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Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito hemocoel

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Abstract

For successful transmission to the vertebrate host, malaria sporozoites must migrate from the mosquito midgut to the salivary glands. Here, using purified sporozoites inoculated into the mosquito hemocoel, we show that salivary gland invasion is inefficient and that sporozoites have a narrow window of opportunity for salivary gland invasion. Only 19% of sporozoites invade the salivary glands, all invasion occurs within 8 h at a rate of approximately 200 sporozoites per hour, and sporozoites that fail to invade within this time rapidly die and are degraded. Then, using natural release of sporozoites from oocysts, we show that hemolymph flow through the dorsal vessel facilitates proper invasion. Most mosquitoes had low steady-state numbers of circulating sporozoites, which is remarkable given the thousands of sporozoites released per oocyst, and suggests that sporozoite degradation is a rapid immune process most efficient in regions of high hemolymph flow. Only 2% of *Anopheles gambiae* hemocytes phagocytized *Plasmodium berghei* sporozoites, a rate insufficient to explain the extent of sporozoite clearance. Greater than 95% of hemocytes phagocytized *Escherichia coli* or latex particles, indicating that their failure to sequester large numbers of sporozoites is not due to an inability to engage in phagocytosis. These results reveal the operation of an efficient sporozoite-killing and degradation machinery within the mosquito hemocoel, which drastically limits the numbers of infective sporozoites in the mosquito salivary glands.

Keywords

Anopheles gambiae; *Aedes aegypti*; Malaria; Sporozoite; Hemolymph; Hemocyte; Phagocytosis; Salivary gland; *Plasmodium*

1. Introduction

Plasmodium parasites, the causative agents of malaria, kill millions of people each year and have a substantial negative impact on the economic and educational development of endemic countries (Hill et al., 2005). For natural transmission of *Plasmodium* to the vertebrate host, the parasite undergoes a series of obligatory developmental, propagative and migrational processes inside a mosquito vector. These include zygote formation and ookinete development in the midgut lumen, oocyst formation and sporogony on the basal side of the midgut, sporozoite

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migration through the hemocoel, and invasion of the salivary glands. The process of sporogony is of particular importance because it results in the production of thousands of sporozoites from a single oocyst, each of which can be infective to the vertebrate host if it safely reaches the salivary glands.

The dramatic increase of parasite numbers within the oocyst prior to venturing into the mosquito's open circulatory system suggests that salivary gland invasion by sporozoites is an inefficient process. From experiments done via natural infections, it has been estimated that only 10–20% of the sporozoites released from oocysts invade the salivary glands (Rosenberg and Rungsiwongse, 1991; Korochkina et al., 2006). Experiments that quantified salivary gland invasion following intra-hemocoelic sporozoite injections agree with these estimates (Barreau et al., 1995). The fate of the 80–90% of sporozoites that are unable to invade the salivary glands is unknown. In limited studies to date, mosquito phagocytosis and occasionally melanization of sporozoites has been observed (Foley, 1978; Hernández-Martínez et al., 2002; Hillyer et al., 2003b).

Given that relatively few sporozoites invade the salivary glands, it is perhaps surprising that the process of migration through the hemocoel has received little attention. Instead, most studies have focused on the interaction between *Plasmodium* and the mosquito midgut (Abraham and Jacobs-Lorena, 2004; Riehle et al., 2006; Whitten et al., 2006), and to a lesser extent the salivary glands (Rodriguez and Hernandez-Hernandez, 2004; Korochkina et al., 2006). One possible explanation for this is the difficulty in quantifying sporozoites in mosquito tissues and in visualizing them as they flow through the hemocoel. However, advances in *Plasmodium* research have resulted in the development of molecular technologies for the quantification of sporozoites (Vernick et al., 1995) and in the recent production of transgenic *Plasmodium berghei* strains that express the fluorescent marker, green fluorescent protein (GFP) (Natarajan et al., 2001; Franke-Fayard et al., 2004). This novel technology has for the first time allowed researchers to accurately visualize live parasites and parasite movement inside mosquito (Frischknecht et al., 2004; Vlachou et al., 2004) and vertebrate host tissues (Frevert et al., 2005).

In the current study, we examined the fate of malaria sporozoites in the vector hemocoel using two distinct mosquito-*Plasmodium* laboratory models with complementary strengths. Firstly, the *Aedes aegypti*-*Plasmodium gallinaceum* model is a robust system with consistent high-intensity infections, although *Ae. aegypti* is not a vector of human malaria parasites. The robust infections obtained in this system make it feasible to purify large numbers of sporozoites from mature midgut oocysts and inoculate them into uninfected mosquitoes to quantify the rates of sporozoite salivary gland invasion and death in the hemocoel. This approach allowed us to measure in vivo sporozoite kinetics by molecular quantification in a pulse-chase experiment, with a discrete zero-time point and without the complication of continuous sporozoite release from midgut oocysts. Second, the *Anopheles gambiae*-*Plasmodium berghei* model has the advantage that this mosquito is a natural vector of human malaria, and there are transgenic tools available for *P. berghei* (Natarajan et al., 2001; Franke-Fayard et al., 2004). With this system, which is not robust enough for large-scale purification of midgut sporozoites, we instead used bloodmeal-induced infections with a fluorescently-labeled parasite to visualize the migration, phagocytosis and destruction of sporozoites during their journey from ruptured midgut oocysts to the salivary glands in live, intact *An. gambiae*.

2. Materials and methods

2.1. Mosquito rearing and maintenance

Mosquito rearing and maintenance was done inside an environmental chamber at 26 °C, 80% relative humidity, with a 12-h light/12-h dark photoperiod. *Anopheles gambiae* (G3 strain) and

Ae. aegypti (Liverpool strain) larvae were hatched in distilled water, maintained in enamel-coated pans and fed a slurry of ground Tetramin fish food (Tetra, Melle, Germany). Pupae were transferred to 3.79-L cartons with a fine mesh marquisette top and, upon emergence, cotton pads soaked in a solution consisting of one part corn syrup and seven parts water were placed over the marquisette and mosquitoes allowed to feed ad libitum.

2.2. *Aedes aegypti*-*Plasmodium gallinaceum*: quantification of sporozoite numbers in the salivary glands and hemocoel

The *Ae. aegypti*-*P. gallinaceum* system was used to quantify the rate of salivary gland invasion by sporozoites and the rate of sporozoite degradation in the hemocoel. *Aedes aegypti* were infected by feeding on White Leghorn chickens with a parasitemia of approximately 10% and a gametocytemia of 2%. Sporozoites were purified from midgut oocysts by an initial centrifugation of abdomens from infected mosquitoes followed by a gradient centrifugation using a cushion of 6.4% Ficoll 400 and 10% Hypaque (Barreau et al., 1995).

Uninfected mosquitoes were cold anesthetized and intrathoracically injected with approximately 10,000 purified sporozoites. At 0, 8, 24, 48 and 168 h post-injection, salivary glands and their respective carcasses (whole bodies minus salivary glands) were separated, RNA isolated using TRIzol (Invitrogen, Carlsbad, CA), and quantification done by RT-PCR as previously described (Vernick et al., 1995). Assays using the calcein-AM/ethidium homodimer-based LIVE/DEAD Viability/Cytotoxicity Assay (Molecular Probes, Carlsbad, CA) showed that prior to injection, greater than 90% of sporozoites were viable. Infections and the maintenance of infected mosquitoes were performed at 26°C and 80% relative humidity.

2.3. *Anopheles gambiae*-*P. berghei*: visualization of sporozoite migration and phagocytosis

The *An. gambiae*-*P. berghei* system was used to visualize sporozoites in the hemocoel and for phagocytosis assays. Infections were performed using two strains of *P. berghei* that express GFP. The first, PbFluspo (Natarajan et al., 2001), was constructed from the NK65 strain of *P. berghei* and constitutively expresses GFP during the sporozoite stage of the parasite. The second, PbGFP_{CON} (Franke-Fayard et al., 2004), was constructed from the ANKA strain of *P. berghei* and constitutively expresses GFP throughout the life cycle of the parasite. Similar results were obtained regardless of the strain used. For *P. berghei* infections, mosquitoes were starved for 12 h and then allowed to blood feed for 15–30 min on an infected Swiss Webster mouse with a parasitemia of approximately 10% and a gametocytemia of approximately 2%. Infections and the maintenance of infected mosquitoes were performed at 21 °C and 80% relative humidity.

Correlative fluorescence and light microscopy was used to visualize parasites: i) through the cuticle of live mosquitoes, ii) in dissected mosquito tissues, and iii) in the hemolymph perfusate (see below). All observations were made using a Nikon Eclipse E600 upright microscope connected to a CoolSNAP_{ES} digital camera (Photometrics, Tucson, AZ). Digital images were taken using MetaVue Imaging Software (Universal Imaging Corporation, Downingtown, PA), HiGauss filtered using Image-Pro Plus (Media Cybernetics, Silver Spring, MD), and histogram stretches and overlays produced using Adobe Photoshop (Adobe Systems, San Jose, CA). Whole mosquitoes and dissected mosquito tissues were observed under bright field illumination and epi-fluorescence. Hemolymph perfusates and hemocytes were observed under differential-interference-contrast (DIC) illumination and epi-fluorescence. Hemocytes were classified using the criteria of Hillyer and Christensen (2002).

For this study, the intensities of infection were purposefully kept relatively low compared with normal laboratory conditions. The mean oocyst number per mosquito was 12 and the median number was eight. Oocyst rupture is asynchronous, so keeping oocyst numbers low prevented

continuous sporozoite release and allowed us to sample mosquitoes at different times following egress. Nevertheless, the infection intensities used in this study are equivalent to or higher than those normally found in nature (Beier, 1998).

2.4. Hemolymph collection and phagocytosis assays

Hemolymph collection was via volume displacement (perfusion). Mosquitoes were cold anesthetized and a tear was made in the last two segments of the abdomen. Using a microinjection needle, mosquitoes were intrathoracically injected with PBS (pH 7.0). The liquid exiting the tear in the abdomen was collected onto a glass slide (diluted hemolymph) and examined by DIC and fluorescent microscopy. Injections were done on the lateral side of the mesothorax through the membrane located between the paratergite, the postspiracular area and the mesepisternum (Knight and Laffoon, 1970).

To test the phagocytic capacity of *An. gambiae*, mosquitoes were injected with live tetracycline-resistant *Escherichia coli* (DH5 alpha strain) that constitutively express GFP or 1 μ m diameter red-fluorescent carboxylate-modified latex microspheres (Molecular Probes, Eugene, OR). This *E. coli* strain and carboxylate-modified latex particles were chosen because they are readily phagocytized by the hemocytes of two other mosquito species and are easily visualized by fluorescent microscopy (Hillyer et al., 2003a, 2003b, 2005). *Escherichia coli* were grown overnight in Luria Bertani's rich nutrient medium (LB broth) at 37°C while shaking at 300 RPM until they reached stationary phase. Individual mosquitoes were cold anesthetized and intrathoracically injected with approximately 18,000 *E. coli* in 0.25 μ l PBS. Latex particles were injected in a similar manner, but at doses of 180,000 and 1,000,000 per mosquito. The injection procedure had no deleterious effect, because fewer than 2% of control mosquitoes died during the first 24 h post-injection.

As a measurement of the phagocytic activity of *An. gambiae* hemocytes following *Plasmodium* natural infections, *E. coli* injections, or latex bead injections, we measured the percentage of circulating hemocytes that engaged in phagocytosis. This measure, the phagocytic index, has previously been used to assay phagocytic activity in mosquito hemocytes (Hillyer et al., 2005). Briefly, individual mosquitoes were perfused onto glass slides and hemocytes allowed to adhere for 5 min. Hemocytes were then observed live with simultaneous GFP epi-fluorescence and low-light intensity DIC. Cells were considered positive when they contained at least one fluorescent *P. berghei*, *E. coli* or latex particle. Sporozoite movement and sporozoite location within the focal plane were two criteria used to distinguish between sporozoites that had been phagocytized versus those that had attached to the hemocyte cell surface. For *Plasmodium* infections, mosquitoes were perfused between days 14 and 20 p.i. Only mosquitoes that had both salivary gland sporozoites and unruptured midgut oocysts were used in the analysis because it was presumed that in these mosquitoes sporozoite release from oocysts was continuous. For *E. coli* or latex bead injections, perfusions were performed at various times post-injection but quantitative analysis was done only at 3 h following challenge.

3. Results

3.1. Sporozoites either rapidly invade the salivary glands or are degraded in the hemocoel

When uninfected *Ae. aegypti* mosquitoes were injected with purified *P. gallinaceum* sporozoites and the number of sporozoites assayed by RT-PCR, 19% of sporozoites invaded the salivary glands within 8 h post-injection (Fig. 1). When the number of sporozoites was assayed at 24–168 h post-injection, no new invasion was detected. Moreover, by 8 h post-injection only 39% of sporozoites were detected in the hemocoel, indicating that 42% of sporozoites had been degraded (Fig. 1). The sharp decline continued until 24 h post-injection, when only 16% of sporozoites were detected in the hemocoel, followed by a steady decline to

1% by 168 h post-challenge. Because the RT-PCR sporozoite quantification assay relies on the detection of rRNA, signal decreases only when *Plasmodium* rRNA is degraded. Thus, decrease in signal indicates that parasites are not just dead, but physically destroyed. The assay measures parasite rRNA in completely solubilized mosquito extract, so any intra- or extracellular parasites, regardless of anatomical location, would be detected.

The rates of both salivary gland invasion and degradation in the hemocoel were bimodal. Invasion only occurred during the first 8 h post-challenge (slope = 233 sporozoites/h) and after 8 h the number of sporozoites in the salivary glands remained constant (slope = -4 sporozoites/h; $R^2 = 0.77$). Moreover, the number of sporozoites in the hemocoel declined rapidly until 24 h post-challenge (slope = -232 sporozoites/h; $R^2 = 0.92$) followed by a slower progression of degradation until only 1% could be detected by 168 h post-challenge (slope = -11 sporozoites/h; $R^2 = 0.91$). The timing of the plateau differed between invasion and degradation, because salivary gland invasion reached a plateau at 8 h but degradation did not reach a plateau until 24 h. However, it is likely that hemocoel sporozoites, between 8 and 24 h post-injection, are incapable of invasion and are in the process of dying, but some rRNA is still available for amplification by RT-PCR.

Salivary gland invasion cannot account for the reduction in parasite numbers in the hemocoel. Overall, only 19% of sporozoites invaded the salivary glands, numbers consistent with published estimates after natural release or inoculation of sporozoites. Because we were unable to detect the remaining sporozoites, based on the characteristics of the detection assay we conclude that they were degraded in the hemocoel.

3.2. Following egress from oocysts, sporozoites migrate with hemolymph flow to all parts of the hemocoel

We infected *An. gambiae* with GFP-expressing *P. berghei* and observed the movement of sporozoites in the hemocoel. Fluorescence microscopy observations through the cuticle of live mosquitoes illustrated that, upon sporozoite release into the hemocoel, parasites are subject to hemolymph flow. They are swept towards the posterior of the insect and enter the dorsal vessel (located along the dorsal midline and underneath the terga) through paired ostia located on the anterior portion of each abdominal segment (Fig. 2). Once in the heart, the majority of sporozoites then rapidly flow with the hemolymph towards the anterior of the mosquito, while some appear to be stationary in the anterior regions of the abdominal segments (Fig. 3A–C). These regions coincide with the location of the ostia and the regions of expanded chambers of the heart formed by the tension caused by the alary muscles. Regions of sporozoite trapping in the heart also coincide with the location of pericardial cells that surround the heart. Little is known about the function of pericardial cells, but in the mosquito *Anopheles quadrimaculatus* they have been observed to phagocytize ammonia carmine dye (Jones, 1954). Although some trapped sporozoites may have been phagocytized by pericardial cells, others were not because: i) they were not completely immobile and can be seen slowly shifting in place, and ii) their location was different from that observed when mosquitoes are injected with fluorescent *E. coli* or latex beads (see below). While fluorescence from sporozoites sometimes mimics the fluorescent foci from beads and bacteria (Fig. 3C), most trapped sporozoites are dispersed throughout the expanded heart chambers (Fig. 3B).

Hemolymph flow across the dorsal vessel is strong and carries sporozoites from the abdomen to the head. On a few occasions hemolymph flow through the dorsal vessel was in the opposite direction, anterior to posterior, which has previously been observed (Jones, 1954). Upon reaching the anterior end of the aorta segment of the dorsal vessel, sporozoites are deposited in front of the brain, and then flow posteriorly into the perivisceral, perineural (ventral) and pericardial (dorsal) sinuses of the hemocoel. In these chambers hemolymph flow is considerably slower and is aided by pressure originating from the peristaltic contractions of

the dorsal vessel as well as by numerous accessory pulsatile organs. In a successful parasite cycle, sporozoites invade the salivary glands. However, numerous sporozoites become trapped in the distal appendages such as legs, antennae, wings and halteres (Fig. 3D–I). Fluorescent intensities of these trapped sporozoites can be very faint, which suggests that many are dead or dying. To quantify the location of sporozoites, only mosquitoes that possessed both unruptured midgut oocysts and salivary gland sporozoites were included in the analysis. These mosquitoes comprise the sample set where salivary gland invasion has begun but not finished, and hence where we would expect to find hemocoel sporozoites. When visualized through the cuticle of intact live mosquitoes, numerous anatomical locations had at least one non-circulating sporozoite (Fig. 4).

When oocysts rupture, thousands of sporozoites are released into the hemocoel (Rosenberg and Rungsiwongse, 1991), and this release is asynchronous because oocysts do not rupture at the same time. In our experiments, when mosquitoes were examined through the cuticle and after dissection at 14–20 days post-infective blood meal, sporozoites could be seen within the salivary glands. Furthermore, midgut oocysts could still be seen and sporozoites were often observed in the distal appendages. Interestingly, few sporozoites were observed circulating through the dorsal vessel. While 87% of mosquitoes that contained both oocysts and salivary gland sporozoites had at least one sporozoite detectable in the hemolymph, only 35% of mosquitoes had one or more sporozoites flowing through the dorsal vessel (Fig. 4). Thus, a key finding was that the majority of mosquitoes had low steady-state levels of hemocoel sporozoites. For example, while 87% of mosquitoes observed during the period of active sporozoite migration and salivary gland invasion had hemolymph sporozoites, only 10% had greater than 100 (Fig. 4). The low numbers of hemocoel sporozoites were unexpected given the numbers of sporozoites released by each oocyst and the multiple oocysts present inside each mosquito. We hypothesize that most of the sporozoites visualized were likely released shortly before observation and that previously released sporozoites had either already invaded the salivary glands or been degraded.

3.3. Phagocytosis is an effector mechanism against *Plasmodium* sporozoites

Because only 10–20% of sporozoites invade the salivary glands and the remaining 80–90% disappear from circulation, we infer that the majority of sporozoites die while migrating towards the salivary glands. The mechanism(s) of sporozoite clearing by mosquitoes are unknown, although a possible mechanism for clearance is phagocytosis by hemocytes. Previous studies observed phagocytosis of sporozoites, but no quantification was done (Foley, 1978; Hernández-Martínez et al., 2002; Hillyer et al., 2003b). We wished to determine the capacity of *An. gambiae* hemocytes to phagocytize *P. berghei* sporozoites following natural infections. These studies were carried out using simultaneous DIC and epi-fluorescence, which allowed for the examination of large numbers of hemocytes from individual mosquitoes (Fig. 5). When we examined the hemolymph of 43 mosquitoes 14 to 20 days p.i. that had greater than 50 sporozoites in the salivary glands (indicating that invasion had begun) and at least one unruptured midgut oocyst (indicating that invasion was still taking place), only $2.09\% \pm 3.17$ S.D. (median = 0.96%) of the hemocytes per mosquito contained at least one sporozoite. When we restricted the analysis to mosquitoes that contained at least one sporozoite in the perfusate, only $2.50\% \pm 3.32$ S.D. (median = 1.45%) of the hemocytes per mosquito contained sporozoites and when only mosquitoes that had greater than 100 hemolymph sporozoites were analyzed, $6.0\% \pm 6.13$ S.D. (median 3.00%) of hemocytes had engaged in phagocytosis. Moreover, when we constrained the analysis to mosquitoes that had sporozoites circulating through the dorsal vessel prior to perfusion, only $3.65\% \pm 4.20$ S.D. (median = 2.38%) of the hemocytes per mosquito contained sporozoites. Of all the hemocytes that contained sporozoites, 96% contained one sporozoite, 4% contained two sporozoites, and no hemocyte was observed with

more than two sporozoites. Hence, only a small proportion of hemocytes phagocytize sporozoites and the overall number of sporozoites eliminated by this mechanism is small.

Because phagocytosis of sporozoites by hemocytes was an infrequent process, we wished to determine whether *An. gambiae* hemocytes had a similar overall phagocytic capacity as hemocytes from other mosquito species. It has been shown in three different mosquito genera that individual hemocytes can phagocytize thousands of injected particulates or bacteria (Hernández-Martínez et al., 2002; Hillyer et al., 2003a, 2003b). We found that circulating *An. gambiae* hemocytes phagocytized injected *E. coli* or latex particles as early as 5 min post-injection, which was the earliest timepoint sampled (Fig. 6A–B). Most hemocytes phagocytized numerous bacteria or latex beads, although hemocytes containing a single foreign particle were also observed. The phagocytic cells readily spread on glass slides and were morphologically and behaviorally indistinguishable from the granulocyte cell population previously described in *Ae. aegypti* and *Armigeres subalbatus* (Hillyer et al., 2003a, 2003b).

To obtain a measure of the phagocytic activity of *An. gambiae* hemocytes, we injected mosquitoes with known doses of *E. coli* or latex particles and calculated the percentage of hemocytes that engaged in phagocytosis at 3 h post-challenge (phagocytic index; Fig. 6C). When we injected mosquitoes with approximately 18,000 *E. coli*, 75% (± 5.58 S.D.) of hemocytes phagocytized at least one bacterium. When instead we injected 180,000 latex particles, 83% (± 9.27 S.D.) of hemocytes engaged in phagocytosis and when the inoculum was increased to 1,000,000 latex beads, 96% (± 5.45 S.D.) of hemocytes participated in this immune process. These results are consistent with earlier findings in *Ae. aegypti* (Hillyer et al., 2005) and show that *An. gambiae* hemocytes are capable of phagocytizing large numbers of foreign particles. Fixed pericardial cells also phagocytized large numbers of *E. coli* and latex particles. Following injections, prominent fluorescent foci formed as distinct lines flanking the lateral sides of the net-like baskets surrounding the heart in the anterior portion of the abdominal segments (Fig. 6D–E). These regions correspond with the location of pericardial cells (Jones, 1954).

4. Discussion

Malaria parasites undergo a complex life cycle inside the mosquito vector. Each step of this life cycle is an opportunity for the mosquito to control and eliminate the infection. To date, most studies of mosquito resistance to *Plasmodium* have focused on the midgut infection stages of the parasite (Levashina, 2004; Vernick et al., 2005). However, the midgut is by no means the sole location where resistance mechanisms can be expressed. During migration from the oocyst to the salivary glands, sporozoites are immersed in the mosquito blood which includes a repertoire of pathogen recognition molecules, immune effector molecules and hemocytes (Hillyer et al., 2003b; Bartholomay et al., 2004; Paskewitz and Shi, 2005).

Previous studies (Rosenberg and Rungsiwongse, 1991; Barreau et al., 1995; Korochkina et al., 2006) as well as this study have shown that salivary gland invasion is an inefficient process. This is not surprising, because the enormous amount of foreign biomass released into the hemocoel following oocyst rupture (thousands of sporozoites per oocyst) would likely provoke a host response. However, until now the fate of non-invading sporozoites was unknown. The quantitative data presented here confirm that only a small percentage of sporozoites invade the salivary glands. Moreover, we believe we show here for the first time that non-invading sporozoites die rapidly following hemolymph exposure. Salivary gland invasion occurs in less than 8 h after sporozoite entry into the hemocoel, and sporozoites that fail to invade the salivary glands within this time are degraded. Because of the speed of degradation and its correlation with a cessation in salivary gland invasion, these results show that sporozoites have a narrow

window of opportunity to invade salivary glands or be destroyed by a powerful response in the hemocoel.

In the current study we visually examined the journey of fluorescent *Plasmodium* sporozoites in the mosquito hemocoel. After release from oocysts, sporozoites are dispersed throughout the hemocoel by hemolymph flow. Sporozoites could often be seen in the distal appendages, but seldom were hundreds of sporozoites found in circulation. This is remarkable given the numbers of sporozoites released per oocyst and oocysts per mosquito. We observed sporozoites with various levels of fluorescence intensity, suggesting that many were in the process of being degraded. The mechanisms of sporozoite degradation are unknown, but the fact that sporozoites can be observed in the appendages when there are few in circulation implies that sporozoite degradation involves cellular and/or humoral factors present at highest concentrations in regions of high hemolymph flow.

Similar to other mosquito systems, *An. gambiae* hemocytes are capable of phagocytizing sporozoites. However, here we show that phagocytosis does not make a major contribution to the elimination of sporozoites from the hemocoel. Because *An. gambiae* hemocytes and pericardial cells are capable of phagocytizing large numbers of *E. coli* and latex particles, the failure to efficiently phagocytize sporozoites is not due to an inability to engage in this immune process. An intriguing possibility is that, rather than functioning in physical clearance as an effector mechanism, phagocytosis of sporozoites may function instead to initiate cellular or systemic immune signaling. By analogy to vertebrate macrophages, the capacity of hemocytes to phagocytize sporozoites may not be as important as the processing of the foreign pathogen once internalized within the immune cell. Thus, if invertebrate hemocytes function similarly as a surveillance arm of immunity, sampling the milieu for foreign antigens and transducing immune signals, a low level of phagocytized sporozoites could nevertheless provide sufficient trigger for a general or specific immune alarm. Consistent with this hypothesis, *Drosophila* hemocytes appear to confer a large proportion of total immune competence for antimicrobial defense (Matova and Anderson, 2006).

Because of the strong force of hemolymph flow across the dorsal vessel into the thorax, hemolymph flow places sporozoites in the vicinity of the salivary glands. Only in this region, where flow speed has been greatly reduced, can sporozoites actively seek and invade the salivary glands. Sporozoites are motile (Vanderberg, 1974; Menard, 2001; Frischknecht et al., 2004) and salivary gland invasion is an active process, involving membrane junctions formed with putative specific receptors on the salivary glands (Pimenta et al., 1994; Brennan et al., 2000; Korochkina et al., 2006). Abolishing sporozoite motility by knockout of the gene for the parasite surface thrombospondin-related anonymous protein (TRAP) prevented sporozoite invasion of the salivary glands (Sultan et al., 1997). It is not difficult to imagine that the mosquito may also have learned the same trick, for example by judicious attack on a critical external component of the sporozoite motility machinery by a hemolymph protease. The incapacitated parasites might then be opsonized for cytolysis, analogous to similar mechanisms directed against the ookinete in the mosquito midgut (Blandin et al., 2004). We are currently investigating the function of several candidate genes whose expression and/or abundance of protein product is altered during the period of sporozoite release. If large numbers of sporozoites are lysed in situ in the hemocoel, processing and disposal of the released foreign proteins could be a distinct challenge for the mosquito.

Our data indicate that following egress from midgut oocysts, sporozoites enter a race in which they must recognize molecular cues on the surface of the salivary glands and invade before succumbing to the mosquito immune response. Most sporozoites fail to complete this journey. But for the mosquito, eliminating a *Plasmodium* infection at the sporozoite stage is an equally daunting task given the numbers of sporozoites, and the mosquito's own circulatory system

that facilitates invasion by placing hemocoel sporozoites near their target organ. Here we show that an endogenous anti-sporozoite response greatly reduces sporozoite burden but does not eliminate the infection, at least in average mosquitoes. If both the molecular mechanism for salivary gland invasion and the immune mechanisms used by mosquitoes to reduce sporozoite numbers are uncovered, mosquitoes may be manipulated in efforts to confer resistance to previously susceptible populations.

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Fig 1.

Plasmodium sporozoites either rapidly invade the salivary glands or are degraded in the hemocoel. Uninfected mosquitoes were each injected with 10,000 sporozoites. Salivary glands and carcass (whole body minus salivary glands) were dissected at different timepoints, and the number of parasites determined by RT-PCR. All invasion (19% of sporozoites) occurred within 8 h and the remaining parasites were quickly degraded.

Fig 2.

Diagrammatic representation of *Plasmodium* sporozoite migration in the hemocoel. Following release, sporozoites flow with the hemolymph and enter the heart through paired ostia located in the anterior portion of each abdominal segment. After exiting from the aorta, sporozoites flow posteriorly and a small percentage invade the salivary glands (SG). Solid gray arrows indicate hemolymph flow and proximity of the arrows are indicative of relative flow speed.

Fig 3.

Plasmodium sporozoites in mosquito tissues. Following egress from oocysts, sporozoites migrate with hemolymph flow and are found in tissues such as the heart (A–C), halteres (D), wings (E), cuticle (F), legs (G), antennae (H) and ovaries (I). In the heart, sporozoites flow with the hemolymph (A), become stationary in the expanded chambers of the heart (B) or become stationary, flanking the expanded chambers of the heart (C). Dotted lines delineate the heart and arrowheads show examples of sporozoites. Bar = 100 μ m.

Fig 4.

Distribution of *Plasmodium* sporozoites in mosquito tissues. Intact mosquitoes were examined to detect sporozoites during the period of active sporozoite migration and gland invasion, indicated by the presence of both midgut oocysts and salivary gland sporozoites within each mosquito. The total number of free circulating sporozoites detected within mosquitoes was low. While 87% of mosquitoes had hemolymph sporozoites (hemolymph bar), only 10% had greater than 100 (black square inside hemolymph bar). Whether the ovary-associated sporozoites were inside or attached to the surface of the ovaries was not determined.

Fig 5.

Phagocytosis of *Plasmodium* sporozoites by circulating hemocytes. A–C) Differential-interference-contrast light micrographs and fluorescence overlays showing that hemocytes phagocytize sporozoites. Bar = 15 μm. D) Only 2% of hemocytes were observed to phagocytize sporozoites and no hemocyte was observed to have phagocytized greater than two sporozoites.

Fig 6.

Phagocytosis of *Escherichia coli* and latex particles by circulating hemocytes and pericardial cells. A–B) differential-interference-contrast light micrographs and fluorescence overlays showing phagocytosis of green fluorescence protein-expressing *E. coli* (A) and red fluorescent latex particles (B) by hemocytes at 5 min (A1; B1), 1 h (A2; B2) and 3h (A3; B3) post-injection. The majority of hemocytes contained multiple foreign particulates. Bar = 15 μ m. C) Phagocytic indices following injection with *E. coli* or latex beads showing that greater than 95% of circulating hemocytes readily engage in phagocytosis. D–E) Fluorescence micrographs through the cuticle of live mosquitoes showing phagocytosis of *E. coli* (D1–D2) and latex beads (E1–E2) by pericardial cells. Fluorescent foci flanked the lateral sides of the heart in the anterior portion of the abdominal segments. Dotted lines delineate the heart and arrowheads mark the junctions between abdominal segments. Bars: D1, E1 = 500 μ m; D2, E2 = 100 μ m.