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Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites

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ABSTRACT

The invasion of red blood cells (RBCs) is an essential event in the life cycle of all malaria-causing *Plasmodium* parasites; however, there are major gaps in our knowledge of this process. Here, we use video microscopy to address the kinetics of RBC invasion in the human malaria parasite *Plasmodium falciparum*. Under in vitro conditions merozoites generally recognise new target RBCs within 1 min of their release from their host RBC. Parasite entry ensues and is complete on average 27.6 s after primary contact. This period can be divided into two distinct phases. The first is an ~11 s 'pre-invasion' phase that involves an often dramatic RBC deformation and recovery process. The second is the classical 'invasion' phase where the merozoite becomes internalised within the RBC in a ~17 s period. After invasion, a third 'echinocytosis' phase commences when about 36 s after every successful invasion a dramatic dehydration-type morphology was adopted by the infected RBC. During this phase, the echinocytotic effect reached a peak over the next 23.4 s, after which the infected RBC recovered over a 5–11 min period. By then the merozoite had assumed an amoeboid-like state and was apparently free in the cytoplasm. A comparison of our data with that of an earlier study of the distantly related primate parasite *Plasmodium knowlesi* indicated remarkable similarities, suggesting that the kinetics of invasion are conserved across the *Plasmodium* genus. This study provides a morphological and kinetic framework onto which the invasion-associated physiological and molecular events can be overlaid.

1. Introduction

Malaria remains one of the most devastating diseases of humankind causing more than a million deaths per year and placing substantial economic and social burdens on developing nations (Sachs and Malaney, 2002; Snow et al., 2005). The protistan parasites *Plasmodium falciparum* and *Plasmodium vivax*, cause the vast majority of disease. A steady pipeline of new anti-malarial drugs and an effective vaccine are required as key tools in long-term strategies to markedly reduce the incidence of malaria.

All *Plasmodium* parasites amplify dramatically during a replication cycle in erythrocytes and it is at this blood-stage stage that clinical symptoms become apparent. While largely intracellular during each replicative cycle, which is 48 h for *P. falciparum*, an invasive form of the parasite known as the merozoite is briefly extracellular as it moves from one red blood cell (RBC) host to the next (Cowman and Crabb, 2006). Merozoite egress and invasion are much studied processes because of their obvious vulnerability to immune or therapeutic intervention. Despite this attention and the identity of many of the molecular players involved in both processes (Cowman and Crabb, 2006; Blackman, in press), fundamental gaps in our knowledge of these events remain. To some degree this relates to the difficulties encountered in studying these processes. This is most notable in simply observing the different steps in invasion in real time where issues such lack of parasite synchrony, poor merozoite viability, the small size of merozoites (1–3 μM), the speed of invasion and the susceptibility of merozoites to photo-damage have conspired against the generation of much knowledge in this regard.

Despite these challenges several 'real time' imaging studies have been published, notably two time-lapse microscopy investigations focussing on the schizont rupture/merozoite egress in *P. falciparum* (Winograd et al., 1999; Glushakova et al., 2005) and a seminal invasion study in the primate malaria species *Plasmodium knowlesi* (Dvorak et al., 1975). As far as we are aware, the latter study was the only work to closely observe and describe the different morphological steps observed in a single invasion event and to examine the kinetics of the process. Briefly, these authors observed that schizonts swelled prior to rupturing, which they did with "explosive suddenness" (Dvorak et al., 1975). When a free merozoite contacted the plasma membrane of a new erythrocyte it did not immediately invade but initiated waves of deformation on the erythrocyte plasma membrane that soon ceased, leaving the mer-

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ozoite attached to the erythrocyte by its anterior end. Invasion then followed, taking 10–20 s to complete. Following this, secondary waves of deformation persisted for 10–15 min during which the infected erythrocyte gradually resumed a normal shape.

By comparison *P. falciparum* merozoites are only about half the length of *P. knowlesi* (1.6 versus 3.7 μm) and are much more short-lived (Aikawa et al., 1978, 1981). Hence, questions remain as to general applicability of these observations in *P. knowelsi*. In this paper, we use time-lapse microscopy to film seven schizont rupture and 19 successful invasion events. Our observations of *P. falciparum* indicate that the morphological steps and kinetics of erythrocyte invasion are remarkably similar to those detected in *P. knowlesi*. This conservation across a large evolutionary distance suggests that the maintenance of invasion rates, including the timing of the individual steps, has reached an optimum. Hence, perturbation of invasion kinetics, as opposed to complete blockage of invasion per se, may have therapeutic potential. We also describe dramatic morphological changes that occur to the infected erythrocyte immediately after it has been invaded. This may offer another target for anti-parasite intervention. This study establishes a morphological and kinetic baseline of cellular behaviour from schizont rupture through to merozoite invasion of new erythrocytes.

2. Materials and methods

2.1. Parasite culture and microscopy

Plasmodium falciparum line 3D7 was cultivated and synchronised as per standard procedures (Trager and Jensen, 1976; Lambros and Vanderberg, 1979). Highly synchronous late schizont stage parasites (5% parasitemia) were diluted 1/10 with culture media and 100 μl were transferred onto a sterile glass coverslip glued over a 10 mm hole in the bottom of a 5 ml plastic petri dish. A tissue moistened with culture media was also placed in the petri dish to maintain humidity. The dish was returned to the 37 °C incubator then onto the preheated (37 °C) stage area of an inverted Zeiss Axiovert microscope. The stage was also supplied with humidified tissue culture gas (5% CO_2, 5% O_2 and 90% N_2). Cells were observed by differential interference contrast (DIC) at 630× with an oil immersion lens and illumination was reduced as much as possible to avoid cell damage. Exposure times typically ranged from 50–100 ms and time-lapse images were acquired every 2 s. Movie files were managed with Image J.

3. Results

3.1. Merozoite disaggregation precedes schizont rupture

Time-lapse imaging every 2 s of mature schizont stage *P. falciparum* parasites reveals that schizont rupture and merozoite dispersal occurs very rapidly as described previously (Fig. 1A–C; Supplementary Movies S1–3) (Dvorak et al., 1975; Glushakova et al., 2005). In the seconds prior to rupture, the diameter of the schizont enlarges slightly and the merozoites become increasingly distinct, probably because they begin separating and the schizont takes on a 'flower'-like appearance as described by Glushakova et al. (2005) (Fig. 1A and B). As recorded for *P. knowlesi*, blebs in the membranes surrounding the merozoites were sometimes observed in *P. falciparum* and rupture and merozoite release proceeded in the direction of the blebs (Fig. 1A, 6 s, arrow) (Dvorak et al., 1975). Occasionally a schizont was observed which only partly filled the erythrocyte and a region of the blood cell's plasma membrane distal to the parasite could be clearly distinguished (Fig. 1C, 0 s, arrow). As the schizont approached rupture the merozoites appeared to disaggregate, presumably due to breakdown of the parasitophorous vacuole membrane (PVM) and fill the erythrocyte (Fig. 1C, 200 s). The merozoites became increasingly mobile until final rupture of the plasma membrane occurred at 230 s (Fig. 1C).

Of seven schizont rupture events observed, the average time taken for a merozoite to contact a RBC that it would successfully invade was 38 s (Supplementary Data S1). The fastest time from rupture to attachment was 3 s and the longest time observed was 90 s. We note that many merozoites contacted but failed to invade RBCs and these detached after a few seconds.

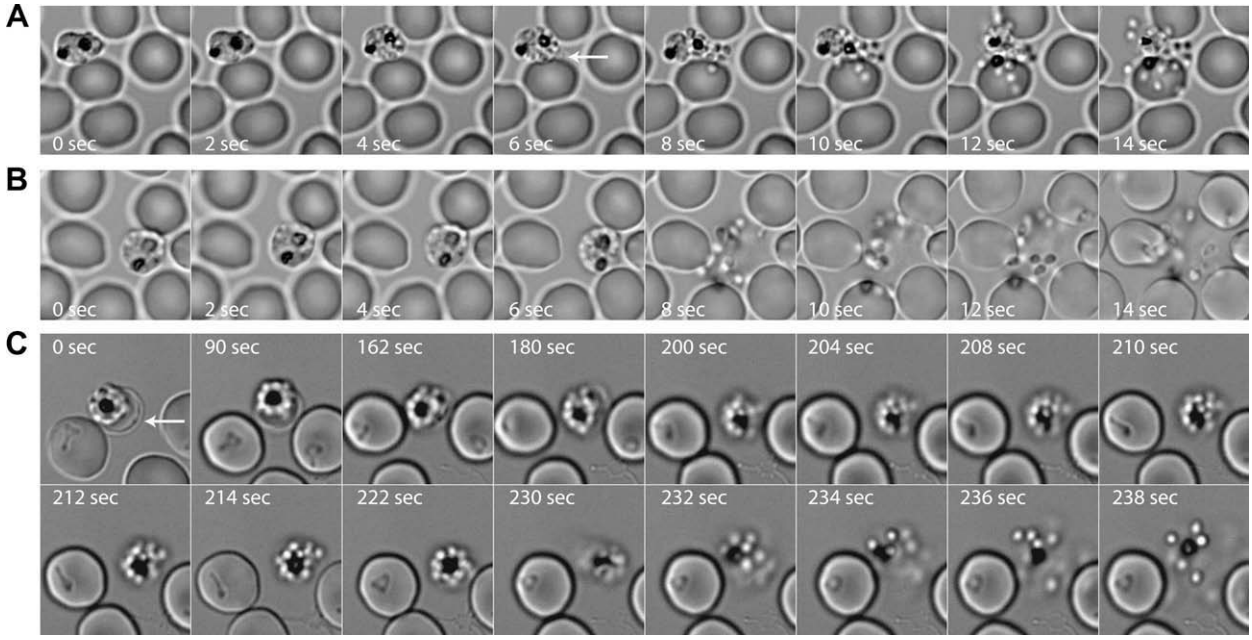

Fig. 1. Time-lapse image sequences of rupturing schizonts in *Plasmodium falciparum*. (A) Rupture and merozoite release appear to emanate from a membranous bleb that bulges out from the erythrocyte's surface (arrow). The schizonts from this doubly infected erythrocyte rupture at different times. (B) Simultaneous double schizont rupture of a doubly infected erythrocyte. (C) The schizont in this singly infected erythrocyte is positioned on one side of the blood cell leaving the cell's plasma membrane clearly visible (arrow). Merozoites begin filling the blood cell at 200 s, probably because the parasitophorous vacuole membrane breaks down prior to host cell rupture (232 s).