Developmentally Regulated Infectivity of Malaria Sporozoites for Mosquito Salivary Glands and the Vertebrate Host

By Musa G. Touray, Alon Warburg, Andre Laughinghouse, Antoniana U. Krettli,* and Louis H. Miller

*From the Laboratory of Malaria Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; and the *Centro de Pesquisas R. Rachou, Fundacao Oswaldo Cruz, 30.190 Belo Horizonte, Minas Gerais, Brazil*

Summary

Sporozoites are an invasive stage of the malaria parasite in both the mosquito vector and the vertebrate host. We developed an in vivo assay for mosquito salivary gland invasion by preparing *Plasmodium gallinaceum* sporozoites from infected *Aedes aegypti* mosquitoes under physiological conditions and inoculating them into uninfected female *Ae. aegypti*. Sporozoites from mature oocysts were isolated from mosquito abdomens 10 or 11 d after an infective blood meal. Salivary gland sporozoites were isolated 13 or 14 d after an infective blood meal. Purified oocyst sporozoites that were inoculated into uninfected female mosquitoes invaded their salivary glands. Using the same assay system, sporozoites derived from salivary glands did not reinvade the salivary glands after inoculation. Conversely, as few as 10 to 50 salivary gland sporozoites induced infection in chickens, while only 2 of 10 chickens inoculated with 5,000 oocyst sporozoites were infected. Both sporozoite populations were found to express a circumsporozoite protein on the sporozoite surface as determined by immunofluorescence assay and circumsporozoite precipitation test using a circumsporozoite protein-specific monoclonal antibody. We conclude that molecules other than this circumsporozoite protein may be responsible for the differential invasion of mosquito salivary glands or infection of the vertebrate host.

Sporozoites are the stage of the malaria parasite that the mosquito inoculates into the vertebrate host while taking a blood meal, and have been the focus of research toward development of a malaria vaccine (1). Under natural conditions, sporozoites invade and reside in the mosquito salivary glands during the life cycle of the parasite in the mosquito until they are subsequently injected into the vertebrate host during blood feeding. After inoculation into the vertebrate host, sporozoites must invade specific cells to establish infection.

The life cycle of *Plasmodium* parasites in the mosquito (2, 3) begins with the ingestion of sexual stages (gametocytes) during blood feeding on an infected vertebrate host (Fig. 1). The male and female gametes fertilize and produce motile zygotes (ookinetes). The ookinetes traverse the midgut wall and become lodged between the midgut epithelium and the basement membrane, where they round up to form oocysts. The nuclei of oocysts divide many times, and the cytoplasm undergoes marked differentiation. Mature oocysts release thousands of motile sporozoites that are dispersed throughout the mosquito hemocoel by the action of its open circulatory system. Sporozoites selectively invade mosquito salivary glands. Transmission to the vertebrate host occurs during blood feeding.

In their avian host, *P. gallinaceum* sporozoites invade cells of the reticuloendothelial system where they undergo schizogony to produce merozoites that subsequently invade and develop in erythrocytes, thereby initiating the clinical course of the disease. During subsequent schizogony, some of the merozoites differentiate to produce gametocytes, the infective stage for mosquitoes.

Thus, sporozoites invade two different cells: one in the mosquito and one in the vertebrate host. Although invasion of mosquito salivary glands requires specific recognition (4), the mechanism by which sporozoites recognize, attach to, and penetrate the glands is unknown. In a first step toward defining the molecular mechanisms underlying salivary gland recognition and invasion, we developed an in vivo assay which could render this complex biological event amenable to experimental analysis. The assay enabled us to identify some profound biological differences in infectivity between sporozoites obtained from mature oocysts and sporozoites obtained from salivary glands.

Materials and Methods

The experimental protocols described below were designed to obtain adequate numbers of viable sporozoites at specific stages of development in the mosquito for inoculation into uninfected mosquitoes and chickens.

Mosquitoes and Malaria. *Aedes aegypti* (Liverpool Black Eye strain) were reared at 27 ± 1°C and 80 ± 5% relative humidity with 12-h cycles of alternating light and darkness. *P. gallinaceum* (8A strain) was maintained in 3-wk-old white Leghorn chickens (Truslow Farms, Chestertown, MD) by intravenous injection of blood taken from a donor chicken with known parasitemia. Mosquitoes were infected by feeding 5–8-d-old female *Ae. aegypti* on infected chickens with a rising parasitemia of 10–25%. Infection in mosquitoes was monitored by examining wet mounts of guts in PBS or stained with mercurochrome and salivary glands in PBS.

Sporozoite Preparations. Depending on the rate of oocyst maturation, oocyst sporozoites were recovered from mosquito abdomens on day 10 or 11 postinfection. Oocysts were considered mature when large numbers of free motile sporozoites were expelled from oocysts under the pressure of a cover slip after dissecting mosquito midguts. Salivary gland sporozoites were collected from mosquito thoraces on day 13 or 14, at which time no intact oocysts were seen on the midgut.

Infected mosquitoes were immobilized by placing them briefly in a plastic bag containing CO_2. They were emersed in a 1% solution of a commercial detergent (Palmolive; Colgate-Palmolive Company, New York) for 30 sec and rinsed in deionized water. The excess water was removed by briefly placing mosquitoes on absorbent paper after which they were transferred to a petri dish kept on wet ice. The mosquito thorax and abdomen were separated with fine forceps (Fig. 1). Dissections were performed in medium M199 (Bethesda Research Laboratories, Gaithersburg, MD) supplemented with 5% heat-inactivated chicken serum kept in an ice bath. Oocyst sporozoites were extracted from dissected abdomens, and salivary gland sporozoites were obtained from dissected thoraces according to the method described by Ozaki et al. (5). The mortality of mosquitoes at 24 h after inoculation with sporozoites prepared by this method was as follows: 90 to 100% when prepared from the abdomens, and about 80% when collected from the thoraces. Therefore, such preparations were further purified on a Ficoll-Hypaque cushion (2.4 parts 9.0% Ficoll 400, Pharmacia Fine Chemicals, Piscataway, NJ, mixed with 1 part 34% Hypaque solution, Winthrop Pharmaceuticals, New York) as follows: 300 μl of the crude sporozoite suspension was layered on a 1-ml cushion in a 1.5-ml microfuge tube and centrifuged 4.5 min (4950 *g*) at 4°C. The top 200 μl of the supernatant, devoid of sporozoites, was discarded. The next 200 μl, containing the interface, was transferred into a new tube, and 1 ml of *Aedes* saline (6) was added, centrifuged for 4.5 min (4,950 *g*) at 4°C, and decanted. The pellet was then resuspended in 1.2 ml of *Aedes* saline, centrifuged as before at 4°C, and decanted. The final pellet was collected in 20–30 μl *Aedes* saline. ~6,000 sporozoites were recovered per abdomen and 10,000 sporozoites were recovered per thorax. Sporozoites were counted using a hemocytometer and phase contrast microscopy. The sporozoite preparations were virtually free of microscopically visible mosquito debris, and sporozoites were viable as judged by their motility when observed under the microscope. This purification procedure significantly reduced mosquito mortality. 90–100% of mosquitoes injected with such sporozoite preparations were alive 24 h later.

Assay For Sporozoite Invasion of Salivary Glands. Uninfected female *Ae. aegypti* (4–5-d-old) were anesthetized by exposing them to CO_2 in a sealed plastic bag. Anesthetized mosquitoes were placed in a petri dish on wet ice. ~ 30,000 sporozoites in about 1 μl *Aedes* saline were injected into the thoracic cavity using glass capillaries pulled with a vertical needle puller (model 700D, David Kopf Instruments, Tujunga, CA). Injected mosquitoes were maintained at 27°C, 90% relative humidity. After 24 h, the salivary glands were dissected, rinsed, and mounted in PBS on microscope slides. The presence of sporozoites was determined by phase-contrast microscopy after crushing the glands by applying pressure on the coverslip.

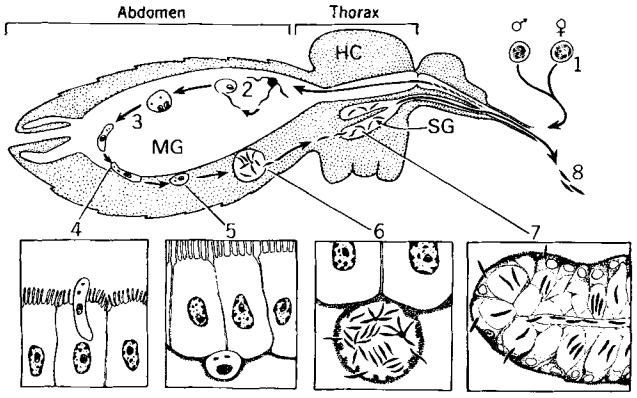

Figure 1. The developmental sequence of *Plasmodium* in mosquitoes. ~ times for *P. gallinaceum* developing in *Ae. aegypti* at 25–27°C are given in parentheses. (*1*) Gametocytes ingested by a female mosquito feeding on a malaria-infected vertebrate. (*2*) Gametes become extracellular. Male gametes fertilize female gametes to produce zygotes (15–60 min). (*3*) Zygotes transform into elongate motile ookinetes (16–24 h). (*4*) Ookinetes penetrate and cross the midgut epithelium (24–30 h). (*5*) Ookinetes reach the basement membrane of the midgut and transform into oocyst (30–40 h). (*6*) Mature sporozoites exit the oocyst (9–11 d). (*7*) Sporozoites invade the salivary glands (10–12 d). (*8*) Mosquito transmits sporozoites during bloodmeals (12 d and thereafter). (Modified from Warburg, A., and L. H. Miller. 1992, *Science [Wash. DC].* 255:448.).

To quantify the number of sporozoites that had invaded, the salivary glands were dissected, rinsed in PBS, and transferred to a microfuge tube in a total volume of 100 μl PBS. Glands were macerated using a loosely fitting teflon douncer. Sporozoites were counted in a hemocytometer, and the mean number per gland was calculated.

Determination of Sporozoite Infectivity in Chickens. A known number of purified sporozoites in 200-μl medium M199 supplemented with 5% heat-inactivated chicken serum was inoculated into 3-wk-old chicks via a wing vein. Daily Giemsa-stained thin smears of peripheral blood were obtained to monitor parasitemia.

Reactivity of Sporozoites with a mAb. The presence of circumsporozoite protein on purified sporozoites was analyzed by indirect immunofluorescent assay (IFA)[1] and circumsporozoite precipitation reaction using a circumsporozoite protein-specific mAb designated N5G3H6. This antibody was raised against *P. gallinaceum* (Brumpt strain) sporozoites extracted from the salivary glands of infected *Ae. fluviatilis* mosquitoes and was of the IgG1 isotype (7). An unrelated IgG1 mAb, 6002, was used in control experiments.

For IFA, approximately 10,000 live sporozoites were incubated with the 10-fold serial dilutions of antibody in 100 μl PBS for 40 min at 25°C. Sporozoites were centrifuged 4.5 min (4,950 *g*) at

[1] *Abbreviation used in this paper:* IFA, indirect immunofluorescent assay.

4°C. The pellet was washed with three changes of 1.5 ml PBS and finally resuspended in 100 μl of 1:50 dilution of FITC-conjugated goat anti-mouse antibodies (Cat. No. 9031 Becton Dickinson Immunocytometry Systems, Mountain View, CA), and incubated at 25°C for 30 min. Sporozoites were washed three times in 1.5 ml PBS, after which they were observed by fluorescence microscopy.

Circumsporozoite precipitation reaction was performed with live sporozoites by mixing sporozoite suspended in 5 μl medium M199 supplemented with 5% chicken serum and an equal volume of antibodies in PBS on a microscope slide (8). The final dilution of antibody in the reaction mixture was 1:20. The reaction mixture was sealed and incubated for 30 min at 37°C. A positive circumsporozoite reaction was characterized by the formation of a tail-like structure on one end of sporozoites.

Results and Discussion

Infectivity to mosquito salivary glands and chickens was determined for both abdominal sporozoites, which contained primarily oocyst sporozoites, and thoracic sporozoites, which contained primarily salivary gland sporozoites. Both sporozoite preparations were separately injected into *Ae. aegypti* female mosquitoes, and their infectivity to salivary glands was determined 24 h later. Between 6.5 and 10.4% of the oocyst sporozoites invaded the salivary glands (Table 1). In contrast, injected salivary gland sporozoites did not reinvade the salivary glands (Table 1). The infectivity of the same two sporozoite preparations to chickens gave the opposite results. As few as 10–50 salivary gland sporozoites were required to induce infection in chickens, while 5,000 oocyst sporozoites infected only 2 of 10 chickens (Table 2). Thus, we showed that newly formed sporozoites in oocysts were able to invade salivary glands of mosquitoes but were only minimally infectious to chickens. After sporozoites have left the oocyst and invaded the salivary glands, they had a different specificity, infecting the chicken but not the salivary glands.

The reason that only 6.5 to 10.4% of inoculated oocyst sporozoites invaded the salivary glands is unknown. Possibilities include premature release of sporozoites from oocysts, death of some sporozoites during preparation, and the possibility that only a fraction of oocyst sporozoites are infectious to the salivary glands. Recently, it has been reported that only 20% of *P. vivax* sporozoites released from oocysts invaded salivary glands of *Anopheles dirus* (9). This finding may, in part, explain our observation that about 10.4% of inoculated oocyst sporozoites were recovered in the salivary glands 24 h later.

Specificity of oocyst sporozoites for salivary glands may indicate that invasion of salivary glands is a receptor-mediated event. This was first demonstrated in experiments with *P. knowlesi* (4), which produces sporozoites in two mosquito species (*An. dirus* and *An. freeborni*), but only the salivary glands of *An. dirus* were invaded by these sporozoites. Salivary glands from each mosquito species were transplanted into the hemocoel of the two mosquito species. Transplanted salivary glands from *An. dirus* became infected in both *An. dirus* and *An. freeborni*. Transplanted salivary glands from *An. freeborni* were not infected in either mosquito. These experiments demonstrated that the salivary glands themselves determined specificity, not some other factor in susceptible mosquitoes such as hemolymph components.

Infectivity of sporozoites for the vertebrate host is usually associated with residence of sporozoites in the mosquito sali-

Table 1. *Sporozoite Infectivity to Salivary Glands of Female Aedes aegypti**

Exp. no.	Source of sporozoites	Day of infection‡	Glands infected/ total glands§	No. sporozoites per gland‖
1	Oocyst	11	9/10	1,950 (12)
	Salivary glands	13	0/10	ND
2	Oocyst	10	5/5	3,000 (13)
	Salivary glands	14	0/10	ND
3	Oocyst	10	7/7	2,555 (9)
	Salivary glands	14	0/10	0 (16)
4	Oocyst	11	10/10	3,117 (17)
	Salivary glands	13	0/10	0 (13)
5	Oocyst	10	10/10	2,233 (9)
	Salivary glands	14	0/10	0 (11)

* 30,000 oocyst (abdominal) or salivary gland (thoracic) sporozoites were injected into each mosquito.
‡ Number of days from the infective blood meal to the preparation of sporozoites.
§ Individual salivary glands were observed for sporozoites by phase contrast microscopy after crushing the glands under a coverslip.
‖ Salivary glands were isolated and homogenized. The total of sporozoites were counted and the mean number per gland determined. The numbers in parentheses = numbers of glands isolated.

Table 2. *Infectivity of Plasmodium gallinaceum Sporozoites to Chickens**

Exp. no.‡	Source of sporozoites	Day of infection§	Sporozoites inoculated per chicken	No. infected/ no. inoculated	Prepatent period (days)‖
1	Oocyst	11	50	0/3	
			500	0/4	
			5,000	0/3	
	Salivary glands	13	50	4/4	9, 9, 9, 10
			500	3/3	7, 7, 8
			5,000	3/3	6, 7, 7
2	Oocyst	10	50	0/3	
			500	0/4	
			5,000	0/3	
	Salivary glands	14	50	3/4	9, 9, 10
			500	4/4	8, 8, 8, 9
			5,000	4/4	7, 7, 7, 7
3	Oocyst	10	50	0/4	
			500	0/4	
			5,000	2/4	9, 9
	Salivary glands	14	10	1/4	11
			100	4/4	7, 8, 8, 8
			1,000	4/4	7, 7, 7, 7

* Sporozoites were either obtained from oocysts or salivary glands of *Aedes aegypti* mosquitoes.
‡ Exps. I, II, and III used the same batch of sporozoites as those used in the experiments in Table 1.
§ Number of days from infective blood meal until preparation of sporozoites.
‖ Prepatent period is the time in days from sporozoite inoculation until the first positive blood film.

vary glands. Sporozoites of the avian malaria parasite *P. gallinaceum* (10) and the rodent parasite *P. berghei* (11) which were derived from oocysts 7 and 10 d, respectively, after an infective blood meal, were not infectious to the vertebrate host. In the same studies, however, both *P. gallinaceum* and *P. berghei* sporozoites obtained from oocysts 9 and 14 d, respectively, after an infective blood meal induced infection after inoculation into the vertebrate host. Furthermore, *P. relictum* (12) and *P. gallinaceum* (13) oocysts maintained in vitro in the absence of salivary glands produced sporozoites infectious to canaries and chickens, respectively. Thus, development of sporozoite infectivity to the vertebrate host appears to be dependent on time and not invasion of and residence in salivary glands.

The differential infectivity of oocyst sporozoites and salivary gland sporozoites may result from a difference in the cell surface ligands of the two stages. The circumsporozoite protein is a major component of the surface of sporozoites (1). Therefore, we tested the possibility of differential expression of a circumsporozoite protein on the surface of oocyst sporozoites and salivary gland sporozoites by IFA and circumsporozoite precipitation test. Both oocyst sporozoites and salivary gland sporozoites showed surface fluorescence using mAb N5G3H6. The highest dilution of antibody to give a positive reaction was $1{:}10^5$ for both oocyst sporozoites and salivary gland sporozoites. In the same experiments, corresponding dilutions of a control mAb did not show any positive reaction with any sporozoite. In two independent experiments, 84 and 75% of oocyst sporozoites gave a positive circumsporozoite precipitation reaction with mAb antibody N5G3H6. In the same experiments, 97 and 93% of salivary gland sporozoites reacted positively. Control mAb 6002 did not induce positive circumsporozoite precipitation reaction in either sporozoite type. Thus, using a circumsporozoite-specific mAb in both IFA and circumsporozoite precipitation tests, we did not detect any major differences between *P. gallinaceum* sporozoites derived from oocytes and salivary glands. This suggests that this circumsporozoite protein alone is not the stage-specific ligand that mediates reception to mosquito salivary glands or the reticuloendothelial cells of the avian host.

Despite the presence of a circumsporozoite protein on both oocyst and salivary gland sporozoites in the present study, differences between the two populations have been previously reported. Polyclonal antibodies raised against salivary gland

sporozoites of *P. berghei* and *P. cynomolgi* reacted by the circumsporozoite precipitation test with salivary gland sporozoites but not with oocyst sporozoites (14, 15). In these earlier studies, the ability of the oocyst sporozoites to invade mosquito salivary glands was not determined, and the specificities of the sera for sporozoite proteins were not tested.

There is more than one protein on the sporozoite surface. The first-described circumsporozoite protein accounts for 10 to 20% of the total proteins synthesized in a sporozoite during residence in mosquito salivary glands and has been a vaccine candidate for malaria control (1, 16). Molecular analysis of the structure of circumsporozoite proteins revealed that they contained an immunodominant repeat region (1) and two highly conserved amino acid motifs termed regions I and II (17). Another sporozoite surface protein has recently been described (18). It also contains a region II-like motif and may also be involved in invasion. The presence of the same sequence motif as region II in vertebrate adhesion molecules properdin and thrombospondin (19) strongly indicated that the circumsporozoite protein may be a ligand for cell binding. Moreover, consistent with the ligand function of the circumsporozoite protein, a peptide from region I of *P. falciparum* sporozoites showed specific and saturable binding to the human hepatoma cell line HepG2-A16 in vitro (20).

Invasion of erythrocytes by another stage of the malaria parasite, the merozoite, is mediated by multiple ligands and host cell receptors. Initial attachment is mediated by a different receptor than junction formation and invasion (21). It would not be surprising, then, for sporozoites to require ligands for host cell invasion, in addition to the circumsporozoite proteins described to date. These requirements may explain the cellular specificity of oocyst sporozoites and salivary gland sporozoites. We now have the stage-specific sporozoites that may offer a reagent for the study of additional sporozoite surface molecules that are required for invasion of the mosquito salivary glands and the target cells in the vertebrate host.

One approach for the control of malaria is the introduction of genes into mosquito populations that render them refractory to malarial infections. This strategy requires the identification of extrinsic factors involved in critical developmental stages of the malaria parasite in the mosquito vector. The present paper identifies invasive capabilities of oocyst sporozoites that are lost once sporozoites have invaded and resided in the salivary glands. These functionally distinct sporozoite populations could serve as tools for identifying specific receptor molecules on the mosquito salivary glands. Genes encoding such receptor molecules may prove invaluable in the biological control of malaria.

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Address correspondence to Dr. Louis H. Miller, Laboratory for Malaria Research, NIAID, Building 4, Room 126, National Institutes of Health, Bethesda, MD 20892.

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