Molecular & Biochemical Parasitology 204 (2015) 26–33

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Isolation of invasive *Plasmodium yoelii* merozoites with a long half-life to evaluate invasion dynamics and potential invasion inhibitors

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 28 October 2015
Accepted 5 December 2015
Available online 9 December 2015

Keywords:
Malaria
Plasmodium yoelii
Merozoite
Invasion
Inhibitors

ABSTRACT

Malaria symptoms and pathogenesis are caused by blood stage parasite burdens of *Plasmodium* spp., for which invasion of red blood cells (RBCs) by merozoites is essential. Successful targeting by either drugs or vaccines directed against the whole merozoite or its antigens during its transient extracellular status would contribute to malaria control by impeding RBC invasion. To understand merozoite invasion biology and mechanisms, it is desired to obtain merozoites that retain their invasion activity *in vitro*. Accordingly, methods have been developed to isolate invasive *Plasmodium knowlesi* and *Plasmodium falciparum* merozoites. Rodent malaria parasite models offer ease in laboratory maintenance and experimental genetic modifications; however, no methods have been reported regarding isolation of high numbers of invasive rodent malaria merozoites. In this study, *Plasmodium yoelii*-infected RBCs were obtained from infected mice, and mature schizont-infected RBCs enriched *via* Histodenz™ density gradients. Merozoites retaining invasion activity were then isolated by passing the preparations through a filter membrane. RBC-invaded parasites developed to mature stages *in vitro* in a synchronous manner. Isolated merozoites were evaluated for retention of invasion activity following storage at different temperatures prior to incubation with uninfected mouse RBCs. Isolated merozoites retained their invasion activity 4 h after isolation at 10 or 15 °C, whereas their invasion activity reduced to 0–10% within 30 min when incubated on ice or at 37 °C prior to RBC invasion assay. Images of merozoites at successive steps during RBC invasion were captured by light and transmission electron microscopy. Synthetic peptides derived from the amino acid sequence of the *P. yoelii* invasion protein RON2 efficiently inhibited RBC invasion. The developed method to isolate and keep invasive *P. yoelii* merozoites for up to 4 h is a powerful tool to study the RBC invasion biology of this parasite. This method provides an important platform to evaluate the mode of action of drugs and vaccine candidates targeting the RBC invasion steps using rodent malaria model.

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1. Introduction

The global mortality rate by *Plasmodium falciparum* was reported to have decreased by 47% between the years 2000 and 2013, with 54% reduction in deaths in the WHO African region. Despite this optimistic progress, malaria infections still prevailed at 128 million estimated cases per year [1] and this underscores the need for continuous and concerted efforts to search for novel and effective vaccine and drug targets to combat the burden of malaria. Malaria parasite infection is initiated when infected mosquitoes inoculate sporozoites into the vertebrate host during a blood feed. The sporozoites travel through the epidermis to reach the blood stream, and then are swept to the liver where they invade hepatocytes within which they remodel, grow and multiply. The result is an amplification in which a single *P. falciparum* sporozoite can develop within 6 days into an estimated 40,000 merozoites per infected hepatocyte [2]. Following hepatocyte rupture these merozoites are released into the blood circulation, where they invade red blood cells (RBCs). Clinical symptoms and pathogenesis manifest during the asexual blood phase after cycles of merozoite invasion and replication within RBCs. Specifically, following invasion of RBCs merozoites undergo a period of growth and concomitant remod-

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http://dx.doi.org/10.1016/j.molbiopara.2015.12.003
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elling of the RBC structure, followed by parasite cell division into a schizont stage harbouring approximately 20 merozoites [3], and finally rupture to release merozoites into the blood circulation for a new round of RBC invasion. The merozoite is external to a RBC for a short time window, and this brief vulnerability following egress is an opportunity to block subsequent invasion of RBCs by either drugs or vaccines targeting the whole merozoite or merozoite antigens.

The target host cell is thought to play a passive role during invasion [4]. The physical mechanisms of the *Plasmodium* merozoite's ability to reach the target RBC has been attributed to the explosive nature of schizont rupture/merozoite release and a close proximity to RBCs due to the high density of RBCs in blood [5]. Video microscopy to capture the RBC invasion by *Plasmodium knowlesi*, *P. falciparum* and *Plasmodium yoelii* revealed that the dynamics of invasion begin with merozoite attachment and rapid deformation of the target RBC, followed by merozoite reorientation to bring the apical end to close apposition with the target RBC surface. The merozoite then forms an irreversible tight/moving junction, and internalizes with the generation of a nascent parasitophorous vacuole enclosing the invaded parasite. Following invasion, the infected RBC is transiently deformed, in a poorly understood process termed echinocytosis [3,6,7].

The invasion steps of a merozoite are facilitated by multiple interactions between RBC receptors and merozoite ligands located on the surface or secreted from apical organelles; namely, micronemes, rhoptries and dense granules. These merozoite surface and secreted proteins compose the leading blood-stage vaccine candidate antigens [8]. For some essential merozoite-specific molecules, cellular localization and protein maturation have been characterized in free and invading merozoites [9,10], but their specific roles are still largely unknown. Recently, a robust method was developed to isolate invasive *P. falciparum* merozoites by forcefully rupturing synchronized mature schizonts *via* passage through a membrane filter [11]. This enabled direct evaluation of invasion events and invasion-inhibitory activity of antibodies and chemical compounds, independent of other inhibition mechanisms that may be associated with intracellular growth or schizont rupture.

A number of parasite gene or protein alteration tools and technologies have been developed and used to evaluate the phenotype of loss or gain of function of merozoite specific molecules in *P. falciparum.* However, genetic modifications and protein regulation experiments in *P. falciparum* are time consuming and laborious. Stable transformation and gene targeting approaches are also established for rodent parasites, such as *Plasmodium berghei* and *P. yoelii*, and are much faster than that for *P. falciparum* [12,13]. Furthermore, while *P. falciparum* invades all sub-populations of RBCs, different strains of *P. yoelii* have different RBC invasion preferences, and this tropism is a major determinant of parasite virulence. For example, the virulent/lethal 17XL line (*Py*17XL) invades all sub-populations of RBCs, whereas a less virulent 17X line (*Py*17X) is able to invade only young RBC sub-populations [14]. Thus, the rodent malaria model provides a platform to understand the role of merozoite surface proteins, RBC invasion and virulence. The complete life cycle in vertebrate and mosquito hosts can be easily maintained in laboratory settings, and allow genetic crosses of selected malaria parasites using the mosquito vector [15,16]. Short term culture from ring to schizont stages is achievable for *P. yoelii* [17], and RBC invasion mechanisms and phenotype can be characterized in detail using ligand-deficient rodent malaria merozoites and/or RBC receptor-deficient transgenic mice [18]. Although the overall cellular dynamics of invasion is similar between *P. falciparum* and *P. yoelii*, a unique phenotype was recently observed in *P. yoelii*; merozoites egressed from RBC transform from non-invasive flat elongated oval to invasive spherical bodies within 60 s after release and are not able to invade RBC during this phase [3], whereas egressed *P. falciparum* merozoites are already in spherical form and are able to invade RBC immediately [3,7]. The importance of the morphological change of *P. yoelii* has not been determined, but we consider that, experimentally, this expanded time window allows the opportunity to analyze the transformation events readying the merozoite to invade RBC, which might occur before rupture in *P. falciparum* and therefore are difficult to observe.

In this study, we have adopted and modified the techniques developed for *P. falciparum* merozoite isolation [11], and establish a method to isolate invasive *P. yoelii* merozoites for both *Py*17XL and *Py*17X. Storage conditions of isolated merozoites were optimized in order to lengthen the time available to perform RBC invasion assays. To authenticate the applicability of our method, we document RBC invasions by transmission electron microscopy, and show that a synthetic peptide derived from *P. yoelii* RON2 amino acid sequence effectively inhibits RBC invasion by *P. yoelii*. Combined with the numerous advantages of using *P. yoelii* to complement basic research in *P. falciparum*, the technique reported here is a milestone in further understanding of the merozoite invasion biology and evaluating novel anti-merozoite vaccines and invasion-inhibitory compounds and their mode of action.

2. Materials and methods

2.1. Parasites and experimental animals

P. yoelii 17XL (*Py*17XL) and 17X (*Py*17X) parasite lines were obtained from the Institute of Tropical Medicine, Nagasaki University participating in the National Bio-Resource Project of the MEXT, Japan. Female 6–8 weeks old ICR mice (SLC Inc., Shizuoka, Japan) were used for parasite propagation. All experiments were approved by the Animal Care and Use Committee of Nagasaki University (Permit number 0912080806-4). *P. falciparum* 3D7A line was obtained from L. H. Miller, NIH [19], and maintained with O^+ RBCs in *Pf*-complete medium; RPMI1640 medium (with L-glutamine, without sodium bicarbonate; Invitrogen, Carlsbad, CA) supplemented with 25 mM HEPES (Sigma, St. Louis, MO), 0.225% sodium bicarbonate (Invitrogen), 50 μg/mL hypoxanthine (Sigma), 25 μg/mL gentamicin (Invitrogen), 0.25% AlbuMax I (Invitrogen) and 5% heat-inactivated pooled type AB^+ human serum, according to standard procedures [20].

2.2. Preparation of schizonts

P. yoelii-infected blood, cryopreserved in liquid nitrogen, was thawed and inoculated intravenously into a donor mouse on day 0. On day 3 or 4 post infection (p.i), 10^7 iRBCs from the donor mouse were inoculated intra-peritoneally into naïve mice and parasitemia growth was monitored by microscopic observation of thin Giemsa-stained smears. For *Py*17X, 300 μL of 6 mg/mL phenylhydrazine-HCl in phosphate buffered saline (PBS) were injected to mice three days prior to parasite inoculation to enrich reticulocytes. When parasitemia reached 70–80% and parasite stages were predominantly segmented schizonts, about 1 mL of whole blood was collected by cardiac puncture from each of anaesthetized mouse into 1 mL of pre-warmed (37 °C) saline sodium citrate (SSC). The pooled blood was centrifuged at 600 × *g* using a swing-rotor centrifuge (KUBOTA 3740, Japan) for 5 min and the supernatant was removed. To remove serum, platelets and buffy coat, the blood was washed 3 times with 10 times the volume of the pellet using incomplete medium; RPMI1640 medium supplemented with 25 mM HEPES, 0.225% sodium bicarbonate, 0.1 mM hypoxanthine, and 25 mg/mL gentamicin. The blood pellet was re-suspended in incomplete medium at up to 25% hematocrit. The schizont-rich fraction from this preparation was obtained using a Histodenz™ (Sigma) density gradient [13,21]. Histodenz™

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