Direct Microscopic Quantification of Dynamics of *Plasmodium berghei* Sporozoite Transmission from Mosquitoes to Mice

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The number of malaria sporozoites delivered to a host by mosquitoes is thought to have a significant influence on the subsequent course of the infection in the mammalian host. We did studies with *Anopheles stephensi* mosquitoes with salivary gland infections of *Plasmodium berghei* sporozoites expressing a red fluorescent protein. After individual mosquitoes fed on an ear pinna or the ventral abdomen of a mouse, fluorescence microscopy was used to count numbers of sporozoites. Mosquitoes allowed to feed on the ear for periods of 3 versus 15 min deposited means of 281 versus 452 sporozoites, respectively, into the skin; this may have epidemiological implications because mosquitoes can feed for longer periods of time on sleeping hosts. Mosquitoes feeding on the ventral abdomen injected sporozoites not only into the skin but also into the underlying peritoneal musculature. Although mosquitoes injected fewer sporozoites into the abdominal tissues, more of these were reingested into the mosquito midgut, probably a consequence of easier access to blood intake from the abdominal area. The most consistent parameter of sporozoite transmission dynamics under all conditions of mosquito probing and feeding was the relatively slow release rate of sporozoites (~1 to 2.5 per second) from the mosquito proboscis. The numbers of sporozoites introduced into the host by mosquitoes and the transmission efficiencies of sporozoite delivery are multifactorial phenomena that vary with length of probing time, skin site being fed upon, and numbers of sporozoites within the salivary glands.

Malaria infection is initiated by an *Anopheles stephensi* mosquito injecting sporozoites into the skin of its mammalian host while probing for a blood meal (1, 15, 16, 21, 25, 28). Continuation of the malaria infection depends upon these sporozoites leaving the skin via dermal blood vessels and then traveling to the liver and developing into exoenzymophagocytic forms (EEF). The relatively large number of studies attempting to quantify the dynamics of this process attests to the importance with which it has been viewed by malariologists; the history and rationale for such attempts have been reviewed previously (6, 24, 26). The approaches used have included assessment of numbers of sporozoites released by mosquitoes into liquid media (reviewed in reference 6) or onto glass slides (12), counting of EEF after mosquito feeding on rodent hosts (26), and determination of sporozoite 18S rRNA (16) or β-galactosidase expressed by transgenic sporozoites (8) within the skin of a mouse after sporozoite introduction by mosquitoes.

Each of these approaches has had both advantages and limitations. We believe that the most biologically appropriate approach requires direct feeding of infected mosquitoes on a living host and that the most accurate and precise means of quantification is direct microscopic observation and counting of injected sporozoites. Aside from the unequivocal nature of direct counts of morphologically recognizable sporozoites, this method allows correlation of specific locations of the injected sporozoites with mosquito feeding behavior and with host pathological changes, especially mosquito probe-induced formation of hematomas in the skin. Timed studies of ongoing sporozoite residence within the skin also allow identification of changes in sporozoite morphology over time (25). Finally, we have recently used fluorescence microscopy to confirm that some sporozoites injected by mosquitoes are reingested with the blood meal into the mosquito midgut (13), a process that affects inoculum size. Accordingly, we assessed delivery of fluorescent *Plasmodium berghei* sporozoites into the skin of laboratory mice by *Anopheles stephensi* mosquitoes. We also investigated the role of variables such as length of mosquito feeding time and location and vasculature of the mosquito feeding site on dynamics of sporozoite injection.

**MATERIALS AND METHODS**

*Sporozoites.* *A. stephensi* mosquitoes were infected with a clone of the rodent malaria parasite *Plasmodium berghei* whose sporozoites constitutively express RedStar, an improved red fluorescence protein (10). We used standard protocols for infecting and maintaining mosquitoes (24), which were infected by feeding upon gametocyte-carrying 6- to 8-week-old Swiss Webster mice (Taconic Farms, Inc., Germantown, NY). Our protocols for maintenance and use of experimental animals were approved by the Institutional Animal Care and Use Committee at New York University School of Medicine, and our animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD). Mosquitoes were used for sporozoite transmission studies 18 days after the infective blood meal.

*Mosquito feeding on ear pinnae.* Mosquitoes fed on mice anesthetized by intraperitoneal injection of ketamine (50 mg/kg of body weight) plus xylazine (10 mg/kg) and acepromazine (1.7 mg/kg); mice were placed on a warming tray. To restrict the area of sporozoite deposition for more efficient counting of sporozoites, the dorsal aspect of one ear pinna was partially masked with tape so that only its edge (8 to 10 mm long and 2 to 3 mm wide) was accessible to a feeding mosquito. Mosquitoes were kept individually in plastic feeding tubes 2.5 cm in length and with an inside diameter of 1.5 cm; one end of the tube was covered with netting through which the mosquito was able to feed, and the other end was closed with a screw-on cap. Each mosquito was allowed to probe and feed on the ear through the netting for up to either 3 or 15 min from the time that probing was first observed. Because mosquitoes often cease probing/feeding activity and...
subsequently return to such activity, we recorded both the total number of probes and the sum of the times during which probing/feeding was observed over the 3- or 15-min observation period for each mosquito.

**Experimental protocol.** After each feeding, the fed-upon region of the ear plus the taped adjacent area ~2.5 mm beyond this were immediately excised. This biopsy specimen was separated into dorsal and ventral leaflets with fine forceps (9), and each leaflet was mounted under a coverslip and examined by fluorescence microscopy to count sporozoites and record their distribution. Preliminary mapping and counting of sporozoites were done with biopsy specimens mounted between two coverslips, thus enabling the skin specimens to be flipped and viewed from both sides. This enabled us to confirm that our procedure allows all sporozoites within the specimen to be viewed and counted and that two separate “blinded” observers got consistent results. Hematomas were identified by transmitted bright-field illumination. In some cases, leaflets of skin were further “blinded” by fluorescein-labeled dextran (500 kDa; Molecular Probes) diluted in medium to 590 to 650 nm (peak of 620).

**RESULTS**

Three-minute mosquito feeding on ear. Data were collected from 40 individual mosquito feeds on mouse ear pinnae over 3 min from the start of the initial probe. Because mosquitoes fed intermittently after initiating probing, the mean total actual probing/feeding time during this 3-min period was only 126.2 s. Mosquitoes with confirmed salivary gland infections probed a mean of 6.4 times during this feeding period (Table 1). After feeding, a mean of 281.1 sporozoites was found within the fed-upon area of the skin (range of 0 to 1,293 and median of 41,651 ± 6,775) after being fed upon.

**Table 1. Mosquito injection of sporozoites into mice**

<table>
<thead>
<tr>
<th>Feeding point and time (min)</th>
<th>No. of micea</th>
<th>Time (s) of actual probing/feeding</th>
<th>No. of probes by mosquito</th>
<th>Total no. of sporozoites initially in salivary glandsb</th>
<th>No. of sporozoites in midgut tissuec</th>
<th>No. of sporozoites reingested by mosquitoes</th>
<th>Total no. of sporozoites released by proboscisd</th>
<th>Total no. of sporozoites released by proboscis per second</th>
<th>No. (%) of mice developing parasitemia after being fed upon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>126.2 ± 6.3</td>
<td>6.4 ± 0.4</td>
<td>11,282 ± 1,809</td>
<td>281.1 ± 44.7</td>
<td>0.5 ± 0.5</td>
<td>281.7 ± 44.7</td>
<td>2.2 ± 0.4</td>
<td>0/40 (0)</td>
</tr>
<tr>
<td>15</td>
<td>40</td>
<td>396.0 ± 28.6</td>
<td>14.5 ± 1.2</td>
<td>13,226 ± 2,312</td>
<td>451.7 ± 70.5</td>
<td>101.2 ± 58.2</td>
<td>552.9 ± 88.4</td>
<td>1.4 ± 0.2</td>
<td>9/40 (22.5)</td>
</tr>
<tr>
<td>Ventral abdomen</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>137.2 ± 8.2</td>
<td>3.8 ± 0.4</td>
<td>41,651 ± 6,775</td>
<td>122.6 ± 35.7</td>
<td>202.3 ± 82.0</td>
<td>324.9 ± 90.2</td>
<td>2.5 ± 0.7</td>
<td>6/27 (22.2)</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>488.0 ± 33.4</td>
<td>8.9 ± 1.1</td>
<td>33,248 ± 6,510</td>
<td>116.1 ± 28.4</td>
<td>279.1 ± 78.2</td>
<td>392.1 ± 89.2</td>
<td>0.8 ± 0.2</td>
<td>13/25 (52)</td>
</tr>
</tbody>
</table>

a Each mouse was fed upon by one mosquito.

b Number found in salivary glands postfeeding plus number reingested into midgut plus number observed in fed-upon tissue.

c Number observed in fed-upon tissue plus number reingested into midgut. **, P < 0.01.

d Not assessed for first mice in series of 30 studied.

*e*, P < 0.05.

f Means ± standard errors are shown.
mosquitoes. Frequency distributions of numbers of sporozoites identified within ear pinnae are shown in Fig. 2A. Only 1 of the 40 mosquitoes reingested sporozoites (n = 22). The salivary glands were calculated to have a mean of 11,282 sporozoites prior to initiation of feeding (range of 927 to 50,750 and median of 6,569 sporozoites per mosquito). Interestingly, the mosquito with the greatest number of salivary gland sporozoites transmitted no detectable sporozoites into the skin. The frequency distributions of this transmission efficiency (number of sporozoites identified in skin divided by number in salivary glands) are shown in Fig. 2B.

The mouse ear pinna is ~0.28 mm in thickness near its periphery (where mosquitoes were permitted to feed) and is made up of dorsal and ventral “leaflets” of skin, separated by a sheet of cartilage. Because the mosquito proboscis is ~2 mm in length, it can pass completely through the ear pinna. We restricted mosquito probing to the dorsal side and then compared both sides of the pinna for proboscis-induced trauma. We observed hematomas in 89% of biopsy specimens. Of these, hematomas were seen within both dorsal and ventral leaflets in 78% of the mice, indicating that the proboscis commonly passed through the entire thickness of the ear. Sporozoites were regularly observed within both leaflets of the ear; we added these to obtain the total for each ear.

Fifteen-minute mosquito feeding on ear. The mean total actual probing/feeding time within the 15-min time frames was 396 s out of a total possible time of 900 s. Mosquitoes probed a mean of 14.5 times (Table 1). Following feeding, a mean of 451.7 sporozoites was identified within the fed-upon area of the skin (range of 0 to 1,921 and median of 315 sporozoites per mosquito). The mean number of sporozoites left in the skin after the 15-min feeding intervals was significantly greater than the mean number left during the 3-min feeding intervals, as calculated by Student’s t test (P < 0.05). Nine of the 40 mosquitoes were found to have reingested sporozoites into their midguts (range of 7 to 1,889 sporozoites). The mean number of reingested sporozoites for all 40 mosquitoes was 101.2, compared with a mean of only 0.5 reingested sporozoites for 3-min feedings. For each of the 40 mosquitoes, the mean total number of sporozoites released from the proboscis (number found in the skin plus number reingested) was 552.9 (range of 0 to 2,224). During the 15-min feeds, a mean of 1.4 sporozoites per second was released from proboscises, compared to 2.2 per second during 3-min feeds. The mean total number of sporozoites released from proboscises during the 15-min feeding intervals was significantly greater than the mean number released during the 3-min feeding intervals, as calculated by Student’s t test (P < 0.01).

Because 22.5% of the mice fed upon for 15 min developed patent parasitemias, despite removal of the fed-upon area shortly after feeding, we recognize that we did not count the sporozoites that had left the skin and induced blood infections. Thus, our count of sporozoites released from the proboscises...
of these nine mosquitoes was to some degree an undercount. However, the mean numbers of sporozoites observed after feeding were not significantly different between mosquitoes that induced blood infections and those that did not, thus implying that the numbers of sporozoites moving from skin to blood over the 15-min period were likely insignificant for the 9 of 40 mice that developed blood infections.

Two of the 40 infected mosquitoes delivered no detectable sporozoites; in each case, there was no evidence of reingested sporozoites, nor did either mouse develop a blood infection, thus implying that no sporozoites had been released from the proboscis in either case. Frequency distributions of numbers of sporozoites found within ear pinnae are seen in Fig. 2A. These results show that the significantly greater mean number of sporozoites injected during 15- versus 3-min feedings (Table 1) is due largely to the greater number of mosquitoes injecting more than 600 sporozoites during 15-min feedings.

We calculated that the salivary glands had a mean of 13,226 sporozoites prior to initiation of feeding (range of 250 to 55,746 and median of 6,398 sporozoites per mosquito). Frequency distributions of transmission efficiency are shown in Fig. 2B. Mosquitoes feeding for 15 min had a much greater likelihood of transmitting greater numbers of sporozoites than those that fed for only 3 min. Figure 2B shows that 35% of the mosquitoes feeding for 15 min injected more than 6% of their total salivary gland load of sporozoites into the skin of the ear, whereas only 15% of mosquitoes feeding for 3 min were able to do so.

After the 15-min feedings, we observed distinct hematomas in 92% of the biopsy specimens; hematomas were seen within both dorsal and ventral leaflets of ear pinnae 78% of the time. As with 3-min feedings, we found sporozoites within both leaflets of the ear and in the dermis and epidermis of the ear. Sporozoites were seen only rarely within hematomas but were common within clusters or tracks adjacent to hematomas.

**Three-minute mosquito feeding on abdomen.** Following removal of biopsy specimens of abdominal skin after mosquito feeding in pilot studies, we noted hematomas on the surface of underlying peritoneal musculature (Fig. 3A). Fluorescence microscopy showed sporozoites within this underlying tissue (Fig. 3B). Accordingly, we took biopsy specimens of both abdominal skin and its underlying tissue to count sporozoites that had been deposited in each. Results of 30 separate 3-min feedings on the ventral abdomen by mosquitoes showed that mosquitoes probed a mean of 3.8 times (Table 1). The mean total probing/feeding time was 137.2 s, and the mean number of sporozoites deposited within fed-upon areas of skin and underlying tissues was 122.6 (range of 0 to 932 and median of 65 sporozoites) per mosquito. Figures 3C and D show examples of microscope fields containing sporozoites within abdominal skin and peritoneal musculature, respectively. Numbers of sporozoites deposited in the skin and underlying tissues per mosquito were approximately equal (means of 61.9 and 64.1, respectively).

As with results for ears, sporozoites were seen only rarely within hematomas but were common within clusters or tracks adjacent to hematomas or along puncture tracks made by the proboscis (Fig. 3B). These puncture tracks often fluoresced as red linear streaks (Fig. 3B), apparently from nonspecific fluorescence associated with tracks made by the proboscis. These tracks also exhibited fluorescence when observed with other filter sets not specific for the RedStar fluorescent protein. Similar penetration tracks made by proboscises have previously been observed by intravital microscopy (25). Further studies with mosquitoes probing at other sites showed that hematomas were induced and sporozoites were deposited in the musculature underlying the backs and thighs of mice after mosquito injection (data not shown). Thus, subcutaneous inoculation of sporozoites appears to be a general phenomenon.

The mean number of sporozoites released from the proboscis was 324.9, with a mean of 2.5 sporozoites released per second from the proboscis.

Of the 30 mosquitoes that fed, 4 transmitted no detectable sporozoites anywhere in the abdomen and none of the mice developed a blood infection; 2 of these 4 mosquitoes reingested sporozoites into their midguts, presumably accounting for their apparent failure to deposit sporozoites. Frequency distributions of sporozoites within fed-upon mice are shown in Fig. 4A. The majority of mosquitoes left fewer than 100 sporozoites within abdominal tissues (less than 0.5% of their total salivary gland load) (Fig. 4B). Fifteen of the 30 mosquitoes reingested sporozoites into their midguts (mean of 202.3, with a range of 0 to 1,931). The salivary glands had a mean of 41,651 sporozoites prior to the initiation of feeding (range was 506 to 168,267, with a median of 33,594 sporozoites per mosquito). Of the fed-upon mice retained to determine whether they developed a patent blood infection, 6/27 (22%) became positive. Nevertheless, the mean number of sporozoites deposited in the abdominal tissues by these 6 mosquitoes was not significantly different from the mean number of sporozoites deposited by the 21 mosquitoes that did not induce blood infections, thus implying that the numbers of sporozoites moving from skin into blood over the 3-min period were relatively trivial.

**Fifteen-minute mosquito feeding on abdomen.** Results of 30 separate 15-min feedings on the abdomen by mosquitoes (Table 1) showed that mosquitoes probed a mean of 8.9 times; the mean total actual probing/feeding time within this 15-min period was 488 s out of a total possible time of 900 s. The mean number of sporozoites deposited within the fed-upon areas of the skin and underlying tissues was 116.1 (range of 0 to 688 and median of 65); a mean of 68.5 sporozoites was found in abdominal skin, while a mean of 47.6 was found in the underlying...
tissues. Seven of the 30 mosquitoes transmitted no detectable sporozoites anywhere in the abdomen; 3 of these mosquitoes had reingested sporozoites into their midguts, thus at least partially accounting for their apparent failure to deposit sporozoites. Twenty of the 30 mosquitoes reingested sporozoites into their midgut (overall mean of 279.1 and range of 0 to 1,408). The salivary glands had a mean of 33,248 sporozoites prior to initiation of feeding (range was 1,137 to 142,808, with a median of 18,800 sporozoites per mosquito). Interestingly, the mosquito with the greatest number of salivary gland sporozoites transmitted only two detectable sporozoites into the skin, and only six additional sporozoites were reingested into the midgut of this mosquito. Frequency distributions of numbers of sporozoites identified within the fed-upon mice are shown in Fig. 4A. The majority of mosquitoes left fewer than 100 sporozoites within the abdominal tissues (less than 0.5% of their total salivary gland load) (Fig. 4B).

Of the fed-upon mice retained to determine whether they developed patent blood infections, 13/25 (52%) became positive (mean prepatent period of 7.0 days). The mean number of sporozoites deposited in the abdominal tissues by these 13 mosquitoes (127.5) was not significantly different from the mean number of sporozoites deposited by the 12 mosquitoes that did not induce blood infections (128.2), thus implying that the numbers of sporozoites moving from skin into blood over the 15-min period were relatively trivial. This conclusion is reinforced by the long mean prepatent period (7.0 days) observed for mice that developed blood infections, compared with the mean 4.6-day prepatent period of control mice fed upon for 15 min but without extirpation of feeding sites after feeding. Figure 5 shows a much lower degree of vascularization in ear pinna (Fig. 5A) than in abdominal skin (Fig. 5B), as seen by fluorescence microscopy after intravenous injection of fluorescein-labeled dextran.

**DISCUSSION**

Previous attempts to better understand the dynamics of sporozoite transmission by mosquitoes have used a variety of approaches, each with both strengths and limitations. In an early study, infected mosquitoes fed on medium through a membrane and sporozoites were collected from the medium after the feeding but no attempt was made to count the sporozoites delivered (7). Subsequent studies counted sporozoites released by mosquitoes into fluid-filled capillary tubes or through membranes into fluid-filled vessels (4, 5, 14, 19) or onto a glass slide (12). These studies were useful in that they allowed direct counts of released sporozoites by microscopy; however, mosquito salivation was almost certainly different from that of mosquitoes probing and feeding on a living host.

An attempt to quantitate sporozoites delivered into living hosts was based on EEF counts in rodents fed on by *P. berghei*-infected mosquitoes (26). Although this was a more “natural” approach, there were limitations to its ability to account for all of the sporozoites delivered. Results were reported as delivery of viable sporozoites, i.e., those successful in reaching the liver and developing to EEF; the study noted that these results represented an undercount of sporozoites actually delivered by the mosquitoes. Nevertheless, this was a first indication that the size of a sporozoite inoculum is likely small relative to the numbers of sporozoites within the salivary glands. The results of all of these studies were in essential agreement, i.e., mos-
quitoses deliver only a small percentage of their total salivary gland load.

Some of the reasons suggested for the relatively low transmission efficiency were that (i) the volume of saliva injected by feeding mosquitoes is only a small percentage of the secretory material present in the salivary gland, (ii) many sporozoites in the gland are in transit through the cytoplasm of gland cells and are thus unavailable for transmission, (iii) the lumen of the salivary gland duct narrows in the proximal regions of the lobes to about 1 μm in diameter, thus limiting the rate at which sporozoites can pass out, (iv) a portion of the sporozoites delivered is reingested by the mosquito, and (v) a portion may be lost via lymphatic drainage. It was concluded that the relatively small numbers of sporozoites transmitted by mosquitoes are due to the anatomy of the salivary gland and its ducts and thus seem as likely to occur in the field as in the laboratory. This anatomical restriction on numbers of sporozoites that can be released from salivary glands of mosquitoes has been confirmed by fluorescence microscopy studies.

After it was demonstrated that mosquitoes inject sporozoites into avascular portions of skin rather than directly into blood, it became experimentally valid to quantify numbers of sporozoites delivered by estimating numbers remaining within skin biopsy specimens taken at the feeding site immediately after mosquito feeding. This led to more-sophisticated approaches to quantifying transmission during mosquito feeding on live rodent hosts. A study to estimate P. yoelii sporozoite numbers injected into a mouse ear used quantitative PCR measurement of sporozoite 18S rRNA extracted from a biopsy specimen. The described sensitivity of this method allowed detection of sporozoites deposited by individual mosquitoes. Results indicated that mosquitoes allowed to feed for 3 min deposited a mean of 123 sporozoites, which was less than 1% of the calculated salivary gland load. Comparable results were obtained with transgenic P. berghei sporozoites expressing β-galactosidase, quantified after homogenization of the biopsy specimen, followed by a colorimetric assay. The reduced sensitivity of this assay required that 10 to 15 mosquitoes be used to detect the presence of sporozoites inject sporozoites into avascular tissue rather than directly into blood. A longer feeding time would have confounded the study by allowing time for some of the injected sporozoites to move into the circulation. Indeed, this is borne out in the current study, in which no mice developed a blood infection when the fed-upon ear site was removed shortly after the 3-min feeding, whereas 22.5% became infected when feeding time was extended to 15 min before removal of this site. An analysis of the overall dynamics of sporozoite transmission is defective, however, if mosquito feeding time is limited only to 3 min. We found that 61% more sporozoites were observed in the ear pinna after 15-min feeding than after 3-min feeding, therefore leading to greater transmission efficiency associated with longer feeding times. Obviously even more sporozoites than this were released by the proboscis during 15-min feedings, as indicated by the facts that many more sporozoites were reingested into the midguts of mosquitoes feeding for 15 min and that small numbers of sporozoites had already moved from skin to blood by the time the bitten portion of skin was removed after 15 min.

These findings may have epidemiological relevance. Short, interrupted feedings by mosquitoes are more likely when humans are active, whereas extended feedings are more likely when human hosts are quiescent and mosquitoes can feed to repletion. Indeed, in Africa most malaria vectors tend to bite at night, when human hosts are asleep.

Not all mosquitoes with salivary gland infections successfully
transmit malaria to mammalian hosts (18). We previously proposed that at least some failures may be due to reingestion of injected sporozoites by the mosquito (13). We now show that in addition to this, some mosquitoes (9% of the 140 feedings evaluated in the current study) failed to (i) transmit sporozoites into the skin, (ii) reingest sporozoites, and (iii) induce blood infections. Why these infected mosquitoes did not transmit sporozoites into the host remains to be determined; this failure was not correlated with salivary gland load in the present study.

Our finding that sporozoites are deposited in the epidermis as well as the dermis was unexpected. Because blood vessels are not present in the epidermis, the fate of sporozoites deposited in this skin layer is unclear. Are these sporozoites able to migrate to dermal blood vessels for subsequent transport to the liver, or are they unable to move beyond the epidermis? The finding that some sporozoites remain for long periods of time within the skin and appear to fragment there led to the suggestion that dendritic cells (DC) may process sporozoite antigen and then travel to draining lymph nodes to induce immunity against sporozoites (25). It will be of interest to evaluate whether the two distinct types of DC found in the dermis versus the epidermis play different roles in the induction of immunity. Because intact sporozoites have also been shown to reach lymphatic vessels within the skin and travel to lymph nodes (1, 2, 28), it is conceivable that such sporozoites may play a role in the induction of immunity without initial processing by DC in the skin.

When mosquito feeds on the abdomen were compared with those on the ear pinna, the abdomen feeds involved fewer probes, fewer sporozoites deposited in the tissues, more reingestion of sporozoites, and more blood infections. The greater intensity of salivary gland infections in mosquitoes used for abdomen feeding studies was a matter of chance associated with normal fluctuations in salivary gland infections within our laboratory system. Yet, in spite of the considerably larger numbers of sporozoites that these mosquitoes had in their salivary glands, they left fewer sporozoites within the abdominal tissues. However, when we also take into consideration the greater numbers of sporozoites reingested by mosquitoes feeding upon the abdomen, the total numbers of sporozoites released from the proboscis are roughly comparable to those found with ear feedings.

While we cannot account for sporozoites that escaped to the bloodstream during the feeding periods, most mice never became infected or had extremely long prepatent periods, suggesting that few sporozoites had left the bite site prior to tissue removal. Furthermore, if significant numbers of sporozoites had moved into the blood, we would have expected to find significantly lower numbers of sporozoites remaining behind in these tissues. However, these abdominal tissues had a mean of 127.5 sporozoites per mouse, compared with 128.2 sporozoites in mice that never developed blood infections.

Differences between results from feedings on ear versus abdomen appear to be driven by the much greater vascularization of abdominal tissues (Fig. 5). Mosquitoes obtained blood from the abdomen more rapidly and had to probe fewer times to fill their midguts. This appeared to have two main consequences: (i) more sporozoites were found in ear tissue because sporozoites tend to be released into avascular tissue during probing rather than during blood ingestion, and (ii) because more blood was ingested by mosquitoes feeding on the abdomen, more sporozoites were reingested by these mosquitoes. Interestingly, results for one parameter of transmission dynamics remained relatively similar under all circumstances, i.e., the total numbers of sporozoites released by the proboscis per second. For the 3-min feedings times, means of 2.2 versus 2.5 sporozoites per second were delivered to ear and abdomen, respectively. For 15-min feedings, means of 1.4 versus 0.8 sporozoites per second were delivered to ear and abdomen, respectively. These relatively slow release rates of sporozoites reflect the previous observation that the narrow lumen (<1 μm) of the distal salivary duct limits the flow of saliva and sporozoites into the proboscis (26).

Because of this relative consistency of sporozoite release per unit of time, one must be cautious about the use of calculated transmission efficiency (number of sporozoites delivered divided by number of sporozoites within the salivary glands) as a constant parameter of sporozoite transmission dynamics. First, transmission efficiencies vary with length of probing and feeding time; the longer that probing/feeding lasts, the more sporozoites will be released by the proboscis, yet numbers of sporozoites in the salivary glands remain constant. Second, transmission efficiencies vary inversely with salivary gland loads. Thus, the much greater numbers of sporozoites in the glands of mosquitoes that we used for abdomen feeds accounts for the much lower calculated transmission efficiency of these mosquitoes. Thus, frequency distributions of transmission efficiencies show a relatively even spread across the entire range for mosquitoes feeding on the ear (Fig. 2B), whereas these values are clustered at the low end for mosquitoes that fed on the abdomen (Fig. 4B).

The wide range of differences in thickness and vascularization of human skin at different body sites (20, 27) is likely a determinant in the ability of mosquitoes to obtain a blood meal and to transmit sporozoites at the different sites. Nevertheless, human skin is generally less than 2 mm thick (20), thus making it possible for the mosquito proboscis to penetrate below the skin at most body sites; this is even more likely in children, who tend to have thinner skin (20). Differences in mosquito delivery of sporozoites to subcutaneous tissues with different degrees of vascularization, e.g., muscle versus adipose tissue, could also play a role in the dynamics of sporozoite transmission.

The results reported here support our hypothesis that most if not all sporozoites are injected into avascular tissue during mosquito probing and that some sporozoites begin to move into the bloodstream within minutes (21). Furthermore, when blood is more readily accessible to mosquitoes, mosquitoes need to probe fewer times to engorge and they reingest more sporozoites in the process. This is associated with the finding that sporozoites may be plentiful in the avascular tissue at the bite site but are generally absent from hematomas from which the mosquitoes take their blood. The higher delivery rate of sporozoites per second during the earlier portion of the feedings (as seen in the 5-min results) is compatible with our conclusion that sporozoites tend to be ejected into skin and subcutaneous tissues while the mosquito is engaged in probing for a blood source rather than during the subsequent imbibing of blood. Thus, the numbers of sporozoites introduced into the host by mosquitoes and the transmission efficiencies of sporo-
sporozoite delivery are multifactorial phenomena that vary with length of probing time, skin site being fed upon, and number of sporozoites within the salivary glands.

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