New Ultrastructural Analysis of the Invasive Apparatus of the *Plasmodium* Ookinete

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Abstract. Invasion of the mosquito midgut by the *Plasmodium* ookinete determines the success of transmission of malaria parasites from humans to mosquitoes and therefore, is a potential target for molecular intervention. Here, we show higher-resolution ultrastructural details of developing and mature *P. gallinaceum* ookinetes than previously available. Improved fixation and processing methods yielded substantially improved transmission electron micrographs of ookinetes, particularly with regard to visualization of subcellular secretory and other organelles. These new images provide new insights into the synthesis and function of vital invasive machinery focused on the following features: apical membrane protrusions presumptively used for attachment and protein secretion, dark spherical bodies at the apical end of the mature ookinete, and the presence of a dense array of micronemes apposed to microtubules at the apical end of the ookinete involved in constitutive secretion. This work advances understanding of the molecular and cellular details of the *Plasmodium* ookinete and provides the basis of future, more detailed mechanistic experimentation on the biology of the *Plasmodium* ookinete.

INTRODUCTION

In recent years, targeting mosquito stages of the parasite by vaccines and engineering transgenic mosquitoes are key strategies for reducing malaria transmission and eliminating the disease.^{1–3} The transmission of malaria begins when a female mosquito takes up a blood meal from *Plasmodium*-infected vertebrate hosts. The gametocyte stages of the parasite enter the mosquito midgut along with the blood meal (and potential transmission-blocking antibodies that might be produced during natural infection or induced by vaccination).^{4–8} Gametocytes are rapidly activated in response to the midgut milieu, emerging within seconds from red cells and forming haploid male and female gametes that merge to form diploid zygotes. Zygotes then transform into ookinetes after 12–24 hours. Ookinetes are elongated, motile cells that penetrate the chitin-containing peritrophic matrix using chitinases and proteases,^{9,10} invade and cross midgut epithelial cells, and finally, come to rest and develop into oocysts at the basal lamina.¹¹ Within oocysts, sporozoites develop by asexual reproduction and fission, eventually releasing thousands of sporozoites that make their way to salivary glands, ready to be injected into new vertebrate hosts during blood meal ingestion.

Fundamental understandings of all aspects of *Plasmodium* cell biology are likely to underlie new contributions to the development of antimalarial strategies. This study focuses on ultrastructural features of the ookinete, with a specific interest in understanding mechanisms of protein secretion and invasion of the mosquito midgut by this developmental stage of *Plasmodium* that may be amenable to vaccine-induced antibody blocking. For example, it has been shown that not only surface proteins, such as P25 and P28 family proteins, but also ookinete-secreted proteins may synergize as transmission-blocking vaccine targets.^{10,12} To this end, new methods to study the ultrastructure of *Plasmodium* ookinetes may provide new insights into how to interfere with the biology of malaria parasites to the goal of malaria control.¹³

Here, we show new transmission electron microscope morphologies of developing and mature ookinete stages of

P. gallinaceum. These studies will help us understand the role of secretory/invasion-related organelles in ookinete invasion of the mosquito midgut and details of the constitutive secretion mechanisms of this parasite developmental stage. Furthermore, we describe an optimized experimental protocol for the *in vitro* culture of *P. gallinaceum* ookinetes in serum-free medium.

MATERIALS AND METHODS

***In vitro* culture of *P. gallinaceum* ookinetes in serum-free media.** A gametocyte-producing line of *P. gallinaceum* strain 8A was maintained by cycling through White Leghorn chickens and *Aedes aegypti* mosquitoes. Procedures were carried out according to animal protocols approved by the Institutional Animal Care and Use Committee at the University of California at San Diego. Buffers and solutions used in this protocol were sterilized by passing through a 0.22-micron filter (Millipore, Bedford, MA), and experiments were carried out under sterile conditions. Briefly, 5- to 6-week-old female chickens were inoculated with P1 parasites (the first blood passage of parasites after mosquito inoculation; 10⁵ parasites) into the wing vein. Parasitemia was monitored after 4 days by Giemsa-stained thin blood smear. At 10% parasitemia, chickens were anesthetized and terminally bled by cardiac puncture using a 10-mL syringe containing heparin (50 units/mL blood; excess heparin is deleterious to the culture). From each chicken, an average of 5 mL blood was obtained. The blood sample was immediately transferred into a pre-warmed (37°C) 50-mL tube containing 35 mL suspended animation buffer (SA buffer, 8.3 mM Tris, 150 mM NaCl, 10 mM glucose, pH 7.4). Cardiac blood samples from three chickens (maximum of 15 mL) were pooled together in the same SA buffer tube and centrifuged (37°C) at 3,000 rpm for 5 minutes, and the supernatant was discarded. To the pellet, 20 mL pre-warmed (37°C) exflagellation media (15% heat-inactivated chicken serum, 0.1 mM xanthurenic acid, 25 mM sodium bicarbonate in 1× SA buffer) were added, vortexed briefly, and incubated at room temperature for 30 minutes with intermittent mixing. The resulting cell suspension was put onto a Ficoll-Hypaque density gradient. Briefly, 10 mL 34% Hypaque were mixed with 17.5 mL 9% Ficoll, and the mixture was loaded onto the bottom of a sterile 50-mL clear centrifuge tube (Oak Ridge centrifuge tube, Nalgene, Nalge

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Nunc International, Rochester, NY). Exflagellation medium containing the cell suspension was layered onto the top of the Ficoll-Hypaque cushion and centrifuged at 6,000 rpm for 15 minutes at 22°C in a swinging bucket rotor (Sorvall HB-4 centrifuge, 2,500 × *g*). The tubes were carefully removed from the centrifuge without disturbing the gradient, the middle gritty layer (contains zygotes) was carefully removed using a glass Pasteur pipette into a 50-mL sterile polypropylene (Falcon, BD, Franklin Lakes, NJ) tube, the layer was diluted five times with SA buffer, and it was centrifuged (4,000 rpm for 5 minutes at 22°C) to pellet the zygotes; 10 mL SA buffer were added to the cell pellet, which was gently resuspended. Wheat germ agglutinin (WGA; selectively binding sialic acid on chicken but not parasite cells) was incubated with the cells (1 mg in SA buffer; Sigma, St. Louis, MO) at 22°C for 20 minutes and then centrifuged at 100 × *g* (low speed spin) in a standard tabletop centrifuge for 3 minutes to remove contaminating chicken red and white cells. The supernatant containing zygotes was transferred to a fresh 50-mL tube, diluted 10 times with SA buffer, centrifuged to pellet the purified zygotes, and resuspended with 25 mL ookinete medium (M199 medium containing 0.2% glucose, 2 mM L-glutamine, 50 U penicillin, 50 µg streptomycin/mL; Life Technologies, Carlsbad, CA). The cultures were incubated at 26°C for 36 hours. The number of ookinetes present per culture, proportion transformation from zygote to ookinete, and number of zygotes and ookinetes were quantified using a hemocytometer. A second purification step was carried out to remove remaining chicken cells by adding biotinylated WGA (1:250 dilution of 1 mg/mL solution) with 30 minutes incubation at room temperature followed by adding streptavidin-coated magnetic beads (1:100 dilution; Invitrogen, Carlsbad, CA). After incubation at 22°C for 30 minutes, the tube was placed in a magnetic block for 30 minutes, and the parasite-containing flow-through was also used for processing.

Transmission electron microscopy. For transmission electron microscopy (TEM) studies, three separate ookinete cultures having > 60% ookinete transformation were pooled, pelleted, and washed 1× with phosphate-buffered saline (PBS). Cells were immediately fixed in Karnovsky's fixative (3% paraformaldehyde, 1.5% glutaraldehyde, and 5% sucrose in 0.1 M cacodylate buffer, pH 7.4) for 2 hours at 22°C followed by overnight incubation at 4°C. After fixation, samples were washed three times in 0.1 M cacodylate buffer followed by 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour. The preparation was then washed three times in 0.1 M cacodylate buffer, treated for 1 hour with 1% uranyl acetate in 10% ethanol, and dehydrated in an alcohol series from lower to higher concentrations (25%, 50%, 75%, 90%, and 100%). Then, samples were treated with 1:1 mixture of oxalic acid/ethanol, gradually infiltrated with LX112 resin, and embedded in the LX112 resin that was allowed to polymerize overnight at 60°C. Sections were cut with a diamond knife, treated in 2% uranyl acetate, and then stained in lead nitrate treatment of 2 minutes. Grids were examined with a JEM-1200 EX transmission electron microscope (Hitachi, Tokyo, Japan).

RESULTS

The *in vitro* culture of *P. gallinaceum* ookinetes in serum-free M199 media yielded an average of 2.4 million ookinetes

(0.6–5.6 million) from a total of 15 mL *P. gallinaceum*-infected blood sample with parasitemia of ~10%. The average proportion of parasites that transformed from zygotes to ookinetes was 46% (range = 33–63%). The results were estimated from 12 independent cultures under similar conditions. These yields were an improvement over previous methods^{14,15} that used higher chicken parasitemia^{14–16} as a source of gametocytes (30–50%) and less chicken serum (10%) for the gametogenesis/fertilization step. Using these previous protocols, we routinely achieved 20–30% zygote to ookinete transformation, whereas the newer modification reproducibly led to higher transformation efficiencies (33–63%).

In vitro cultures that produced more than 60% ookinete transformation were used for TEM studies. In this method, Karnovsky's fixative and LXR 112 resin were used to prepare the ookinete cells, which resulted in better structural and organelle integrity and resolution than previously observed in *Plasmodium* (including the incorrect depiction of rhothies in the apical complex)^{17–28} and *Haemoproteus*.²⁹

The TEMs (Figures 1 and 2) revealed key features of the morphological differentiation of secretory organelles and apical conoid structures that differed significantly between developing (Figure 1) and mature (Figure 2) ookinetes. These cell developmental processes are necessary for ookinete invasion of the mosquito midgut, but molecular details by which these processes are regulated remain obscure. The apical end of the ookinete shows highly adapted morphological features (Figure 2), showing the protrusion of the outer membrane at the apex to form a cone or sucker-like structure. This structure (magnified in Figure 2B) is likely to be the extruded polar rings as previously commented on in the work by Garnham and others,¹⁸ but here, we improved ultrastructural resolution. The morphology of this structure suggests a potential function in midgut epithelial cell attachment and the means by which micronemal contents are released to hydrolyze the chitin- and chitin binding protein-containing peritrophic matrix^{30–33} to facilitate the invasion process as previously observed in the work by Sieber and others.³⁴

Among the unique and hitherto subcellular structures of unknown function in apicomplexan parasites including the ookinete stage of *Plasmodium* is the crystalloid body.³⁵ In *Plasmodium*, these transient structures are only present in ookinetes and young oocysts, and they have been suggested to have an important role in malaria transmission, including the localization of LCCL proteins, homologues of which have been implicated in sporozoite motility and infectivity.³⁶ The developing ookinete has repeatedly been observed to contain the crystalloid body (Figure 1) composed of hundreds of particles geometrically arranged in a geometric pattern reminiscent of viral particles but likely being pseudocrystallized protein structures.³⁵ Figure 2A shows a dense spherical structure, more or less in the apical part of the ookinete. These structures, devoid of typical features of crystals, do not seem to be comprised of malaria pigment/hemozoin. Whether these bodies are membrane-bound is unclear, and their functional roles remain to be determined.

The most definitive images presented here depict the presence of numerous micronemes in the apical end of the ookinete (Figure 3A), consistent with the known enormous, constitutive protein secretion capability of the ookinete during the simultaneous processes of motility and invasion.¹² A dense set of variably staining and shaped micronemes, both cigar-shaped

FIGURE 1. Ultrastructural feature of developing or immature *P. gallinaceum* ookinete (Scale bar: 500 nm). The TEM of a longitudinal ookinete section shows the electron dense area of the apical complex (AC), small protein-containing spheres arranged as a honeycomb-like structure called crytalloids (CR), hemozoin (HZ), nucleus (NU), and elongated secretory micronemes (MI).

and rounded, abut microtubules known to be involved in microneme mobilization and secretion.²⁶ The high-quality visualization of these structures, distinctly seen in Figure 3B, may be the result of improved TEM methods described herein.

DISCUSSION

Using an optimized method for the *in vitro* production of large numbers of *P. gallinaceum* ookinetes and improved TEM fixation and imaging methods, we show here unique features of mature ookinetes, including a novel secretion apparatus involving the polar rings, a dense set of micronemes abutting microtubules, and improved ultrastructural resolution of all aspects of ookinete morphology.

Over the past four decades, production of the various species of *Plasmodium* ookinetes (*P. falciparum*,^{26,37,38} *P. vivax*,³⁹ and rodent-infecting parasites including *P. berghei*, *P. yoelii*, and *P. yoelii nigeriensis*^{40–42}) in *in vitro* systems has been a cumbersome procedure and limited by quantities of parasites obtainable by *in vitro* culture for detailed analysis.

FIGURE 2. Ultrastructural features of a mature *P. gallinaceum* ookinete. (A) TEM of a longitudinal section of a mature *P. gallinaceum* ookinete and (B) a magnified image of the unique apical structure (Scale bar: A, 1 μ m; B, 200 nm). The pictures show the apical complex (AC), hemozoin (HZ), mitochondria (Mt), nucleus (NU), and an extended apical pellicle (EP), which has a large opening (apical pore) likely to be involved in secretion. *The darkly stained circular bodies are regularly seen structures of unknown function.

Herein is described an improved method of *in vitro* production of *P. gallinaceum* ookinete in serum-free media.^{14,43} In contrast to *P. falciparum*, *P. gallinaceum* apparently does not need serum proteins, such as mammalian host-derived plasminogen, for ookinete development.^{44,45} Although previous work used parasitemias ranging from 30% to 50% as source of parasites for *in vitro* parasite preparation, we found that a parasitemia not exceeding ~10% was more efficient in yielding *P. gallinaceum* gametocytes. Other details of parasite preparation were also slightly modified, including the following: dilution of parasitemic blood with SA buffer (reduced to 1/10); processing blood sample collection more quickly (using 50-mL tubes and completing in < 30 min); an increased volume of exflagellation solution (3 $\times$ blood volume); higher concentrations of serum (15%) and xanthurenic acid (0.1 mM) were added followed by brief gentle vortexing; and the incubation time for exflagellation was extended to 30 minutes. These small changes, detailed here, allowed us to significantly improve *P. gallinaceum* ookinete production in serum-free media. These procedures should allow new avenues to improve transmission-blocking vaccine and drug discovery experimentation to transmission-blocking vaccine and drug approaches as well

FIGURE 3. TEM of an oblique section of *P. gallinaceum* ookinete showing (A) micronemes and (B) the involvement of microtubules (MTB) for the micronemal (MI) secretion (Scale bar: 200 nm).

as allow for new fundamental studies of this neglected aspect of the malaria parasite life cycle.

Since the first electron microscopic studies of *P. gallinaceum* ookinete morphologies by Garnham and others¹⁷ appeared in 1962, numerous additional reports on the fine structure of ookinete morphology among different species of *Plasmodium* have been published.^{17–19,27,46} Here, we show, using improved techniques for electron microscopy, finer details of the ultrastructure of *Plasmodium* ookinetes than previously appreciated. These improved results were primarily because of better fixation procedures producing intact subcellular structures. Previously, similar fixation and embedding in LXRI12 was used to study the TEM of knob and knobless *P. falciparum*-infected erythrocytes.⁴⁷ The ultrastructure findings of the apical complex structures required new fixation and embedding methods and materials. It is noteworthy to observe that the apical end of the mature ookinete protrudes as a sucker-like structure, which might enable effective attachment and targeted secretion of enzymes, such as proteases and chitinases, to dissolve down peritrophic matrix (PM) barrier as previously visualized.^{27,34} This extension of apical structure, not previously observed in studies of the fine structure of *P. falciparum* and *P. gallinaceum* ookinetes, was previously reported in ookinetes of the rodent-infecting parasite *P. yoelii*, both in free ookinetes in culture and during penetration of the basement membrane of the mosquito midgut.^{26,48–51}

The *Plasmodium* life cycle is complex, with sequential, pre-programmed developmental stages in vertebrate and invertebrate hosts. Although the merozoite and sporozoite stage parasites responsible for red cell and liver cell invasion, respectively, contain three distinct organelles—micronemes, rhoptries, and dense granules—the ookinete only seems to have one such organelle, micronemes. Nonetheless, there are organelles in the ookinete that do require additional explanation (for example, the dense spherical structures at the apical end of the parasite). These organelles are unlikely to be homologous to dense granules, because the ookinete does not form a parasitophorous vacuole in the mosquito midgut epithelium. The multiple crystalloid bodies in ookinetes, earlier thought to be virus-like particles, have recently been shown to contain important proteins such as the LCCL family proteins involved in midgut invasion in *P. berghei*.⁵¹ Recently, the proteome of crystalloid bodies in *Eimeria tenella*, an

apicomplexan protozoan that causes chicken coccidiosis, was analyzed and confirmed as a reservoir of proteins necessary for invasion and energetic and metabolic functions,⁵² but such findings need to be corroborated and extended in *Plasmodium*. Recent proteomic studies of *Plasmodium* zygote and ookinete stage proteins^{53,54} will facilitate functional analysis of these sexual stage proteins by use of improved electron microscopic techniques.

The electron micrographs presented here are consistent with the notion that the ookinete is a highly efficient secretion machine that constitutively secretes high levels of chitinases, proteases, and possibly, other unknown factors in a non-cell–cell contact manner, allowing the parasite to cross the chitin- and proteinaceous-containing PM *en route* to oocyst development.^{33,55} These structures likely allow the ookinete to recognize and penetrate the midgut epithelial cell as well. In ookinetes, micronemes are the only known pathway for secretion of cell surface-associated or secretory proteins, such as circumsporozoite and TRAP-related protein (CTRP), chitinase, and WARP (von Willebrand factor A domain related protein).^{12,32,56–58} Proteins essential for ookinete invasion are thought to be localized in micronemes,⁹ and therefore, soluble micronemal proteins are presumably important potential targets of transmission-blocking immunity.^{3,12} We and others⁴⁹ have shown that a single ookinete possesses numerous micronemes, corresponding to the most abundant, densely packed organelle found in the cell (Figure 3A). These dense, protein-packed organelles are enclosed by a lipid membrane,⁵⁹ but whether all micronemes contain the same set of proteins is unknown. The large number of micronemes combined with the extraordinary constitutive secretory capacity of the ookinete suggests that any neutralizing antibodies targeting micronemal proteins may be needed in a very high titer for effective transmission blocking.

In conclusion, these new studies of ookinete ultrastructure add to our knowledge of the fine structure and components of the *Plasmodium* ookinete, *Plasmodium*, and more generally, apicomplexan cell biology, and they provide a greater level of insight into the interaction of *Plasmodium* with its definitive host, the mosquito. With improved methods for obtaining *P. falciparum*^{26,37,38} and *P. vivax* ookinetes *in vitro*,^{39,60} studies of these human-infecting parasites are now possible as well.

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