Bypassing the Midgut Results in Development of *Plasmodium berghei* Oocysts in a Refractory Strain of *Anopheles gambiae* (Diptera: Culicidae)

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**ABSTRACT** The L35 strain of *Anopheles gambiae* Giles was genetically selected for its ability to melanize and kill malaria parasites. A wide range of *Plasmodium* species are subject to this response when orally ingested, including the rodent malaria, *P. berghei*. However, when we directly injected *P. berghei* into the hemocoel, we found that parasites developed normally to the oocyst stage. This work suggests that the parasite melanization response depends on the interaction of the ookinetes and the midgut. This result is surprising because it contrasts with a genetically validated model system, where injection of CM-Sephadex beads directly into the hemocoel results in bead melanization.

**KEY WORDS** melanization, Sephadex beads, mosquito, malaria

Immune processes in *Anopheles gambiae* are of interest because this mosquito is one of the major vectors of *Plasmodium falciparum*, a causative agent of human malaria. These protozoan parasites must survive for longer than a week in the body of the mosquito as the parasites go through a complex developmental sequence carried out in several different tissues. During this time, the parasites must overcome a number of barriers, both physical and chemical, that can slow or prevent their development (Ghosh et al. 2000).

Melanization of parasites within mosquitoes is an interesting example of an immune response that can result in the complete blockage of parasite development. The best characterized refractory mosquitoes belong to the genetically selected L35 strain of *A. gambiae*. This strain melanizes late ookinetes and early oocysts as they reach the basal side of the midgut epithelium (reviewed in Paskewitz and Gorman 1999). To facilitate study of this phenomenon, a model melanization system was developed using Sephadex beads as targets. One type of Sephadex bead, a carboxy-methylated derivative, is melanized in the L35 strain but not in a second strain of *A. gambiae* that is susceptible to and does not melanize malaria parasites (4a rr strain) (Paskewitz and Riehle 1994). Although beads and parasites are introduced into the mosquito in different ways (via injection or by feeding, respectively), the CM-Sephadex bead model system was validated by genetic mapping, which demonstrated that a major quantitative trait locus affecting *Plasmodium* melanization also affected bead melanization (Gorman et al. 1997, Zheng et al. 1997). A particularly useful attribute of the model is the ability to inject beads and then remove them to examine and identify the proteins that bind to them.

Key components of the melanization reaction seem to originate from the hemolymph (Paskewitz et al. 1988, 1989) or at least to occur in both the hemocoel and the extracellular spaces of the midgut. Because beads are melanized in the hemocoel, and the two melanization phenotypes are genetically related, we predicted that injection of parasites directly into the hemocoel also would result in melanization of these targets. However, herein we report the development of *P. berghei* in the L35 strain of *A. gambiae* after directly injecting cultured ookinetes into the mosquito hemocoel. The ookinetes developed to oocysts, whereas melanized ookinetes or oocysts were never observed. This suggests that interaction between parasite and midgut is a critical aspect of the L35 strain’s ability to recognize and respond to malaria parasites as foreign invaders.

**Materials and Methods**

G3 and L35 strains of *A. gambiae* were used for these experiments. The L35 strain melanizes ookinetes of *P. berghei* after they cross the midgut, whereas most of these parasites develop normally in the G3 strain. Mosquitoes were reared as described previously (Paskewitz et al. 1999).

A transgenic strain of *P. berghei* that produces green fluorescent protein throughout the life cycle (Franke-Fayard et al. 2004; gift of Dr. Andy Waters [Leiden University Medical Center, Leiden, The Netherlands] provided by Dr. George Dimopolous [Johns Hopkins University, Baltimore, MD]) was used to facilitate identification of parasites within the mosquito. Ookinetes were produced as follows. Gametocytes of...
P. berghei were produced during normal infections in BALB/c mice. Peak microgametocyte exflagellation activity typically occurred 7 or 8 d after intraperitoneal injection of a frozen parasite stabilate. On the day of culture, exflagellation of the microgametocytes was confirmed and then anesthetized mice were used for feeding mosquitoes. After feeding was complete, the mice were bled by retroorbital puncture into heparinized 1.5-ml centrifuge tubes. The blood was diluted to a final concentration of 1.5 in ookinete medium (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum and 50 μg/ml gentamicin, pH 8.4) and cultured at 21°C for 20–24 h (procedure modified from Winger et al. 1988).

We used the method of Winger et al. (1988) to partially purify and enrich the cultured ookinetes. Briefly, the blood culture containing ookinetes was centrifuged at 400 × g in a refrigerated centrifuge for 5 min, and the supernatant was discarded. All subsequent manipulations were performed at 4°C. The cell pellet was resuspended in 20 volumes of 0.17 M NH₄Cl for 10 min to lyse the red blood cells. An equal volume of PBS, pH 7.4, was added to stop further lysis, and the sample was centrifuged at 100 × g for 5 min. The pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged again at 100 × g for 5 min. The final pellet was resuspended in 10 μl of PBS. Fluorescence microscopy was used to estimate the number of ookinetes produced.

For one experiment, we added CM-Sephadex beads to the parasites after the 24-h culture period. Parasites and beads were then taken through the purification and enrichment procedure. This process allowed us to test whether inhibitory substances produced from the blood or parasites might affect the melanization system.

Cultured ookinetes (0.2 μl of PBS containing 30–40 cultured ookinetes) or ookinetes, and beads were injected into the L35 mosquito thorax by using a calibrated glass needle. Needles were prepared by pulling microcapillary tubes and calibrated with a Hamilton microsyringe. Injected L35 mosquitoes were reared at 21°C for up to 2 wk. The mosquitoes were dissected at the following times after infection: 24 h, 2 d, 7 d, and 14 d. The thorax and abdomen were opened in a drop of saline, and fluorescence microscopy was used to identify the presence of parasites. We compared the development of oocysts in the orally infected G3 mosquitoes with those in our experimental group (parasites in the experimental group were ≈24 h behind those developing in the G3 strain because of the over-night culture step). We also verified the melanization of these parasites in L35 mosquitoes that were orally infected rather than injected, to eliminate the possibility of contamination of the mosquito strain. Finally, we scored the CM-Sephadex beads for melanization by dissection at 24 h after injection, by using the methods previously described (Paskewitz and Riehle 1994).

Results and Discussion

First, the infection rates of orally infected versus ookinete-injected mosquitoes were compared. Live oocysts were readily found at 48 h postinjection in 93% of injected mosquitoes (n = 13 mosquitoes). Melanized parasites were found on the midgut at this time in 88% of the orally infected mosquitoes (n = 25 mosquitoes). We did not attempt to quantify the numbers of live oocysts in the injected samples because some adhere to tissues, and they are harder to find. However, we found many oocysts developing within the hemocoel of the L35 strain of An. gambiae at 24 h, 2 d, 7 d, and 14 d postinjection (Fig. 1). We have never seen normal oocysts when the L35 strain feeds upon P. berghei-infected mice. Instead, the parasites are melanized at the late ookinete/early oocyst stage, after they cross the midgut and emerge to develop under the midgut basal lamina (Fig. 2). We examined the body cavity and tissues to determine whether melanized ookinetes might be visible in the injected mosquitoes, but none were found.
We also tested the effect of adding CM-Sephadex beads along with parasites during the purification procedure to be sure that no inhibitory substances were produced during this process. After the purification, we coinjected beads and parasites and then removed and scored the beads 24 h later. We found that these beads were melanized as efficiently as control beads (Fig. 3), whereas parasites were not melanized.

The lack of congruence between the results of CM-Sephadex bead and parasite injections into the hemocoel was a surprise. One hypothesis to consider is that the parasites may change during passage through the midgut in such a way that melanization can be triggered once the extracellular environment of the midgut basal labyrinth is reached. The CM-Sephadex bead surface may mimic some aspect of this change. Ookinetics seem to secrete a subtilisin-like protease and the Pbs21 surface protein into the cytoplasm of invaded cells (Han et al. 2000), suggesting that the parasite surface may be dynamic in this environment. In addition, a number of changes occur in the midgut epithelium during interaction with *Plasmodium* ookineties. These include changes in expression of immune-related and other genes (Dimopoulos et al. 2002), nitric oxide generation (Luckhart et al. 1998), and possible triggering of apoptosis as the parasites pass through cells (Zieler and Dvorak 2000). Any of these responses might in turn cause changes in the parasite surface or secretions.

Another hypothesis is that there are differences in the two compartments (midgut and hemocoel) that cause the parasites to interact with the melanizing system differently in each location. These differences might be in the concentrations or absolute presence or absence of chemicals that trigger or inhibit melanization on various surfaces. To illustrate the point, consider the possibility that an inhibitor of melanization
occurs in the hemocoel but not in the midgut. If the inhibitor interacted with the surface of the parasite but not the bead, then the predicted results would be similar to those we observed.

That the midgut is a key barrier for *Plasmodium* development has been suggested by other experiments. In some parasite–mosquito combinations, the parasites are unable to develop past stages found in the bloodmeal (Vaughan et al. 1994). Because the gut of all animals constitutes a point of contact with potential pathogens and parasites, it is not surprising that the mosquito midgut would be armed with an array of defensive mechanisms. The unexpected complexity of the melanization response is a fascinating window into one of these mechanisms.

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