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Plasmodium yoelii: Axenic development of the parasite mosquito stages

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Abstract

Study of the parasite mosquito stages of *Plasmodium* and its use in the production of sporozoite vaccines against malaria has been hampered by the technical difficulties of in vitro development. Here, we show the complete axenic development of the parasite mosquito stages of *Plasmodium yoelii*. While we demonstrate that matrigel is not required for parasite development, soluble factors produced and secreted by *Drosophila melanogaster* S2 cells appear to be crucial for the ookinete to oocyst transition. Parasites cultured axenically are both morphologically and biologically similar to mosquito-derived ookinetes, oocysts, and sporozoites. Axenically derived sporozoites were capable of producing an infection in mice as determined by RT-PCR; however, the parasitemia was significantly much less than that produced by mosquito-derived sporozoites. Our cell free system for development of the mosquito stages of *P. yoelii* provides a simplified approach to generate sporozoites that may be for biological assays and genetic manipulations.

Index Descriptors and Abbreviations: *Plasmodium yoelii*; Parasite mosquito stages; Malaria; Axenic; cs, circumsporozoite protein; cDNA, complementary deoxyribonucleic acid; DAPI,4′,6-diamidino-2-phenylindole; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; EMEM, Earle's minimum essential medium; EtBr, ethidum bromide; FDA, Food and Drug Administration; Hep17, hepatocyte erythrocyte protein, a relative molecular weight 17 kDa; HRP, horseradish peroxidase; KDa, kilodalton; MSP1, merozoite surface protein 1; Mab, monoclonal antibody; NYS1, anti-circumsporozoite antibody, reactive to oocysts and sporozoite developmental forms; NYSL3, anti-Hep17 antibody, reactive to hepatic and asexual erythrocytic stage parasites; PBS, phosphate-buffered saline; *P. yoelii*, *Plasmodium yoelii*; Pys21, *Plasmodium yoelii* 21 kDa ookinetes surface protein; Pys25, *Plasmodium yoelii* 28 kDa ookinetes surface protein; RBC, red blood cell; rMSP1, recombinant MSP1,19 kDa C-terminal fragment of MSP1 fused to GST of *Schistosoma japonicum*; RNA, ribonucleic acid; rRNA, ribosomal RNA; RT, room-temperature; RT-PCR, reverse transcriptase-polymerase chain reaction; S2 cells, *Drosophila melanogaster* S2 cells; wt, wild-type

1. Introduction

Malaria, a disease caused by members of the genus *Plasmodium*, kills more than two million people each year. Although efforts toward developing an effective subunit or DNA based malaria vaccines are ongoing, immunization with the irradiated sporozoite vaccine in humans has been shown to generate up to nine months of protection in humans against sporozoite challenge (Edelman et al., 1993). However, vaccination with irradiated sporozoites remains impractical due to the technical difficulties of producing adequate numbers of sporozoites under conditions necessary for FDA approval (Luke and Hoffman, 2003). An alternative approach may be to generate sporozoites in vitro.

In vitro generation of sporozoites requires replication of the stages of sporogony that naturally take place in the anopheline mosquito vector (Al-Olayan et al., 2002; Arrighi and Hurd, 2002). The onset of the *Plasmodium* parasite mosquito stages occurs upon the entry of gametocytes into the mosquito gut following ingestion of an infected blood-meal (Beier, 1998). Subsequently, gametes are released and fertilized to form zygotes that then transform into ookinetes. The

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complexity of this phenomenon is such that from a large number of gametocytes taken with the blood-meal, only a fraction of the resulting ookinetes transform into oocysts. Despite the intricacies of the parasite mosquito stages, in vitro development has been achieved in *Plasmodium gallinaceum* (Warburg and Miller, 1992), *P. falciparum* (Warburg and Schneider, 1993), and *Plasmodium berghei* (Al-Olayan et al., 2002). Yet, since *Plasmodium yoelii* is a widely used malaria rodent model, we undertook an effort not only to develop the in vitro culture but also went further to examine the utility of axenically developed parasites. In addition, we were successful in simplifying the culture system and reduced the steps required to obtain sporozoites.

2. Materials and methods

2.1. Parasites/animals/cells

Plasmodium yoelii 17XNL (non-lethal strain) clone 1.1 were cycled between *Anopheles stephensi* mosquitoes and outbred-CD1 mice (Charles River Laboratories, Wilmington, MA, USA). Infected mosquitoes were maintained at 22 °C and 75% humidity for 14 days on sugar and water. After infections, mosquito parasite burdens were monitored by examining five mosquito midguts for oocysts. Sporozoites were isolated from infected salivary glands using Renografin 60 gradient centrifugation as previously described (Lau et al., 2001). Initially, mosquito-derived sporozoites were injected via the tail vein into CD1 mice to establish the cycle and subsequently *P. yoelii* infections were maintained in mice by blood passage. Gametocyte development was monitored by Giemsa stain (Sigma, St. Louis, MO, USA) using blood smears prepared from tail snips of infected mice, starting on days 4 and 5 post-infection.

Female Outbred-CD1 mice 6–8 weeks old or BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were used for infected blood cultures and infectivity assays.

Drosophila melanogaster S2 cells were maintained at 28 °C in supplemented Schneider's *Drosophila* medium following the manufacturer's protocol (Invitrogen, Grand Island, NY, USA).

2.2. In vitro development of parasite mosquito stages

Initially, *P. yoelii* ookinetes, oocysts and sporozoites were produced using a previously published protocol (Al-Olayan et al., 2002) with a minor modification to accommodate temperature differences between *P. berghei* and *P. yoelii* (e.g., 22 °C) (5 Replicates). Subsequently, cultures were progressively modified to simplify the culture protocol. Briefly, infected blood from three mice was collected, diluted 1:10 in ookinete medium (Al-Olayan et al., 2002) and incubated overnight at 22 °C. Ookinete cultures were centrifuged at 1000*g* for 5 min and the pellet was resuspended in 50 ml Red Blood Cell Lysis Buffer (Sigma) and incubated at 25 °C for 30 min. The culture was centrifuged again at 1000*g* for 5 min. The pellet was washed once in phosphate buffer saline (PBS) (Biofluids, Rockville, MD, USA) and resuspended in 1 ml PBS. Ookinetes (1.0×10^6 cells/chamber) were resuspended in a 1:1 mixture of oocyst medium (Al-Olayan et al., 2002) and S2 cells (Invitrogen) conditioned medium (conditioned medium was obtained from 1.0×10^6 cells/ml centrifuged at 250*g* for 5 min) in a total volume of 400 µl/chamber in 8 well Lab-Tek chamber slides with cover (Nalge Nunc International, Naperville, IL, USA). The slides were incubated at 22 °C. Wells were refilled with a (1:1) mixture S2 cell conditioned media and oocyst media every 2–3 days.

2.3. Western blot analysis

At 2–3 day intervals, the contents of one chamberslide was collected and centrifuged for 2 min at 14,000*g*. The parasites were washed once in PBS and resuspended in 50 µl Tris–glycine SDS sample buffer (Invitrogen). Proteins (15 µg/lane) were processed for Western blot analysis and separated on Novex 18% Tris–glycine gels (Invitrogen) in Tris/glycine/SDS buffer (Bio-Rad Laboratories, Hercules, CA, USA) and transblotted in Tris/glycine buffer (Bio-Rad) to PVDF membranes following the manufacturer's protocol (Invitrogen). Membranes were individually probed with α-Pys21, α-Pys25 Mabs (gifts from Drs. Takafumi Tsuboi and Motomi Torii) (Tsuboi et al., 1997) and NYLS1 Mab (a gift from Dr. Yupin Charoenvit) (Charoenvit et al., 1987) antibodies. Next, the antibody–antigen complex was visualized using a Western Breeze Chemiluminescent kit as per manufacturer's instructions (Invitrogen).

2.4. Indirect fluorescent antibody test (IFAT)

At 2–3 day intervals, the contents from one slide was collected and centrifuged for 2 min at 14,000*g* then washed once in PBS and resuspended in 300 µl PBS. Three cell suspension aliquots of 20 µl each were transferred onto several slides. The slides were incubated at 25 °C in a humidity chamber overnight. Samples were fixed in ice-cold methanol and rinsed three times with PBS. Samples were probed individually with antibodies (see above). Mouse IgG1, Kappa (MOPC21) (Sigma) or PBS served as controls and all slides were incubated at 37 °C for 1 h. Slides were washed three times with PBS, then incubated with a 1:40 dilution of anti-mouse IgG (H + L) FITC-conjugated antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and 0.02% Evans blue in PBS at 37 °C for 30 min in the dark. The secondary antibody was removed and slides were then rinsed three times in PBS. Samples were mounted in Vectorshield mounting media with DAPI (Vector Laboratories, Burlingame, CA).

2.5. Culture productivity

Axenic mosquito stage parasite culture was performed in 8-well Lab-Tek chamber slides as described above. The