

## NONINFECTIOUS SPOROZOITES IN THE SALIVARY GLANDS OF A MINIMALLY SUSCEPTIBLE ANOPHELINE MOSQUITO

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**ABSTRACT:** In studies to evaluate vector-malaria parasite relationships, we have found that *Anopheles albimanus* is minimally susceptible to the rodent malaria parasite *Plasmodium yoelii*. Normally, less than 10% of *A. albimanus* develop oocyst infections compared to 80–100% for *Anopheles stephensi* and *Anopheles freeborni* mosquitoes. Although sporozoites produced in *A. albimanus* invade the salivary glands, they are not infectious to BALB/c or ICR mice. In 11 experiments with sporozoites from *A. albimanus*, intravenous inoculations of up to 24,000 sporozoites in individual mice failed to produce host infections. In contrast, inoculation of 300 sporozoites obtained from the salivary glands of *A. stephensi* and *A. freeborni* always infected mice. The noninfectious sporozoites from *A. albimanus* were morphologically similar to the infectious sporozoites from *A. stephensi* and yielded 4+ circumsporozoite precipitin reactions when incubated with a monoclonal antibody against the circumsporozoite protein of *P. yoelii*. The presence of noninfectious sporozoites in the salivary glands of *A. albimanus* suggests that this minimally susceptible vector either possesses a toxic factor that abolishes sporozoite infectiousness or lacks a critical substance needed by the sporozoite to become infectious. Sporozoite infectiousness was neither attenuated by incubation of infectious sporozoites with *A. albimanus* salivary glands nor restored when noninfectious sporozoites were incubated with *A. stephensi* salivary glands. These studies provide a starting point for defining the biological basis of sporozoite infectivity.

*Plasmodium yoelii*, isolated in the Central African Republic and originally described as a subspecies of *Plasmodium berghei* (Landau and Chabaud, 1965), was given species status by Killick-Kendrick (1974b). As the natural vector is unknown, several vector competence studies have described the varying degrees of *P. yoelii* development in different anopheline species (Wéry, 1968; Killick-Kendrick, 1971; Landau and Boulard, 1978; Vaughan et al., 1994). Some anopheline mosquito species do not support the full sporogonic development of *P. yoelii*. Wéry (1968) reported that sporogonic development was arrested at the oocyst stage in *Anopheles atroparvus* and *Anopheles freeborni*. *Plasmodium yoelii* sporozoites successfully invaded *Anopheles aztecus* salivary glands, but the sporozoites were not infectious when transmitted to a mouse. Similar findings of noninfectious sporozoites have been reported in various anophelines using *Plasmodium berghei berghei* (Yoeli, 1973), *Plasmodium vinckei brucechwatti* (Killick-Kendrick, 1975), *Plasmodium vinckei vinckei* (Bafort, 1971), and *Plasmodium ath-eruri* (Killick-Kendrick, 1974a).

In general, the infectivity of sporozoites in the salivary glands is influenced only by time (Porter et al., 1954; Vanderberg, 1975) and temperature (Ball and Chao, 1964; Vanderberg and Yoeli, 1966). However, the existence of noninfectious *P. yoelii* sporozoites in the salivary glands of *A. aztecus* suggests that additional mechanisms in mosquito vectors may influence the infectiousness of sporozoites. Observations concerning vector modulation of parasite development have recently been reported between *Lutzomyia longipalpis* and *Leishmania* spp. *Lutzomyia longipalpis* saliva reportedly influences *Leishmania*

spp. stages, which occur in the sandfly midgut (Charlab and Ribeiro, 1993), and may also play a role in the severity of *Leishmania donovani chagasi* infections in humans (Warburg et al., 1994). Although several studies have identified mosquito factors that appear to assist malaria parasite development (Nijhout, 1977; Shahabuddin et al., 1993) and transmission (Champagne, 1994), no studies have examined how sporozoite infectiousness may vary as a function of conditions present in taxonomically diverse *Anopheles* species.

The present study was initiated to determine whether the infectiousness of a malaria parasite to its vertebrate host could be altered during the course of sporogonic development in a minimally susceptible vector species. Initially, we found that only a small proportion of *A. albimanus* develop oocysts when infected with *P. yoelii*. To obtain enough sporozoites for comparative studies, a strain of *A. albimanus* was selectively bred for susceptibility to infection with *P. yoelii*.

### MATERIALS AND METHODS

*Plasmodium yoelii yoelii* 17X lethal (L) and nonlethal (NL) strains were maintained by serial blood passage in BALB/c and ICR mice. Gametocyte senescence was controlled by cycling parasites through mosquitoes every fourth blood passage (Vanderberg and Gwadz, 1980).

Sporogonic development was examined in 3 species of anopheline mosquitoes: *Anopheles albimanus*, *A. freeborni* (Marysville), and *A. stephensi*.

Mice used for mosquito infections were bled 3–4 days after blood passage and exflagellation (wet mount) was assessed. Mice possessing mature gametocytes were anesthetized (0.05 mg pentobarbital per g body weight intraperitoneally [i.p.]) and placed on the screened tops of cages containing mosquitoes. Two mosquito species were infected simultaneously from the same mouse (coinfection) to reduce intermouse variability in gametocyte infectiousness. Mosquitoes were allowed to engorge and unfed mosquitoes were removed. Mosquitoes were then maintained at 24 C, 60% relative humidity. Oocyst infection rates were determined between days 8 and 10 as described by Vaughan et al. (1992).

The salivary glands of mosquitoes from coinfection experiments were dissected 15–18 days after infection. All *A. albimanus* were dissected, whereas only 5–10 *A. stephensi* were dissected due to consistently high infection rates. The salivary glands from each mosquito species were dissected in M199 (Mediatech, Washington, D.C.), pooled into separate glass vials (Kontes, Sigma, St. Louis, Missouri) containing M199, and kept on ice. Upon completion of dissection, glands were homogenized

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and a portion of the homogenate was removed to determine sporozoite number by hemocytometer (Hausser, Thomas, Swedesboro, New Jersey) under a phase-contrast microscope (Vaughan et al., 1992). Sporozoites were kept on ice less than 30 min from time of dissection until known numbers of sporozoites were inoculated intravenously into mice. Mouse infections were assessed by Geimsa stain 6 days after sporozoite inoculation and continued every other day up to day 14.

Selection of a susceptible line of *A. albimanus* to *P. yoelii* 17XNL was initiated by transferring fully engorged *A. albimanus* females into individual, screen-topped, pint-sized cages containing an oviposition cup. Eight days after infection, females that oviposited were dissected to determine the presence of oocysts. Eggs and larvae from oocyst-infected females were pooled to establish the next generation ( $F_n$ ). Sufficient numbers of infected females were obtained by the second generation to set-up several lines from individual females. One line consisted of the progeny from 1  $F_2$  female with 32 oocysts and another line was established from the progeny of 5 infected  $F_2$  females with lower numbers of oocysts (range 1–13).

Sporozoite characterization studies on noninfectious sporozoites from selected and nonselected lines of *A. albimanus* and infectious *A. stephensi*-derived sporozoites included measurement comparisons and circumsporozoite protein (CS) assays. Sporozoites used for measurement studies were dried on poly-L-lysine (Sigma)-coated slides and stained with Giemsa (Sigma). Measurements were obtained at 1,000 $\times$  under oil immersion on a light microscope. Mean lengths of lethal and nonlethal strains of *P. yoelii* 17X sporozoites obtained from *A. stephensi* and *A. albimanus* were compared by analysis of variance (ANOVA) (SPSS, Inc., Chicago, Illinois). Circumsporozoite precipitation (CSP) experiments, conducted once using *P. yoelii* 17XNL sporozoites from both nonselected and selected *A. albimanus* lines and once using 17XL sporozoites from nonselected *A. albimanus*, followed the protocol described by Vanderberg et al. (1969) using NYS1, a monoclonal antibody directed against SSP1 protein on *P. yoelii* 17XNL sporozoites (Charoenvit et al., 1987). The experiments were done to measure the presence of CS as well as to observe whether the sporozoites were motile. The indirect fluorescent assay (IFA) using NYS1, conducted twice with each *P. yoelii* 17X strain, followed methods described by Charoenvit et al. (1987). One of the 2 trials used *P. yoelii* 17XNL sporozoites derived from the selected line of *A. albimanus*.

The influence of salivary gland extracts on sporozoite infectivity was tested using 2 approaches. One approach examined the ability of *A. albimanus* to reduce sporozoite infectiousness by incubating *P. yoelii* 17XNL sporozoites from *A. stephensi* with 50 homogenized *A. albimanus* salivary glands in 20  $\mu$ l of M199 (Gibco) containing 3% bovine serum albumin (BSA; Sigma) (M-BSA) for 1 hr at room temperature (RT). The positive control consisted of *P. yoelii* 17XNL sporozoites from *A. stephensi* incubated for 1 hr at RT in 20  $\mu$ l of M-BSA. The second approach examined whether factors in *A. stephensi* salivary glands could "activate" *A. albimanus* sporozoites by incubating *P. yoelii* 17XNL sporozoites from the salivary glands of the selected *A. albimanus* line with 50 homogenized *A. stephensi* glands in 20  $\mu$ l of M-BSA for 1 hr at RT. The positive and negative controls consisted of infectious *A. stephensi*-derived sporozoites and noninfectious *A. albimanus*-derived sporozoites, respectively, incubated for 1 hr at RT in 20  $\mu$ l of M-BSA.

## RESULTS

In 9 of 10 coinfection experiments, oocyst prevalences for *P. yoelii* 17XL and 17XNL in *A. albimanus* did not exceed 10% (Table I). Prevalences were considerably higher in *A. stephensi*, the species used as the control. In 3 other coinfection experiments using *A. freeborni* as the control species, oocyst prevalences in *A. albimanus* were consistently below 5% (data not shown).

Sporozoites from *A. stephensi* consistently infected mice, but sporozoites from *A. albimanus* never produced infections, despite inoculations of up to 24,000 sporozoites (Table II). In similar coinfection experiments, 50 sporozoites from *A. freeborni* salivary glands produced a mouse infection, but 4 trials

TABLE I. Oocyst infection rates in coinfecting *Anopheles stephensi* and *A. albimanus* using 17X lethal and nonlethal strains of *Plasmodium yoelii*.

Parasite strain	Coinfection experiment no.	Oocyst prevalence (%)	
		<i>A. stephensi</i>	<i>A. albimanus</i>
Lethal	1	5/5 (100)	2/11 (20)
	2	11/11 (100)	1/10 (10)
	3	11/11 (100)	0/9 (0)
	4	8/10 (80)	0/11 (0)
Nonlethal	1	5/5 (100)	1/12 (8)
	2	5/5 (100)	1/13 (8)
	3	5/5 (100)	0/13 (0)
	4	3/6 (50)	0/12 (0)
	5	2/6 (33)	1/12 (8)
	6	5/10 (50)	0/11 (0)

using 500, 1,000 (2 $\times$ ), and 5,000 sporozoites from *A. albimanus* did not produce any infections in BALB/c mice (data not shown).

Oocyst prevalences in *A. albimanus* increased from 3% to 11% in the first selected generation ( $F_1$ ), then up to 22% in the second selected generation ( $F_2$ ).  $F_3$  generation prevalences (%) were similarly high whether the progeny were derived from 1  $F_2$  female (41%) or a pooled group of eggs from 5 infected  $F_2$  females (38%). The  $F_4$  generation attained an oocyst prevalence of 70%.

No significant differences in mean lengths were detected for sporozoites from both lethal ( $F = 0.06$ ,  $df = 1, 23$ ,  $P = 0.8$ ) and nonlethal strains ( $F = 0.43$ ,  $df = 1, 35$ ,  $P = 0.52$ ) of *P. yoelii* 17X when passaged through *A. stephensi* and *A. albimanus*. Sporozoites obtained from the salivary glands of *A. stephensi* and *A. albimanus* produced CSP reactions of 4+, thereby indicating no difference in the release of CS protein between infectious and noninfectious sporozoites, respectively. Additionally, IFA experiments indicated the strong presence of CS protein on infectious sporozoites from *A. stephensi* and noninfectious *A. albimanus* sporozoites. For both sporozoite populations, the monoclonal antibody NYS1 produced a heavy fluorescent staining over the entire sporozoite surface.

The infectivity of sporozoites from *A. stephensi* salivary glands was not altered after incubation in *A. albimanus* salivary gland homogenates (Table III). Additionally, noninfectious *P. yoelii* sporozoites from the salivary glands of the selected *A. albimanus* line did not become infectious when incubated with *A. stephensi* salivary gland homogenates (Table III).

## DISCUSSION

*Plasmodium yoelii* sporozoites obtained from *A. albimanus* salivary glands consistently failed to produce infections, even when 24,000 sporozoites from the selected line of *A. albimanus* were inoculated into 1 mouse. Conversely, sporozoites from coinfecting *A. stephensi* and *A. freeborni* always produced infections in mice. Noninfectious sporozoites have been reported earlier in the salivary glands of various anophelines, but the observations were either based on single cohort infections and small sample sizes (Wéry, 1968; Bafort, 1971; Yoeli, 1973), or the results were described with no indication of sample size or

TABLE II. Infectiousness of *Plasmodium yoelii* (17X lethal and nonlethal) sporozoites obtained from the salivary glands of *Anopheles stephensi* and *A. albimanus* and inoculated into mice.

Parasite strain	Co-infection experiment no.	Mouse type	<i>A. stephensi</i>		<i>A. albimanus</i>	
			No. sporozoites inoculated	Infection outcome (±)	No. sporozoites inoculated	Infection outcome (±)
Lethal	1	BALB/c	8,000	+	200	-
	2	BALB/c	1,000	+	100	-
	3	BALB/c	500	+	100	-
	4	BALB/c	1,500	+	100	-
Nonlethal	1	BALB/c	300	+	300	-, -*
	2	BALB/c	400	+	400	-
	3	BALB/c	500	+	400	-
	4	ICR	10,000	+	6,000†	-
	5	ICR	24,000	+	24,000†	-, -*

\* The reported numbers of sporozoites were inoculated into each of 2 mice.

† Sporozoites obtained from the selected line of *A. albimanus*.

number of infections (Yoeli, 1973; Killick-Kendrick, 1974a, 1975). Small sample sizes from single cohort infections make it difficult to determine whether any sporozoites in the salivary glands were capable of inducing infections in vertebrate hosts (Wéry, 1968). The results from our study, based on observations from infections using both lethal and nonlethal strains of *P. yoelii* 17X and a selected line of *A. albimanus*, have provided the sporozoite densities necessary to establish the presence of noninfectious sporozoites in *A. albimanus* salivary glands.

Initial studies comparing noninfectious sporozoites from *A. albimanus* with infectious sporozoites were impaired by small numbers of noninfectious sporozoites due to low oocyst prevalences. This limitation was overcome by pooling larvae from oocyst-infected *A. albimanus*, i.e., mass selection, and producing a strain of *P. yoelii*-susceptible *A. albimanus* in 4 generations. Subsequently, noninfectious *P. yoelii* sporozoites from *A. albimanus* were found to be similar to infectious sporozoites from *A. stephensi* salivary glands in both size and ability to produce CS protein. The presence of *P. yoelii* sporozoites in *A. albimanus* salivary glands indicated the sporozoites were alive and capable of gland invasion, but were somehow attenuated in their infectiousness to mice. These studies, although basic, provide a foundation from which further comparisons between infectious and noninfectious sporozoites can be made at the biochemical or molecular level.

The presence of noninfectious *P. yoelii* sporozoites in *A. albimanus* salivary glands also provided an opportunity to pursue what causes these sporozoites to be noninfectious. There are 2 general mechanisms by which *P. yoelii* sporozoites can be rendered noninfectious in *A. albimanus*. One possible mechanism involves the action of some toxic or antagonistic mosquito factor, which renders healthy sporozoites noninfectious (Bafort, 1971). Another mechanism may involve the lack of some substance or metabolite in the mosquito needed to "activate" sporozoites (Bafort, 1971). These mechanisms can influence parasite development in any location throughout the mosquito (Weathersby, 1952, 1975). Preliminary in vitro studies focused

TABLE III. The effect of *Anopheles stephensi* and *A. albimanus* salivary gland homogenates on *Plasmodium yoelii* 17XNL sporozoite infectivity.

Sporozoite source	Salivary gland homogenate source	Experiment no.	Experimental group	No. of sporozoites	Infection outcome (±)	
<i>A. albimanus</i> *	<i>A. stephensi</i>	1	Test§	615	-	
			- Control‡	615	-	
			+ Control§	1,000	+	
<i>A. stephensi</i>	<i>A. albimanus</i>	1	Test†	10,000	+	
			+ Control§	10,000	+	
			2	Test†	6,000	+
				+ Control§	6,000	+
				Test†	2,000	+
3	+ Control§	2,000	+			

\* Sporozoites obtained from the selected line of *A. albimanus*.

† Test = *P. yoelii* 17XNL sporozoites from 1 species of mosquito with 50 homogenized glands of the other mosquito species in 20 µl of M199 containing 3% BSA for 1 hr at RT.

‡ - Control = *P. yoelii* 17XNL sporozoites from *A. albimanus* salivary glands incubated for 1 hr at RT in 20 µl M199 containing 3% BSA.

§ + Control = *P. yoelii* 17XNL sporozoites from *A. stephensi* salivary glands incubated for 1 hr at RT in 20 µl M199 containing 3% BSA.

on whether salivary gland factors influenced sporozoite infectiousness. The influence of "toxic" factors in *A. albimanus* saliva was addressed by incubating *A. stephensi*-derived sporozoites in *A. albimanus* salivary gland extract. Conversely, the incubation of *A. albimanus*-derived sporozoites in *A. stephensi* salivary gland extract examined the possible restoration of an essential sporozoite "activating" factor that might be absent from *A. albimanus* saliva. Neither approach had any effect on the infectiousness nor noninfectiousness of the respective sporozoites. Whereas these results indicate that the site of action may not be in the salivary glands, the influence of salivary gland enzymes on sporozoite infectivity cannot be completely ruled out.

Differences in the levels of various salivary gland enzymes are beginning to be identified between various anopheline species (Cupp et al., 1994). Apyrase levels are considerably higher in *A. freeborni* (Ribiero et al., 1985) and *A. albimanus* (Cupp et al., 1994) compared with *A. stephensi* (Ribiero et al., 1985). As *P. yoelii* sporozoites from *A. freeborni* salivary glands were infectious while *A. albimanus*-derived sporozoites were not infectious, apyrase does not appear to influence the infectivity of *P. yoelii* sporozoites. However, other salivary enzymes may be involved. *Anopheles albimanus* salivary glands have recently been identified as active sites of salivary peroxidase and catechol oxidase activity (Ribiero and Nussenzweig, 1993). Future studies could evaluate the peroxidase activity levels in *A. stephensi* and *A. freeborni* salivary glands and compare activity levels with *A. albimanus* as a possible means to describe the alteration of *P. yoelii* sporozoite infectiousness.

An alternate site where *P. yoelii* sporozoites could be rendered noninfectious is the mosquito hemolymph (Weathersby, 1975). Plasmodial parasites are continuously exposed to hemolymph for up to 2 wk in developing oocysts and 1-3 days outside the oocysts, prior to salivary gland invasion. However, whereas early studies identified factors in mosquito hemolymph that are toxic to sporozoites (Weathersby, 1975), no further studies have

characterized the biochemical nature of these toxic factors. Future studies should include species comparisons of anopheline hemolymph as a means to define the site of action where *P. yoelii* sporozoites are rendered noninfectious in *A. albimanus*.

Despite the questions that remain from this study concerning where and how *P. yoelii* sporozoite infectivity is altered in *A. albimanus*, the isolation of noninfectious *P. yoelii* sporozoites from *A. albimanus* salivary glands provides a useful system to begin elucidating the influence of mosquito vectors on the infectiousness of sporozoites.

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