. Attempts to obtain further development of the parasite in vitro have met with very limited success [5].
Mosquitoes can be infected experimentally by feeding them through a membrane with blood containing gametocytes or ookinetes [6–8]. The injection of *P. gallinaceum* infected blood, ookinetes or oocysts into the hemocoel of *Aedes aegypti* resulted in development of sporozoites [9, 10].
This paper reports for the first time the successful development of *Plasmodium*, from ookinetes to infective sporozoites, in mosquitoes fed only on defined media with ookinetes. A detailed account of this work is in preparation.

Materials and methods

P. berghei (ANKA strain) gametocytes from infected Syrian hamsters were cultured in vitro for 18 h at 21 °C [8]. Ookinetes that developed were centrifuged on a 36% percoll gradient (Sigma). The greyish layer that formed at the bottom of the gradient was suspended in 36% percoll, overlayed on 45% percoll and recentrifuged. The interface layer from this centrifugation was washed with Ham's F-12 nutrient mixtures containing 10% fetal bovine serum (FBS), 25 mM HEPES buffer, and 0.3% sodium bicarbonate (washing medium). All isolation and washing procedures were carried out at 4 °C [11]. For presentation to mosquitoes, the ookinetes were suspended in either 9% sucrose solution or in formulated Leibovitz's L-15 medium (L-15B) [12, 13]. Ten mM sodium bicarbonate was added to the medium before it was offered to the mosquitoes; no ATP was added [14].
As a control, isolated ookinetes were suspended in uninfected, defibrinated hamster blood diluted with equal parts of Ham's F-12 nutrient mixture containing 10% FBS.
In each of six experiments, batches of 40, 4–6-day-old female *Anopheles stephensi* mosquitoes, previously fed only on a sugar solution, were placed in small cages, and offered the ookinetes suspended in either the blood control, 9% sucrose in water, or L15-B medium. Feeding was through Baudrousche membranes, which are prepared from bovine intestine. They are available from Long & Long Co., Beleville, New Jersey. The concentration of ookinetes varied from 0.9×10^6 to 1.3×10^6 per ml. Mosquitoes that did not feed were discarded. The remainder were kept at 21 °C and provided daily with fresh cotton pads soaked in either 5% sucrose solution for the blood control group and the group fed ookinetes suspended in 9% sucrose, or in L15-B medium plus 5% sucrose for the group fed ookinetes suspended in L15-B medium. 11–14 days after feeding, about 4–19 mosquitoes from each group were dissected and the midgut examined for oocysts. The numbers of fed and infected mosquitoes, as well as the number of oocysts that developed in the midgut wall, were recorded. After 17–19 days the heads and thoraxes of additional

The effect of feeding *A. stephensi* with suspensions of *P. berghei* ookinetes in different media on the infection rate of mosquitoes, the number of developed oocysts and the infectivity of the subsequently ground mosquitoes for injected hamsters.

	Experiment number												Mean ratio between trial and control		No. batches inoculated/infective for hamsters
	1		2		3		4		5		6				
Diet	% females infected (No. dissected)	No. oocysts/infected female	% females infected (No. dissected)	No. oocysts/infected female	% females infected (No. dissected)	No. oocysts/infected female	% females infected (No. dissected)	No. oocysts/infected female	% females infected (No. dissected)	No. oocysts/infected female	% females infected (No. dissected)	No. oocysts/infected female	% females infected	No. oocysts/infected female	
L15-B	75 (8)	38.7	82 (11)	5.7	47 (19)	12	100 (4)	121	–	–	–	–	1.0	2.3	2/2
9% sucrose	–	–	–	–	–	–	22 (18)	1.8	7 (14)	5	33 (7)	4	0.2	0.2	2/0
Blood (control)	50 (6)	6.0	82 (11)	7.8	100 (6)	8.8	100 (6)	150	82 (11)	9.6	100 (5)	106	–	–	5/5

mosquitoes were removed, macerated in wash medium and injected intracardially into healthy hamsters.

Results and discussion

The results of the comparison between a blood meal and blood-free diets, all containing ookinetes, on the development of *Plasmodium* in the mosquito, are presented in the table. The results include the calculated mean ratio between experimental and control diets, and the infectivity of subsequently ground mosquito tissues for hamsters.

Mosquitoes fed ookinetes in L15-B medium were infected to the same extent (ratio of 1, table) as the controls, which were fed ookinetes in blood. However, the average number of oocysts in the L15-B groups (4 experiments) was 2.3 times more than that in the control. Hamsters were readily infected with the ground tissues of mosquitoes infected with ookinetes suspended in L15-B. When the mosquitoes were fed with ookinetes suspended in an isotonic sucrose solution, the percentage of infected mosquitoes and the number of oocysts per infected mosquito were only one fifth of that found in the controls. No infectivity of these mosquitoes was found in two experiments checked.

P. berghei ookinetes have been shown to be capable of penetrating the mosquito midgut wall and completing their intra vector developmental cycle in mosquitoes fed only on defined media with ookinetes. This differs from earlier investigations with *P. gallinaceum* in *A. aegypti*, in which red blood cell factor(s) were found to be necessary for the development of sporozoites from zygotes[9]. This difference in results may be due to the use of a different species of malaria vector and/or parasite, different media, or a different initial developmental stage.

The fact that sucrose alone barely supports *Plasmodium* development may be connected with its low nutritive value, or because it does not trigger some essential physiological change(s) in the parasite and/or its vector.

Infecting mosquitoes via the natural route (feeding) with isolated parasites suspended in a fully defined medium enables the specific nutritional requirements for parasite development in the invertebrate host to be studied, with the eventual goal of maintaining the entire parasite sporogenic cycle in vitro. The method of artificially feeding purified pathogens to their vectors in a defined medium can probably be used in the study of the physiological relationships and requirements of other pathogens.

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