RE-INGESTION OF *PLASMODIUM BERGHEI* SPOROZOITES AFTER DELIVERY INTO THE HOST BY MOSQUITOES

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Abstract. Malaria-infected mosquitoes feeding on a mammalian host inject sporozoites into the skin to induce a malaria infection. The numbers of sporozoites ultimately able to reach the liver may be important determinants of the characteristics of the ensuing blood infection. Because feeding mosquitoes not only inject sporozoites into the host but concomitantly ingest blood to obtain their bloodmeal, some sporozoites are re-ingested by the feeding mosquito. We studied transmission of fluorescent *Plasmodium berghei* sporozoites injected into mice by *Anopheles stephensi* mosquitoes and found that the numbers of sporozoites re-ingested by mosquitoes are comparable to numbers previously reported to be delivered directly into mice. Thus, re-ingestion of sporozoites likely plays a significant role in transmission dynamics of malaria by mosquitoes, and may account for the failure of some sporozoite-infected mosquitoes to induce a blood infection.

INTRODUCTION

Not all mosquitoes with infectious sporozoites in their salivary glands are successful in initiating malaria infections while feeding on a mammalian host.\(^1,2\) Accordingly, experimental mosquito challenge of immunized humans normally uses several infected mosquitoes to ensure 100% infection of the non-immunized control subjects.\(^3,4\) The assumption has been that transmission failure by some infected mosquitoes is due to their inability to introduce sporozoites into the mammalian host. Sporozoites are injected by mosquitoes into avascular skin tissue of mice.\(^5,6\) Multiple studies have shown that the numbers of sporozoites delivered or involved in initiating an infection within the host represent only a tiny percentage of the sporozoites within the salivary glands of the mosquito (reviewed in \(^7\)). Because this appears to be a phenomenon dictated by the particular anatomy of the mosquito salivary glands and ducts, we proposed that it is as likely to occur in the field as in the laboratory.\(^7\) Thus, if significant numbers of sporozoites released from the proboscis are immediately re-ingested by the mosquito, the numbers available to initiate the mammalian infection could be significantly reduced, thereby altering the subsequent course of the infection or preventing it from becoming established in the first place.

Estimates of the numbers of sporozoites injected by individual mosquitoes into the skin of mice have been made by evaluating skin biopsy specimens post-feeding, using real-time polymerase chain reaction (PCR) assessment of parasite 18S rRNA.\(^8\) This study reported small numbers of infected mosquitoes that fed but did not leave behind detectable numbers of sporozoites within the skin. However, an inability of mosquitoes to inject or to leave behind residual sporozoites in the host's skin does not necessarily imply an actual failure to deliver sporozoites. Such failure could also be caused by the re-ingestion of these sporozoites by the mosquito after their delivery into the skin. This possibility must be considered as a component of the quantitative dynamics of sporozoite delivery by mosquitoes.

Many years ago, Yorke and MacFie\(^9\) demonstrated the presence of sporozoites in the bloodmeal of an infected *Anopheles maculipennis* shortly after it had fed. Re-ingestion of sporozoites into the midgut of feeding mosquitoes was confirmed with *An. gambiae* and *An. stephensi* that had been experimentally infected with *Plasmodium falciparum* and then allowed to feed on a rat.\(^10\) Our long-term interest in the kinetics of mosquito delivery of sporozoites into the skin of rodents\(^5,7\) prompted us to re-examine the phenomenon of sporozoite re-ingestion by mosquitoes. Because previous studies\(^8,10\) may have failed to differentiate between sporozoites that had been re-ingested by mosquitoes after deposition into the host versus sporozoites released into the midgut lumens from ectopic oocysts emptying into the interior of the midgut,\(^11\) we took precautions to resolve this question. *An. stephensi* mosquitoes infected with *P. berghei* sporozoites that express enhanced green fluorescent protein (EGFP) were used for these studies because they are readily detectable with great sensitivity by fluorescence microscopy.\(^5,12\) The methodology described in the current article allows us to distinguish between sporozoites ingested into the midgut after their delivery into the mammalian host versus those released directly into the midgut from ectopic oocysts.

MATERIALS AND METHODS

Mice, mosquitoes, and malaria parasites. Mice were female BALB/c 6–8 weeks old obtained from Taconic Farms Inc. Our protocol for maintenance and care of experimental animals was approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. Our animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD). Mosquitoes were *An. stephensi* infected with a clone of *P. berghei* whose sporozoites constitutively express EGFP.\(^12\)

Microscopy. We used a Leica MZ16FA fluorescence stereoscopic microscope with a 2.0X stereoscopic objective lens (Leica Microsystems, Wetzlar, Germany). Illumination for fluorescence studies was with an EXPO X-Cite 120 F1 illumination system and with a GFP2 filter set (restricting excitation to 480 ± 20 nm and fluorescence signal emission to wavelengths longer than 510 nm). Images were acquired with a Leica DFC350 FX digital camera and saved as digital files for further analysis and processing. We used Leica FW4000 software for documentation and analysis.

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**Experimental studies.** Mosquitoes were allowed to feed for 3–4 minutes on mice anesthetized by IP injection of ketamine (50 mg/kg) plus xylazine (10 mg/kg) and acepromazine (1.7 mg/kg). Midguts were then removed through the posterior segment of the abdomen, placed on a microscope slide, and examined by fluorescence microscopy to detect the presence of sporozoite-containing oocysts on the midgut wall. Midguts were then treated (see later in this article) to quench fluorescence from endogenous oocysts and sporozoites on the exterior of the midgut wall. The head and thorax of the mosquito were washed and moved to a clean slide for dissecting out the salivary glands and determination of their infection with sporozoites.

**Quenching of enhanced green fluorescent protein fluorescence.** To distinguish newly ingested salivary gland sporozoites within the lumen of the midgut from endogenous sporozoites released from oocysts on the hemocoel side, midguts were dissected out and treated with 0.4 mg/ml Crystal Violet to quench fluorescence of oocysts and sporozoites at the exterior of the midgut. We used an aqueous dilution of a starting solution of 3 mg/ml of Crystal Violet (DIFCO—Gram Crystal Violet Primary Stain [Becton-Dickinson and Co., Franklin Lakes, NJ]). Midguts were treated for 2–3 minutes to quench EGFP emission, then washed with water, dried gently with bibulous paper, placed on a microscope slide, and pulled apart with fine forceps to release ingested blood. Each midgut was then covered with a 22-mm² coverslip and observed by fluorescence microscopy. Total number of sporozoites within the bloodmeal of each midgut was determined by counts of sporozoites in randomly sampled fields under the coverslip. We found that after such treatment the fluorescence of endogenous sporozoites at the exterior of the midgut became undetectable (Figure 1A and 1B), whereas newly re-ingested sporozoites released from the bloodmeal mass in the midgut lumen remained EGFP-positive (Figure 1C). As a control, cohorts of these mosquitoes that had not been fed on a mouse were treated in the same way to quench EGFP-emission by external oocysts and sporozoites. The midguts of these mosquitoes were then observed for the presence of fluorescence from sporozoites. In all cases (fed and non-fed mosquitoes), salivary glands were checked to establish that all mosquitoes had gland infections with sporozoites.

**Source of bloodmeal.** To test whether the numbers of re-ingested sporozoites were influenced by the portion of the body on which mosquitoes had probed and fed, we compared mosquitoes that had been fed on the ear pinna versus the ventral abdomen.

**Control: interference effects of blood.** To see if ingested blood from the midgut had an effect on reliability of counts of sporozoites within the bloodmeal mass, we prepared a suspension of sporozoites from salivary gland dissections and did counts to determine the concentration of sporozoites per 1 μL in the suspension. We compared this with counts made when aliquots from the same sporozoite suspension were mixed with the midgut contents of non-infected mosquitoes that had just taken bloodmeals on mice not infected with malaria.

**Control: distinguishing between endogenous oocyst versus re-ingested salivary gland sporozoites.** To further confirm that the luminal sporozoites that we observed were salivary gland sporozoites re-ingested by the mosquito rather than endogenous oocyst sporozoites already residing within the midgut, we used two procedures previously shown to differ-

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**Figure 1.** Micrographs of EGFP—*Plasmodium berghei* oocysts and sporozoites. A, Midgut with fluorescing oocysts (bright circles on midgut wall fluoresce bright green under fluorescence microscopy). B, Same midgut (at same magnification) after treatment to quench fluorescence. C, Fluorescing sporozoites from lumen of midgut after quenching of fluorescence on exterior wall of midgut.
entiate between these developmental stages of sporozoites. These were (1) assessment of gliding motility of sporozoites on microscope slides in the presence of 2% bovine serum albumin (BSA), which is characteristic of sporozoites of salivary gland but not oocyst origin and (2) assessment of the circumsporozoite precipitation (CSP) reaction, which also is characteristic of sporozoites of salivary gland but not oocyst origin. The CSP reaction was done by adding 6 μg of monoclonal antibody 3D11 to each slide preparation of sporozoites in the presence of 2% BSA. For each experiment, a suspension of dissected-out salivary gland sporozoites and a suspension of dissected-out midgut sporozoites from the same cohorts of mosquitoes were assessed in parallel for motility and CSP-reactivity as baseline controls. Slides were coded and read blindly.

RESULTS

We found that mosquitoes ingested a mean of 98.8 ± 13.3 (SE) sporozoites per mosquito (N = 84) during feeding on mice. Distribution of sporozoite numbers per mosquito is shown in Figure 2. There were no detectable, ingested sporozoites in the midgut lumens of ~10% of the mosquitoes. Approximately half of the mosquitoes ingested 100 or fewer sporozoites; there was a progressive decrease in the numbers of mosquitoes found to ingest larger numbers of sporozoites. No sporozoites were ever detected in the midgut lumens of the 30 infected, control mosquitoes that had not been allowed to feed on mice. These data include only mosquitoes that were shown to possess both midgut infections with oocysts and salivary gland infections with sporozoites. To investigate whether significant numbers of re-ingested sporozoites might have been omitted from our totals because of loss through the anus, we used capillary heparinized, hematocrit tubes to collect drops of blood from the posterior of mosquitoes during and after bloodmeals. No sporozoites were ever seen within drops of blood emerging from the anus during and post-bloodmeal.

We further confirmed that the luminal sporozoites that we observed were salivary gland sporozoites re-ingested by the mosquito rather than endogenous oocyst sporozoites from the lumen of the midgut by assessing the developmental phenotypes of these sporozoites (Figure 3). Evaluation of motility of

![Figure 2](image1.png)

**Figure 2.** Distribution of numbers of *Plasmodium berghei* sporozoites re-ingested by *Anopheles stephensi* mosquitoes after feeding on mice (total number of mosquitoes = 84).

![Figure 3](image2.png)

**Figure 3.** Developmental phenotypes (gliding motility and CSP reactivity) of *Plasmodium berghei* sporozoites recovered from midgut lumen of *Anopheles stephensi* mosquitoes post-bloodmeal. Phenotypes observed are identical to those of sporozoites obtained directly from salivary glands rather than from oocysts.
these sporozoites in the presence of 2% BSA (Figure 3A) indicated that 74.5% of these sporozoites recovered from the midgut lumen after a bloodmeal exhibited classic gliding motility. As baseline controls, 83.5% of sporozoites from dissected-out salivary glands obtained from a cohort of these mosquitoes exhibited gliding motility, whereas 0% of sporozoites from midguts of non-fed mosquitoes exhibited gliding (N = 25 mosquitoes for each group). It was important to examine these sporozoites as soon as possible after the bloodmeal because sporozoite motility was lost after extended residence in the midgut lumen.

In a similar manner, evaluation of the CSP reactivity of these sporozoites in the presence of 2% BSA and anti-sporozoite monoclonal antibodies (Figure 3B) indicated that 67.7% of these sporozoites recovered from the midgut lumen after a bloodmeal exhibited CSP reactivity. As baseline controls, 69.4% of sporozoites from dissected-out salivary glands obtained from a cohort of these mosquitoes had positive CSP reactions whereas only 0.8% of sporozoites from midguts of non-fed mosquitoes exhibited CSP reactivity.

In an attempt to determine whether the site of the bloodmeal influenced the numbers of sporozoites re-ingested, we allowed infected mosquitoes to feed on either the ear pinna or the ventral abdomen of mice for our standard 3–4-minute feeding time (N = 26 mosquitoes for each group). Those fed on the ear ingested a mean of 83.8 ± 15.8 (SE) sporozoites, whereas those fed on the abdomen ingested a mean of 114.1 ± 30.4 (SE) sporozoites but the differences between the means were not significant as determined by Student paired t test (P = 0.32).

We tested the possible role that ingested blood cells might have on interfering with our ability to visualize and count sporozoites from within the midgut lumen. We found virtually no differences between counts of sporozoites in aliquots of a suspension of sporozoites from dissected-out salivary glands versus counts taken when the corresponding aliquots of sporozoites were added to appropriate amounts of the bloodmeal contents of non-infected mosquitoes.

DISCUSSION

We have clearly demonstrated that some *Plasmodium* sporozoites injected by mosquitoes feeding on a mouse host are immediately re-ingested by the mosquito into its midgut, thus confirming and extending prior conclusions by others. However, we were not confident that we could successfully use a previously described procedure to remove ingested blood from the lumen of the midgut without contaminating the sample with exogenous sporozoites from the hemocoel side of the midgut. In that study, potentially contaminating midgut oocyst infections tended to be light (Beier JC, personal communication), whereas they were heavy within the present study. Accordingly, we developed a procedure that allowed us to successfully quench fluorescence from exogenous sporozoites from the exterior of the midgut, with no discernable effect on fluorescence of sporozoites within the interior of the midgut.

Another of our technical concerns related to a report that some oocysts may develop in ectopic sites within the midgut, resulting in the release of some oocyst sporozoites directly into the midgut lumen, a concern not previously dealt with by others who assumed that all sporozoites found in the midgut lumen had been re-ingested after release from the mosquito proboscis. Not all workers have been able to demonstrate ectopic development of oocysts, so we felt obliged to do controlled studies to establish whether this was occurring within our own system. Our studies failed to demonstrate sporozoites associated with midguts of the non-fed mosquitoes studied after treatment of their midguts to eliminate fluorescence from external oocysts. Because this treatment does not eliminate fluorescence from sporozoites within the midgut lumen, we would have observed such sporozoites from ectopic oocysts, if they had been present in any of the 30 infected but non-fed mosquitoes so examined.

One of the strongest arguments that had been offered on behalf of the release of sporozoites into the midgut interior from ectopic oocysts was that “feces” collected from mosquitoes fed on mice were able to induce parasitemia after injection into uninfected mice. However, an alternate explanation, as demonstrated by our own study, is that the sporozoites collected in the feces were from salivary glands and had been re-ingested by the feeding mosquitoes after delivery into the mouse. Mosquitoes sometimes concentrate the cellular component of their bloodmeal by releasing tiny drops of plasma-rich fluid from their anus during feeding. However, it required collection of such fluid from hundreds of mosquitoes to obtain infective sporozoites in this prior study, so passage of sporozoites via the feces must have been a relatively rare event. We examined the fluid released from the posterior of individual mosquitoes post-feeding and never found any sporozoites by microscopy.

In further confirmation that the intra-luminal sporozoites we observed had been released from salivary glands and re-ingested by the feeding mosquitoes, we found that these intraluminal sporozoites were fully motile and exhibited full CSP reactivity, whereas sporozoites from the midguts of non-fed mosquitoes exhibited neither motility nor CSP reactivity. The acquisition of motility and CSP reactivity by salivary gland sporozoites is an unambiguous way of differentiating them from oocyst sporozoites. We conclude that sporozoites from ectopic oocysts could not account for the sporozoites that we observed within the bloodmeal mass in the lumen of the midgut after feeding.

The re-ingestion of some sporozoites by mosquitoes during feeding is not a surprising phenomenon. Some saliva injected into the host during mosquito engorgement is likely to be re-ingested with the blood back into the midgut. The food canal within the proboscis, through which blood is sucked into the mosquito, has more than 100 times the cross-sectional area of the parallel channel for saliva down through which saliva flows into the tip of the food canal. It has been estimated that the rate of blood flow into *Aedes aegypti* is of the order of 10^5–10^6 times greater than the opposing flow rate of saliva down the salivary channel and into the bite site. One should thus expect some sporozoites within secreted saliva to be re-ingested during mosquito engorgement.

Re-ingestion of sporozoites is likely enhanced when the mosquito is actively imbibing blood rather than injecting sporozoites into avascular tissue while probing for a blood source. Accordingly, one might expect more sporozoites to remain in the skin during extended, non-productive probing by the mosquito than after rapid localization and ingestion of a blood source. Thus, if mosquitoes tend to probe for longer periods of time in less vascularized tissue before they are able
to encounter a blood source, one might expect greater likelihood of sporozoite transmission into such less vascularized areas of skin. To see if there were differences in numbers of sporozoites re-ingested after feeding on different sites, we compared infected mosquitoes that had fed on the ear pinna versus the ventral abdomen. Greater numbers of sporozoites were found in the midguts of mosquitoes that had fed on the abdomen but we were unable to show that these differences were statistically significant. Further work is needed in this area.

As previously noted, it has been reported that some individual, infected mosquitoes fed but did not leave behind detectable numbers of sporozoites within the skin6 or were unable to induce a blood infection1,2 and it was concluded that there was no transmission of sporozoites in these cases. However, the possibility of re-ingestion of all or most of the injected sporozoites cannot be ruled out in cases of transmission failure. The numbers of sporozoites re-ingested by mosquitoes appear to be of the same order of magnitude as the numbers that remain in the mammalian host and initiate the infection.4,6,8,9,19,20 Thus, this phenomenon of sporozoite re-ingestion likely plays a significant role in transmission dynamics of malaria by mosquitoes. The technique that we have described to measure sporozoite re-ingestion will be a useful addition to other quantitative measures of the kinetics of sporozoite transmission during the infection of the mammalian host by mosquito-injected sporozoites.

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