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INGESTION OF *PLASMODIUM FALCIPARUM* SPOROZOITES DURING TRANSMISSION BY ANOPHELINE MOSQUITOES

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Abstract. We investigated the process of sporozoite transmission during blood feeding for *Anopheles gambiae* and *An. stephensi* experimentally infected with *Plasmodium falciparum*. When infective mosquitoes were fed 22–25 days postinfection on an anesthetized rat, sporozoites were detected in the midgut of 96.5% of 57 *An. gambiae* (geometric mean [GM] = 32.5, range 3–374) and in 96.2% of 26 *An. stephensi* (GM = 19.5, range 1–345). There were no significant differences between species either in salivary gland sporozoite loads or in the number of ingested sporozoites. There was a significant linear relationship between sporozoite loads and the numbers of ingested sporozoites for both *An. gambiae* ($r = 0.38$) and *An. stephensi* ($r = 0.69$). Subsequently, *An. gambiae* were tested for sporozoite transmission by allowing them to feed individually on a suspended capillary tube containing 10 μl of blood. A total of 83.3% of 18 infective mosquitoes transmitted a GM of 5.9 (range 1–36) sporozoites. The same mosquitoes contained a GM of 23.4 (range 2–165) ingested sporozoites. The number of ingested sporozoites was related to sporozoite loads ($r = 0.42$) but not to the number of sporozoites ejected into capillary tubes. Ingested sporozoites remained in the midgut up to 10 hr after feeding. The comparable numbers of sporozoites ingested by infective mosquitoes in both experiments indicates that the actual number of sporozoites transmitted to the vertebrate host during blood feeding is significantly reduced by the blood ingestion process. The detection of ingested sporozoites by simple methods that avoid contamination by mature oocyst or hemolymph sporozoites may facilitate determinations of the minimal numbers of sporozoites released during blood feeding either by naturally or experimentally infected mosquitoes.

Malaria parasite transmission occurs when sporozoites are released during salivation by blood feeding infective mosquitoes. Various methods have been used to quantify the numbers of sporozoites released during blood feeding. By all accounts, individual mosquitoes vary greatly in their potential for sporozoite output, but most mosquitoes probably release fewer than 50 sporozoites per feeding.[1–6] The actual number of sporozoites released, which represents only a small fraction of the sporozoites present in the salivary glands,[7] is probably restricted by the diameter of the salivary duct.[8] The conservative release of sporozoites by blood feeding mosquitoes, whereby salivary glands are not depleted of sporozoites even in vectors that feed up to 15 times,[9] allows mosquitoes to remain infective for life. A potentially important consideration is that some of the sporozoites inoculated into the vertebrate host during bloodfeeding may be withdrawn with the blood ingested by the mosquito. Yorke and Macfie in 1924 demonstrated sporozoites in the blood meal of a single infected *Anopheles maculipennis* two hours after feeding.[10]

This study investigates aspects of sporozoite ingestion during the blood feeding process for two species of anopheline mosquitoes experimentally infected with *Plasmodium falciparum*. The objective was to determine the frequency and numbers of sporozoites ingested during blood feeding in relation to sporozoite loads and the number of sporozoites transmitted in vitro.

MATERIALS AND METHODS

Anopheles gambiae (G3 strain) and *An. stephensi* (Dutch strain) were experimentally infected with the NF54 strain of *P. falciparum*.[3] For all experiments, mosquitoes were deprived of sugar solution, but not water, overnight to enhance the blood feeding response.

TABLE 1

Frequency and number of Plasmodium falciparum *sporozoites in the midgut immediately after sporozoite-positive mosquitoes fed on an anaesthetized rat*

Species	No. tested	% with sporozoites in the midgut	Geometric mean number of sporozoites			
			Salivary glands	Blood meal	Remaining in midgut	Total in midgut
Anopheles gambiae	57	96.5	832.7	29.3	5.4	32.5
Anopheles stephensi	26	96.2	448.8	16.6	4.4	19.5

Infected *An. gambiae* and *An. stephensi*, 22–25 days postinfection, were fed on anesthetized Lewis rats to determine the proportion of infected mosquitoes ingesting sporozoites and the number of sporozoites ingested during normal blood feeding. Individual mosquitoes were dissected to isolate the fresh blood meal within the midgut, the remaining midgut tissue, and the salivary glands. The following precautions were taken to avoid contaminating the midgut contents with sporozoites from the hemolymph. Tergal and sternal plates at the base of the first abdominal segment were cut gently. The abdominal cuticle was removed without damaging the alimentary tract. Holding the mosquito from the wings, the midgut was rinsed several times in a depression slide containing RPMI-1640 medium (Gibco, Grand Island, NY). Next, an incision into the anterior region of the foregut separated the midgut from the alimentary tract. The excised midgut was held at either the foregut or the Malphigian tubules and suspended in 10 μl of RPMI medium contained in the well of a depression slide. The contents of the midgut, which immediately emptied into the well, were mixed and spotted onto a microslide. The remaining midgut was transferred to a 200-μl glass microtissue grinder (Kontes, Vineland, NJ) containing 10 μl of RPMI medium, triturated, and spotted onto a microslide. Salivary glands were dissected on a clean slide, transferred to a micro-tissue grinder, homogenized in 70 μl of RPMI medium, and a 5-μl aliquot was spotted on a microslide and air-dried.

Thus, each mosquito was represented by three spots on a single slide; one for sporozoites from the salivary glands, one for the gut, and one for the blood contents of the gut. Sporozoites were counted at 400× after staining with fluorescein isothiocyanate–conjugated monoclonal antibody 2A10.[11] Sporozoite loads, the number of sporozoites in the salivary glands, were determined by counting sporozoites in the 5-μl aliquot and multiplying the result by 14, the dilution factor.[4] As a control, midguts from infected unfed mosquitoes were also dissected, rinsed several times in RPMI medium, triturated, and checked for the presence of sporozoites as described above.

Individual infected *An. gambiae* were tested for in vitro transmission and ingestion of sporozoites by allowing them to feed on a capillary tube containing 10 μl of a 10% suspension of human erythrocytes in RPMI medium.[3,4] After each mosquito became engorged (approximately 5 min), blood from the capillary tube was expelled onto a microslide. The mosquito was then dissected and checked for the presence of salivary gland sporozoites. Infective mosquitoes were then processed as described above to determine sporozoite loads, the number of sporozoites ingested during the feeding process, and the number of sporozoites transmitted in vitro.

To investigate the fate of ingested sporozoites, infected *An. gambiae* were fed on an anesthetized rat and killed at 2, 4, 6, 8, 9, 10, 11, 12, 14, 17, 22, and 24 hr after feeding. Midgut contents of mosquitoes with salivary gland sporozoites were examined for sporozoites as described above.

Data on sporozoite loads, ingested sporozoites, and in vitro transmitted sporozoites were log transformed to normalize variance prior to testing by analysis of variance and regression analysis.

RESULTS

Plasmodium falciparum sporozoites were detected in the midguts of 96.5% (55 of 57) of *An. gambiae* and 96.2% (25 of 26) of *An. stephensi* immediately after they had fed on an anesthetized rat (Table 1). The total number of ingested sporozoites, representing those in the blood meal and those remaining in the midgut, ranged from 3 to 374 (geometric mean [GM] = 32.5) for *An. gambiae* and from 1 to 345 (GM = 19.5) for *An. stephensi*. Sporozoites remained in the midgut

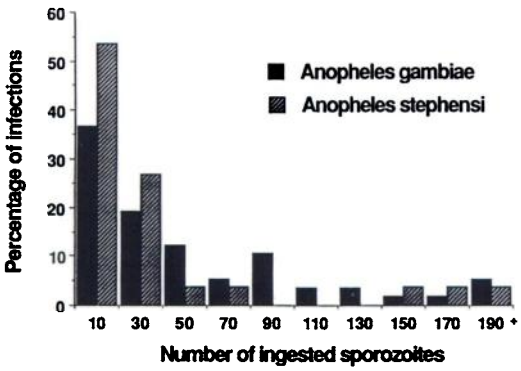

FIGURE 1. Frequency distribution of the number of *Plasmodium falciparum* sporozoites detected in the midguts of infective *Anopheles gambiae* (n = 57) and *An. stephensi* (n = 26) immediately after feeding on an anesthetized rat. Midpoint values for intervals of 20 are shown on the x-axis.

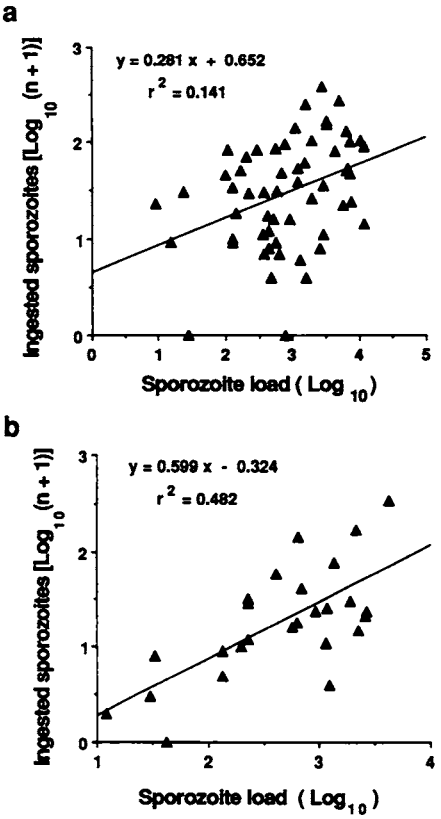

FIGURE 2. Relationships between the number of ingested *Plasmodium falciparum* sporozoites and the number of sporozoites in the salivary glands (sporozoite loads) for **a**, *Anopheles gambiae* (n = 57) and **b**, *An. stephensi* (n = 26) fed on an anesthetized rat.

after blood was expelled in 52.6% (30 of 57) of the *An. gambiae* and in 73.1% (19 of 26) of the *An. stephensi*; for these mosquitoes, the GM number of sporozoites was 5.4 and 4.4, respectively. In infected mosquitoes not allowed to blood feed, there were no sporozoites in the midguts of five *An. gambiae* and five *An. stephensi*. There were no significant differences between species in either the number of sporozoites in the salivary glands ($F = 2.47$, degrees of freedom [df] = 1,81, $P = 0.1156$), or in the total number of ingested sporozoites ($F = 2.42$, df = 1,81, $P = 0.1194$) (Figure 1). The total number of ingested sporozoites was associated with sporozoite loads for *An. gambiae* ($r = 0.38$, df = 55, $P = 0.0004$) and *An. stephensi* ($r = 0.69$, df = 24, $P < 0.0001$) (Figure 2).

Sporozoites were transmitted in vitro by 83.3% of 18 infective *An. gambiae*, containing a GM of 1,421 (range 70–14,810) salivary gland sporozoites. The number of sporozoites transmitted ranged from 1 to 36 (GM = 5.9). Immediately after feeding, sporozoites were also detected in the midguts of 88.8% of the 18 mosquitoes and the GM number of ingested sporozoites was 23.4 (range 2–165). Of the 18 mosquitoes, the three that did not transmit contained ingested sporozoites, while the two without ingested sporozoites transmitted in vitro. No sporozoites were detected in the midguts of five unfed infective mosquitoes from the same cohort.

Of the total sporozoites either transmitted in vitro or ingested (GM = 28.8), the latter represented 66.6% of the total sporozoites released during feeding. Figure 3 compares the frequency distributions of transmitted and ingested sporozoites. The number of sporozoites transmitted in vitro was not related to either sporozoite loads ($r = 0.03$, df = 16, $P = 0.9875$) or the number of ingested sporozoites ($r = 0.32$, df = 16, $P = 0.1911$). However, the number of ingested sporozoites was related to sporozoite loads ($r = 0.42$, df = 16, $P = 0.0549$).

Sporozoites were present in the midguts of 90.5% of 21 infective *An. gambiae* examined at intervals of 2, 4, 6, 8, 9, and 10 hr after blood feeding. There were no time-related differences in the numbers of sporozoites detected ($F = 0.38$, df = 5,15, $P = 0.8540$). No sporozoites were detected after 10 hr, when a total of 23 infective mosquitoes were examined at 11, 12, 14, 17, 22, and 24 hr after blood feeding.

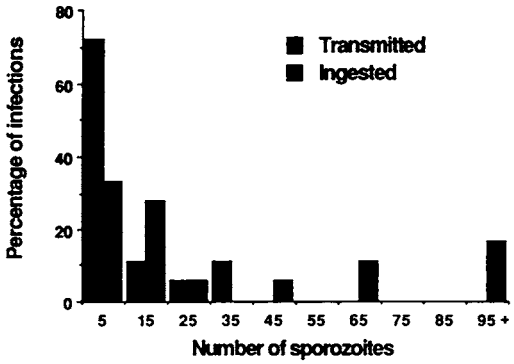

FIGURE 3. Frequency distribution of the number of *Plasmodium falciparum* sporozoites transmitted in vitro in relation to the number of ingested sporozoites for 18 infective *Anopheles gambiae*. Midpoint values for intervals of 10 are shown on the x-axis.

DISCUSSION

The ingestion of sporozoites by mosquitoes during blood feeding is a normal process associated with transmission. Nearly all of the infective mosquitoes of the two vector species tested contained ingested *P. falciparum* sporozoites immediately after feeding. In the only other observation of this phenomenon, York and Macfie stated that "when the mosquito feeds, salivary secretion is first poured out into the wound and then partly withdrawn with the blood into the stomach."[10] This explanation is consistent with mosquito feeding mechanisms,[12] salivary gland and duct structure, and the salivation process.[13]

Sporozoite ingestion during blood feeding effectively removes infective sporozoites from the vertebrate host and significantly decreases the efficiency of transmission. There is an important distinction between the total number of sporozoites inoculated by the vector and the number of sporozoites that remain in the vertebrate host after blood feeding. Our technique of feeding infected mosquitoes on a vertebrate host and then detecting ingested sporozoites represents a suitable, non-manipulative method for estimating minimal numbers of sporozoites released during blood feeding.

To answer the more difficult and epidemiologically significant question of how many sporozoites remain in the vertebrate host, we conducted parallel in vitro transmission experiments. As in previous studies,[3,4] few sporozoites (GM = 5.9) were transmitted. These represented only one-third of the total sporozoites released (i.e., two-thirds were ingested). This may be a realistic estimate because there were no significant differences in the number of sporozoites ingested between mosquitoes tested for in vitro transmission (GM = 23.4) and those fed on rats (GM = 32.5). Certainly, the total number of sporozoites released during in vitro feeding (GM = 28.8, median = 34) is consistent with the median of 22 sporozoites reported by Ponnudurai and others.[5] In their studies, *P. falciparum*-infected *An. stephensi* fed through a mouse skin membrane on continuously stirred blood, a method designed to simulate normal blood feeding but to inhibit the ingestion of sporozoites.

There was no relationship between the number of sporozoites transmitted in vitro and sporozoite loads, as in previous studies[3-5]. This may be because most of the released sporozoites were ingested, as evident from the significant relationships between the number of ingested sporozoites and sporozoite loads. For mosquitoes fed on rats, the amount of variation in the number of ingested sporozoites explained by sporozoite loads was greater for *An. stephensi* (48%) than for *An. gambiae* (14%). The stronger relationship for *An. stephensi* may be a function of feeding behavior. Compared with *An. gambiae*, *An. stephensi* spends a longer time feeding (i.e., approximately 1 min longer)[14] and ingests more blood.[15] Since sporozoites are salivated in clumps or bundles,[3,5,6] the greater feeding capacity of *An. stephensi* may allow this species to ingest a more representative sample of the total sporozoites released during feeding. However, since the total number of sporozoites remaining in the host is not a function of either the number ingested or the sporozoite load, it is inappropriate to suggest that one vector species is more or less infective than another based on feeding time or volume of blood ingested.

Sporozoites ingested during blood feeding remained in the midgut for up to 10 hr, without significant decreases in numbers. Their disappearance after 10 hr was probably associated with increases in bacterial activity rather than with increases in the predominant digestive enzyme, trypsin (unpublished data). We also excluded the possibility that ingested sporozoites could leave the midgut, and possibly migrate to the salivary glands, by feeding uninfected *An. stephensi* on large numbers of sporozoites contained in a blood mixture, detecting and counting sporozoites in the midgut, and then checking the mosquitoes

(n = 47) after 24 hr for the presence of *P. falciparum* circumsporozoite protein by enzyme-linked immunosorbent assay;[16] there was no evidence of sporozoites.

The simple methods described for detecting and quantifying ingested sporozoites may have practical applications for estimating minimal numbers of sporozoites transmitted during blood feeding. For example, such techniques could be used in conjunction with malaria vaccine challenge studies. Current methods provide no indication of the number of sporozoites transmitted by infected mosquitoes,[3] and some feedings by fewer than five gland-positive mosquitoes do not produce infections in individuals serving as controls.[17] The methods could also be used for naturally infected mosquitoes. Since sporozoites remain detectable in the midgut up to 10 hr, any freshly fed mosquitoes captured indoors in the morning, and confirmed to have salivary gland sporozoites, could be examined. If the technique is used properly, there is minimal risk of contamination by sporozoites from the hemolymph or mature oocysts. The technique would be especially valuable for malaria field studies in areas such as Africa, where vector species are highly anthropophilic.

In conclusion, the ingestion of sporozoites by infective mosquitoes during blood feeding is a normal process that serves to decrease the number of viable sporozoites capable of initiating malaria infections in the vertebrate host. The impact on transmission and host infections may be dramatic because mosquitoes ingest up to two-thirds of the sporozoites released during feeding. Answering the question of how many sporozoites remain in the host after blood feeding is difficult, but the number of sporozoites ingested can provide reasonable estimates of the minimal number of sporozoites released during blood feeding by infective mosquitoes.

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