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Minimum requirements for ookinete to oocyst transformation in *Plasmodium*

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Abstract

During their passage through a mosquito vector, malaria parasites undergo several developmental transformations including that from a motile zygote, the ookinete, to a sessile oocyst that develops beneath the basal lamina of the midgut epithelium. This transformation process is poorly understood and the oocyst is the least studied of all the stages in the malaria life cycle. We have used an in vitro culture system to monitor morphological features associated with transformation of *Plasmodium berghei* ookinetes and the role of basal lamina components in this process. We also describe the minimal requirements for transformation and early oocyst development. A defined sequence of events begins with the break-up of the inner surface membrane, specifically along the convex side of the ookinete, where a protrusion occurs. A distinct form, the transforming ookinete or took, has been identified in vitro and also observed in vivo. Contrary to previous suggestions, we have shown that no basal lamina components are required to trigger ookinete to oocyst transformation in vitro. We have demonstrated that transformation does not occur spontaneously; it is initiated in the presence of bicarbonate added to PBS, but it is not mediated by changes in pH alone. Transformation is a two-step process that is not completed unless a range of nutrients are also present. A minimal medium is defined which supports transformation and oocyst growth from 7.8 to 11.4 μm by day 5 with 84% viability. We conclude that ookinete transformation is mediated by bicarbonate and occurs in a similar manner to the differentiation of sporozoite to the hepatic stage.
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Keywords: *Plasmodium berghei*; Ookinete; Oocyst; Basal lamina; Laminin; In vitro; Transformation

1. Introduction

Malaria remains a major life-threatening disease in tropical and sub-tropical parts of the world, causing 300–500 million clinical cases, over one million deaths and severe economic and social losses (Sachs and Malaney, 2002). The causative agents of the disease, apicomplexans of the genus *Plasmodium*, are amongst the most studied parasites because of their devastating impact on human health and welfare.

Plasmodium parasites cycle between a vertebrate host and mosquito vector, experiencing changing environmental conditions as they develop from invasive to intracellular forms in the vertebrate, and invasive, intracellular and extracellular forms in the mosquito. Despite the importance of mosquito stages for malaria transmission, the majority of research on *Plasmodium* is focused upon the asexual stages that invade vertebrate erythrocytes; studies facilitated by the development, in 1976 (Trager and Jensen, 1976), of an in vitro culture technique that has rendered these stages more accessible to experimentation (Hurd et al., 2003).

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In the mosquito, gametocytes complete their differentiation, fertilisation occurs and the zygote transforms into a motile ookinete within 24 h of having imbibed a blood meal. This extracellular ookinete migrates out of the blood bolus, traverses the midgut epithelium and transforms into a vegetative oocyst in the basal subepithelial space between the midgut epithelium and the basal lamina (BL). The young oocyst grows and undergoes sporogony, producing sporozoites that migrate to the salivary glands. Experimentation with systems that substitute for conditions in the vector for a period of some 50 years finally led to the development of methods to culture all the mosquito stages of several malaria species. Species that have been successfully cultured from gametocyte to sporozoite include *Plasmodium falciparum* (Warburg and Schneider, 1993), *Plasmodium gallinaceum* (Warburg and Miller, 1992), *Plasmodium berghei* (Al-Olayan et al., 2002a) and *Plasmodium yoelii* (Porter-Kelley et al., 2006). For one of these species, *P. berghei*, a method for the efficient production of ookinetes in vitro has been developed (Rosales-Ronquillo and Silverman, 1974; Weiss and Vanderberg, 1977) and this has provided material for detailed studies of gametogenesis, fertilisation and zygote development. Several studies have demonstrated that initiation of *Plasmodium* gametogenesis requires specific triggers. The change in environment from a mammalian to insect host provides cues such as a drop in temperature, and a mosquito factor identified as xanthurenic acid (Nijhout and Carter, 1978; Arai et al., 2001) which can regulate gametogenesis. These triggers, or substitutes for them, are present in the in vitro systems used to produce ookinetes (Weiss and Vanderberg, 1977; Carter et al., 2003).

The next critical event in the development of the sporogonic stages of *Plasmodium* is that of the transformation of ookinete to oocyst. Our knowledge of this phase of development is minimal and the process of rounding up of the ookinete to form an oocyst had not been studied in vitro previous to this work. We have identified components of the culture system that are essential requirements to initiate and sustain the formation of young oocysts from ookinetes.

It had been proposed that contact with components of the BL may act as a trigger for ookinete transformation in vivo (Weathersby, 1952; Meis and Ponnudurai, 1987; Ramasamy et al., 1997; Adini and Warburg, 1999; Schneider and Shahabuddin, 2000; Dessens et al., 2003; Arrighi et al., 2005; Vlachou et al., 2006) and culture systems that allow the transformation of ookinetes to oocysts, oocyst growth and sporozoite production have included a BL substitute, Matrigel and a co-cultured cell line such as *Drosophila melanogaster* S2, that may also contribute molecules present in the mosquito BL (Warburg and Miller, 1992; Warburg and Schneider, 1993; Al-Olayan et al., 2002a). Matrigel, an extracellular matrix derived from Engelbreth–Holm–Swarm mouse sarcoma, is comprised of laminin (56%), collagen IV (31%), enactin (8%), heparan sulfate proteoglycans and growth factors (BD Biosciences). Several ookinete proteins, such as the surface proteins P25/P28 (Siden-Kiamos et al., 2000), secreted ookinete adhesive protein (SOAP) (Dessens et al., 2003), circumsporozoite- and TRAP-related protein (CTRP) (Dessens et al., 1999) and von Willebrand factor A domain-related-protein (WARP) (Yuda et al., 2001), that are reportedly essential for ookinete and oocyst development in vivo, have been shown to bind to BL components such as laminin and collagen (Adini and Warburg, 1999; Arrighi et al., 2005). This binding could provide a signal that induces transformation, thus adding weight to the hypothesis that contact with the BL may be essential for parasite development (Dessens et al., 2003). Alternatively, other components of the culture system such as those present in the insect cell medium used as a substitute for haemolymph, may be essential requirements for ookinete transformation.

In vivo, ookinete transformation is a transient and unsynchronised event but in vitro we can observe the process occurring in thousands of ookinetes at any one time. Additionally, controlled interventions that enable careful dissection of putative transformation signals can be made and the minimal nutritional/environmental requirements for sporogonic development can be assessed. Using ookinetes that had been obtained in vitro (Carter et al., 2003) as a starting point, we have been able to describe the process of transformation and to identify a novel transient stage that we have called a transforming ookinete or took. We have demonstrated that, contrary to previous suggestions, BL components, and in particular laminin, are not required for transformation and early oocyst growth and that transformation appears to be a two-step process in which initiation is bicarbonate-dependent and complete transformation requires a range of nutrients.

2. Materials and methods

All reagents were purchased from Sigma unless otherwise specified.

2.1. Parasites and ookinete harvesting

Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 using approved protocols. CD mice were treated with phenylhydrazine 2 days prior to infection by i.p. inoculation with *P. berghei* (clone 259cl2 expressing green fluorescent protein (Franke-Fayard et al., 2004)) obtained from a donor mouse between the second and sixth passage from cryopreserved stock. Infected blood was collected from mice with a parasitaemia of 10–20% via cardiac puncture into a heparinised syringe. Gametocytaemic blood was passed through a pre-equilibrated, 5 ml sterile column containing 1 ml glass wool and 3 ml of Whatman CF11 cellulose powder (Beckton & Dickenson) to remove white blood cells. The blood was collected into T25 culture flasks with RPMI 1640 medium supplemented with 24 mM sodium bicarbonate, 0.4 mM hypoxanthine, 10,000 U penicillin/10 mg streptomycin and 20% FBS in a 1:10 ratio (blood:

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