In Vitro Generation of Plasmodium falciparum Ookinetes

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Abstract. Plasmodium transmission from the human host to the mosquito depends on the ability of gametocytes to differentiate into ookinetes, the invasive form of the parasite that invades and establishes infection in the mosquito midgut. The biology of P. falciparum ookinetes is poorly understood, because sufficient quantities of this stage of the parasite species have not been obtained for detailed study. This report details methods to optimize production of P. falciparum sexual stage parasites, including ookinetes. Flow cytometric sorting was used to separate diploid/tetraploid zygotes and ookinetes from haploid gametocytes and unfertilized gametes based on DNA content. Consistent production of 10^5–10^6 P. falciparum ookinetes per 10 mL culture was observed, with ookinet transformation present in 10–40% of all parasite forms. Transmission electron micrographs of cultured parasites confirmed ookinete development.

INTRODUCTION

Malaria affects more than 500 million people and kills an estimated 900,000 people each year.¹ Interest in the potential of transmission-blocking vaccines to control or even eradicate malaria has recently increased; such vaccines work by inducing antibodies against components of the parasite’s sexual stage forms, hence reducing infectivity of the reservoir, humans, for the obligate vector, Anopheles mosquitoes.²–¹¹ More detailed understanding of Plasmodium falciparum transmission stage biology, particularly ookinetes, would directly contribute to human transmission-blocking vaccine development.

Plasmodium sexual development occurs in the mosquito midgut. Mature gametocytes taken up with the mosquito blood meal emerge from erythrocytes as gametes. Male microgametes fertilize female macrogametes to generate zygotes. The parasite transforms into a motile, constitutively secreting and invasive ookinete at the same time that it is undergoing genetic recombination.¹²–¹⁶ Sexual stage-specific antigens are potential targets for transmission-blocking antibodies, which has been most robustly shown in animal models of malaria.¹⁶,¹⁷ Because of experimental challenges, the biology of few sexual stage antigens of P. falciparum is understood in any detail.¹⁸ Recent successes have been achieved in generating P. falciparum sexual stage parasites in vitro, including a recent study by Ghosh and others.¹⁹,²⁰ However, a detailed study of the biology of transmission-blocking antigens is still limited by the inability to generate large quantities of P. falciparum sexual stage parasites, particularly ookinetes, in vitro.

Here, we report optimization of methods that allow for the consistent generation of 10^5–10^6 P. falciparum ookinetes per 10 mL in vitro culture.¹⁹,²¹–²⁵ Flow cytometry sorting based on detection of DNA content allowed for the enrichment of different sexual stage parasite forms that allow for the direct study of the different stages of P. falciparum sexual stage parasites.

HIGH-YIELD OOKINETE PRODUCTION

The P. falciparum strain NF54 was maintained in continuous asexual culture according to standard protocol.²⁶ Human blood used for in vitro culture was freshly drawn from volunteers after informed consent according to a protocol approved by the University of California, San Diego Human Subjects Protection Program. Gametocytes were cultured as previously described²⁷ (Appendix), and the overall procedure is schematized in Figure 1. Morphologically mature microgametocytes were seen as early as 12 days for microgametocytes and 14 days macrogametocytes and as late as 22 days for both. To compensate for this lack of synchronization, gametocyte cultures were started 2–3 days apart to ensure that cultures containing mature microgametocytes could be mixed with cultures containing mature macrogametocytes.²⁸–²⁹

On days 14–17 of culture, mature macrogametocytes were tested for the ability to exflagellate and emerge under standard conditions (Appendix).³⁰ Two gametocyte cultures with microgametocytes confirmed to be functional (≥4 exflagellation centers per 40x field) were combined with two gametocyte cultures with emergence-competent macrogametocytes (≥80% macrogamete emergence). The combined parasites were centrifuged, and the pellet was resuspended in heat-inactivated AB+ human serum to 10–20% hemocrit at 19–23°C (ambient laboratory temperature) for 30 minutes for gamete maturation and zygote fertilization. Sexual stage parasites were centrifuged, and the pellet was resuspended in freshly prepared filter-sterilized ookinet medium (RPMI–1640, 25 mM Hepes, 2 mM l-glutamine, 2 g/L NaHCO₃, 50 mg/L hypoxanthine, 15% heat-inactivated AB+ human serum or heat-inactivated fetal bovine serum [FBS], pH 8.2–8.4, with NaOH) to 10–20% hemocrit. Centrifugation steps were done at 19–23°C and 800 x g without brake. Parasites in ookinet medium were transferred in 10-mL aliquots to 25-cm² flasks and gently rocked at 19–23°C for 36–48 hours, although ookinetes could be seen in culture for up to 72 hours in ookinet medium.

The generation of large quantities of P. falciparum ookinetes was dependent on the presence of mature micro- and macrogametes, which, in turn, was dependent on the production of mature micro- and macrogametocytes (Figure 2). Gametocyte cultures contained up to 8.5% gametocytemia, and 50–90% of these gametocytes were stage V gametocytes (Table 1). Optimized cultures had an average ookinet density of 25%, with a yield of 5–50 × 10⁶ ookinetes per 10 mL of ookinet...
culture (Table 2). This yield is at least 4- to 8-fold better than previously described methods.23,31

**Purification of P. falciparum Sexual Stage Parasites**

*P. falciparum* sexual stage cultures were produced as mixtures of uninfected red blood cells, asexual stage parasites, gametocytes, macrogametes, zygotes, and ookinetes. Stage-specific enrichment was approached using multiple purification methods, including single-step density centrifugation, magnetic separation, discontinuous density gradient centrifugation, and flow cytometry sorting. The majority of uninfected erythrocytes were removed from sexual stage parasite cultures by single-step density gradient centrifugation (Lympholyte-H; Cedarlane Laboratories, Burlington, NC) according to manufacturer’s instructions. Parasites were collected from the gradient interface, washed twice in ookinete medium, and further purified by magnetic separation. Purification of parasites using density gradient removed approximately 90% of red blood cells. Further purification of parasites using magnetic separation resulted in removal of approximately 95–99% of red blood cells and facilitated sorting of parasites by flow cytometry. A MidiMACS magnetic separator with an LD-50 column was used to positively select for condensed hemozoin-containing gametocytes, macrogametes, zygotes, and ookinetes as well as any remaining asexual stage schizonts, according to manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).32 Magnetic purification of sexual stage parasite cultures was done at 19–23°C using ookinete medium instead of the manufacturer’s recommended MACS® buffer. Magnet-retained sexual stage parasites were washed, eluted from the magnetic column, and then, centrifuged at 800 × g. The cell pellet was resuspended in 100–500 μL of ookinete culture medium and then placed on a 6–11% or 11–16% Nycodenz gradient (Sigma-Aldrich, St. Louis, MO).30,33 Briefly, 400 μL of 6% Nycodenz, 400 μL of 11% Nycodenz, and 400 μL of 16% Nycodenz were layered in a 1.5-mL microcentrifuge tube; 200 μL of resuspended parasites were layered on top of the three-step gradient and centrifuged at 10,000 × g for 10 minutes at 4°C with no brake. The 6–11% contained self-adherent macrogametes, consistent with previous
This difference in ploidy was exploited to separate gametocytes. Asexual stage-to-gametocyte transformation efficiencies for five representative cultures are shown. Optimized = use of conditions to increase parasite density and addition of RBC groups. Increased density = cultures exhibiting ≥80% macrogamete emergence and exflagellation, defined as <80% mature gametocytemia oocysts. Low gametocyte* = cultures with less than optimized parameters resulted in sexual stage parasite cultures (Figure 3). Region R3 showed enrichment for gametocytes, and R4 showed enrichment for non-adherent macrogametes. The finding that macrogametes could be separated from gametocytes by DNA content was surprising but consistent with previous fluorometric studies.9,14 Region R6 was enriched for a mixture of zygotes and ookinetes (Figure 3). The finding that the majority of zygotes and ookinetes had more than or equal to four times as much DNA as region R3 is consistent with our current understanding that meiotic division occurs shortly after gamete fusion.9,13,14,19–21 It is possible that further refinements of flow cytometric sorting (for example, incorporation of forward- and side-scatter parameters to separate round forms from elongated forms based on size or clumping) might be able to separate zygotes from ookinetes.

**TABLE 1**

<table>
<thead>
<tr>
<th>Culture number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asexual parasitemia</td>
<td>0.3%</td>
<td>0%</td>
<td>0%</td>
<td>0.4%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Immature gametocytemia</td>
<td>3%</td>
<td>0.5%</td>
<td>2.2%</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>Stage V gametocytemia</td>
<td>5%</td>
<td>6%</td>
<td>6.3%</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Total gametocyte yield</td>
<td>20 × 10⁶</td>
<td>3.3 × 10⁶</td>
<td>7.5 × 10⁶</td>
<td>5 × 10⁷</td>
<td>10 × 10⁷</td>
</tr>
</tbody>
</table>

Gametocytemia of unpurified *P. falciparum* gametocyte cultures was determined when the majority of gametocytes were mature, as early as 16 days and as late as 22 days of culture; 1,100–2,400 cells were counted in 10 fields. Average yields were 2–20 × 10⁶ gametocytes per 25 mL culture. Asexual stage-to-gametocyte transformation efficiencies for five representative gametocyte cultures used for oocyst preparation are shown.

**FIGURE 2.** Gametocyte and gamete maturity significantly affect oocyste production. Oocyste density in sexual stage cultures depends on gametocytemia and gamete maturation. Sexual stage parasite cultures containing 19–42% ookinetes were consistently produced by mixing ≥5% macrogametocytemic cultures with ≥2% microgametocytemic cultures at a final hematocrit of 10–20%. Optimized = use of conditions to increase parasite density and addition of RBC groups. Increased density = cultures exhibiting ≥80% macrogamete emergence and exflagellation, defined as <80% mature gametocytemia oocysts. Low gametocyte* = cultures with less than optimized parameters resulted in sexual stage parasite cultures (Figure 3). Region R3 showed enrichment for gametocytes, and R4 showed enrichment for non-adherent macrogametes. The finding that macrogametes could be separated from gametocytes by DNA content was surprising but consistent with previous fluorometric studies.9,14 Region R6 was enriched for a mixture of zygotes and ookinetes (Figure 3). The finding that the majority of zygotes and ookinetes had more than or equal to four times as much DNA as region R3 is consistent with our current understanding that meiotic division occurs shortly after gamete fusion.9,13,14,19–21 It is possible that further refinements of flow cytometric sorting (for example, incorporation of forward- and side-scatter parameters to separate round forms from elongated forms based on size or clumping) might be able to separate zygotes from ookinetes.

**TABLE 2**

<table>
<thead>
<tr>
<th>Gametocyte (%</th>
<th>39%</th>
<th>45%</th>
<th>37%</th>
<th>32%</th>
<th>13%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gametocytes (%)</td>
<td>24%</td>
<td>16%</td>
<td>12%</td>
<td>31%</td>
<td>67%</td>
</tr>
<tr>
<td>Oocyste yield</td>
<td>20 × 10⁶</td>
<td>3.3 × 10⁶</td>
<td>7.5 × 10⁶</td>
<td>5 × 10⁷</td>
<td>10 × 10⁷</td>
</tr>
</tbody>
</table>

Purified sexual stage parasites were stained with Leukostat and examined by light microscopy to determine sexual stage parasite densities. Approximately 10²–10⁷ parasites in 10 fields were counted. Yields were 5–60 × 10⁶ oocystes per 10 mL culture. Transformation efficiencies for five representative cultures are shown.

parum zygotes and ookinetes from other sexual stage forms using flow cytometric sorting. Parasites from the 11–16% Nycodenz gradient interface were washed and stained with Vybrant DyeCycle Green stain according to manufacturer’s instructions (Molecular Probes; Invitrogen, Carlsbad, CA). Approximately 10²–10⁷ parasites were then passed through a 30-μm filter to remove clumps of self-adherent cells and were sorted by fluorescence intensity using a MoFlo high-speed sorter (Dako, Glostrup, Denmark). Stained parasites were analyzed by flow cytometry and divided into four subgroups based on fluorescence (Figure 3). Region R3 showed enrichment for gametocytes, and R4 showed enrichment for non-adherent macrogametes. The finding that macrogametes could be separated from gametocytes by DNA content was surprising but consistent with previous fluorometric studies.9,14 Region R6 was enriched for a mixture of zygotes and ookinetes (Figure 3). The finding that the majority of zygotes and ookinetes had more than or equal to four times as much DNA as region R3 is consistent with our current understanding that meiotic division occurs shortly after gamete fusion.9,13,14,19–21 It is possible that further refinements of flow cytometric sorting (for example, incorporation of forward- and side-scatter parameters to separate round forms from elongated forms based on size or clumping) might be able to separate zygotes from ookinetes.

**PFS25 AND CHITINASE (PFCHT1) DETECTION IN GAMETOCYTES AND OOKINETES**

To see if Pfs25 or PfCHT1 could be used as markers to classify elongated parasites as gametocytes or ookinetes (Figure 4A and B), gametocytes and *in vitro*-generated ookinetes were examined by an immunofluorescence assay (IFA) (Figure 4C–H) using previously characterized antibodies to Pfs25 and chitinase,9,10 two proteins presumed to be zygote/ookinete and ookinete-specific, respectively (Appendix).9,13,14,19–21 By IFA, immunoglobulin G (IgG) negative control antibody did not generate fluorescence signal in either gametocytes or ookinetes, whereas antibodies to *P. falciparum* chitinase (PfCHT1) and Pfs25 produced fluorescent signals in gametocytes (as previously described for Pfs25) and ookinetes (Figure 4C–H). The fluorescence signal was observed to be stronger in ookinetes than in gametocytes, with a distinct pattern consistent with surface localization of Pfs25 notable in ookinetes but not gametocytes.

Western immunoblots using the monoclonal antibody 1C3 to the *P. falciparum* chitinase PfCHT1 detected the protein in mixed gametocytes, untransformed gametes, and early zygotes.
directly and in 72-hour ookinete cultures (Figure 4I), with an increased intensity of PfCHT1 seen in mature ookinetes.

The quantitative and non-qualitative aspects of these findings indicate that neither the presence of chitinase nor Pfs25 protein, as detected by IFA or Western immunoblot, unequivocally distinguishes *P. falciparum* ookinetes from gametocytes.

ULTRASTRUCTURAL ANALYSIS OF *P. FALCIPARUM* SEXUAL STAGE PARASITES

Transmission electron microscopy (TEM) of sexual stage cultures was done to determine whether elongated, banana-shaped parasites were either gametocytes or ookinetes (Figure 5 and Appendix). Parasites submitted for TEM were
enriched by magnetic and density gradient separation techniques. Approximately 30% of parasites were ookinetes as determined by light microscopic examination of Leukostat-stained slides. Qualitative analysis of cultivated parasites by TEM showed the presence of round parasite sections, which could have been cross-sections of macrogametes, zygotes, gametocytes, or ookinetes, as well as elongated parasite sections, which could have been gametocytes and ookinetes. Cross-sections of gametocytes and ookinetes could be identified by the presence of subpellicular microtubules, which supports the characteristic banana-shaped forms. Gametocytes had a surrounding erythrocyte membrane, whereas cross-sections of ookinetes did not.\textsuperscript{41,49–55}

Examination of erythrocyte-free elongated parasites showed definitive ookinete ultrastructure, including the apical complex and a pellicle, which was not observed in gametocytes (Figure 5). The apical complex consists of an apical polar ring and micronemes. The polar ring serves to anchor the subpellicular microtubules.\textsuperscript{55} Micronemes are round, protein-dense, membrane-bound organelles that have been shown to contain proteins secreted through their ducts near the apical end.\textsuperscript{56,57} The ookinete pellicle includes the parasite plasma membrane, the inner membrane complex, and subpellicular microtubules.\textsuperscript{55}

The method presented here consistently produced large quantities of sexual stage forms of the human malaria parasite \textit{P. falciparum}. Currently, standard methods for consistently generating \textit{P. falciparum} zygotes and ookinetes require feeding gametocytes to \textit{Anopheles} mosquitoes followed by dissection of these sexual stage forms from mosquito midguts. Despite electron microscopic proof of \textit{P. falciparum} ookinete production, a limitation of the present report is that the ability of \textit{in vitro}-generated \textit{P. falciparum} ookinetes to produce oocysts in mosquitoes was not verified. Nonetheless, this improved production of \textit{P. falciparum} sexual stage parasites is a significant step to understanding biological details of ookinetes specific to this human malaria parasite. This work comes at a critical juncture as interest increases in malaria control.

\textbf{Figure 5.} Ultrastructural features of the \textit{P. falciparum} ookinete. (A) Sagittal section of an ookinete shows micronemes (m), seen as electron-dense round or cigar-shaped organelles, and a hemazoin crystal (h). (Scale bar: 1 μm.) (B) For comparison, a gametocyte within an infected erythrocyte (e) is shown with a discrete digestive vacuole (dv) with hemozoin (h). (Scale bar: 1 μm.) (C) Tangential section of the apical end of an ookinete showed microtubules (→), which converge at the polar ring (pr). This section showed narrow ducts of micronemes (m) that appear to track to the apical end (*→). (Scale bar: 200 nm.) (D) Transverse, midline section of an ookinete apical end showed the apical pore (ap), micronemes (m), and microtubules (→). (Scale bar: 200 nm.) (E) Transverse section of an ookinete showed micronemes (m) as well as the apical pore (ap) located between two electron-dense regions representing the inner membrane complex (IMC) underlying the plasma membrane (pm). (Scale bar: 200 nm.) (F) Transverse cross-section near the apical end of an ookinete shows micronemes (m) as well as microtubules (→) that circumferentially line the subpellicular space of the parasite. (Scale bar: 200 nm.)
efforts based on the potential of Plasmodium transmission-blocking vaccines.

Received August 1, 2010. Accepted for publication September 13, 2010.

Acknowledgments: This work was supported by US Public Health Service Grants T32GM007198 (to V.B.), K24AI068903 (to J.M.V.), and R01AI45999 (to J.M.V.). The authors thank M. G. Farquhar, K. Kudlicka, and T. Meerloo, Core Electron Microscopy Facility (US Public Health Service Grants R01CA100768, R01DK017724, and R01DK017780). The authors thank C. A. Spina, J. Nordberg, and M. O’Keefe of the flow cytometry core of the Center for Acquired Immunodeficiency Syndrome (AIDS) Research at the University of California San Diego (supported by Grant S10RR027933 and P30AI036214). The authors thank S. L. Hoffman for critical comments on the manuscript and Sanaria (Rockville, MD) for this NF54 strain of P. falciparum. The authors thank R. E. Sinden for assistance with advice on the interpretation of electron micrographs. The authors thank S. R. Abeles, J. W. M. Theisen, N. V. Dharia, and T. A. Aguilera for scientific discussion. We particularly thank Paula Maguina for the logistical and scientific support for the work carried out here.

Disclaimer: The authors declare no conflict of interest.

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REFERENCES


APPENDIX: METHODS

P. falciparum gametocyte cultures. The NF54 isolate of P. falciparum used in these experiments was a gift from Stephen Hoffmann (Sanaria, Rockville, MD). NF54 is one of the 13 initial Nijmegen Falciparum (NF) isolates derived from a Dutch patient. This strain was maintained in continuous asexual culture according to standard protocol with the exception that no antibiotics were used in the complete medium: RPMI 1,640, 25 mM Hepes, 2 mM l-glutamine, 2.4 g/L NaHCO3, 50 mg/L hypoxanthine, and 10% heat-inactivated AB+ human serum (Interstate Blood Bank, Memphis, TN).

Asynchronous asexual stage cultures at 8–15% parasitemia were used to start gametocyte cultures. Gametocyte cultures were started by diluting asexual stage parasite cultures into 0.9 mL of freshly washed packed human red cells to generate a final concentration of 0.4–0.6% parasitemia in 15 mL total volume. Dilution of asexual cultures at 8–10% parasitemia generated gametocyte cultures with the highest yields. Spent gametocyte culture medium was removed and replaced daily with 15 mL of 37°C complete medium until cultures reached 5–10% parasitemia, usually by day 3–4. At this point, spent gametocyte culture medium was replaced with 25 mL of 37°C complete medium for the remainder of the culture period. Additionally, approximately 10 mL of spent medium were left in the culture flask during each medium change. Gametocyte cultures were maintained in a low oxygen environment by gassing the cultures with filtered 5% O2, 5% CO2, and 90% N2.

Exflagellation and emergence assays. Mature macrogametocytes were tested for the ability to emerge using a modified exflagellation protocol: instead of examining slides for emergence in real time, blood smears were made 1 hour after gametogenesis, fixed with methanol, and stained with a modified Wright stain for parasite invasion of the mosquito midgut. 52–54 This strain was maintained in continuous asexual culture according to standard protocol with the exception that no antibiotics were used in the complete medium: RPMI 1,640, 25 mM Hepes, 2 mM l-glutamine, 2.4 g/L NaHCO3, 50 mg/L hypoxanthine, and 10% heat-inactivated AB+ human serum (Interstate Blood Bank, Memphis, TN).

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Leukostat-stained light microscopy. A modified Wright stain using Leukostat dyes was used to stain parasite thin smears. Smears were stained for 15 seconds in Leukostat 1 (0.1% eosin Y, 0.4% NaHPO4, and 0.1% formaldehyde), rinsed in distilled water, and stained for 30 seconds in Leukostat 2 (0.04% methylene blue, 0.04% Azure A, KH2PO4, and NaHPO4). Slides were then rinsed in distilled water and left to air dry.

Immunofluorescence of cultured parasites. Cultured parasites were fixed and permeabilized on glass slides and probed with antibodies against chitinase (1C3), Phs25 4B7 (MRA-28; deposited by David C. Kaslow, Malaria Research and Reference Reagent Resource Center, Manassas, VA), or...
mouse IgG negative control as previously described\textsuperscript{42} with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary and 3 μM DAPI (Molecular Probes; Invitrogen, Carlsbad, CA).

**TEM of cultured parasites.** Sexual stage parasite samples were depleted of uninfected red blood cells and asexual parasite forms by centrifugation on Lympholyte-H single-step density gradient and washed three times with ookinete medium. Parasites were then centrifuged and resuspended in 500 L of ookinete medium. These cells were further prepared for TEM according to standard protocol.\textsuperscript{58} Resulting parasite blocks were cut with a Reichert ultramicrotome, stained with 1% uranyl acetate and lead nitrate, examined using a JEOL 1200EX II transmission electron microscope (JEOL, Peabody, MA), and photographed using a Gatan digital camera (Gatan, Pleasanton, CA).