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Morphological evidence for proliferative regeneration of the *Anopheles stephensi* midgut epithelium following *Plasmodium falciparum* ookinete invasion

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Abstract

Ookinetes are motile invasive stages of the malaria parasite that enter the midgut epithelium of the mosquito vector via an intracellular route. Ookinetes often migrate through multiple adjacent midgut epithelial cells, which subsequently undergo apoptosis/necrosis and are extruded from the midgut epithelium into the midgut lumen. Hundreds of ookinetes may simultaneously invade the midgut epithelium, causing destruction of an appreciable proportion of the total number of midgut epithelial cells. However, there is little evidence that ookinete invasion of the midgut epithelium *per se* is detrimental to the survival of the mosquito vector implying that efficient mechanisms exist to restore the damaged midgut epithelium following malaria parasite infection. Proliferation and differentiation of precursor stem cells could replace the midgut epithelial cells destroyed and lost as a consequence of ookinete invasion. Although the existence of so-called ''regenerative'' cells within the mosquito midgut epithelium has long been recognized, there has been no previously published evidence for proliferation/differentiation of these putative precursor midgut epithelial cells in mature adult female mosquitoes. In the current study, examination of Giemsa-stained histological sections from *Anopheles stephensi* mosquito midguts infected with the human malaria parasite *Plasmodium falciparum* provided morphological evidence that regenerative cells undergo division and subsequent differentiation into normal columnar midgut epithelial cells. Furthermore, the number of these putatively proliferating/differentiating regenerative cells was significantly higher in *P. falciparum*-infected compared to uninfected mosquitoes, and was positively correlated with both the level of malaria parasite infection and midgut epithelial cell destruction. The loss of invaded midgut epithelial cells associated with intracellular migration by ookinetes, therefore, appears to trigger, and to be compensated by, proliferative regeneration of the mosquito midgut epithelium.

Keywords: *Anopheles stephensi*; Cell division and differentiation; Goblet cell; Malaria; Midgut epithelium; Mitosis; Mosquito; Ookinete; Parasite-vector interaction; Regenerative cell; *Plasmodium falciparum*

1. Introduction

Malaria parasites undergo a complex obligate developmental cycle within the mosquito vector that enables transmission between vertebrate hosts (reviewed in Baton and Ranford-Cartwright, 2005b). Motile invasive-stage malaria parasites, known as ookinetes, develop within the midgut lumen of adult female mosquitoes from gametocyte-stage parasites ingested during bloodfeeding on an infectious vertebrate host. The ookinetes actively migrate through the bolus of ingested vertebrate blood and invade the surrounding posterior midgut epithelium of the mosquito vector, eventually transforming into non-motile oocyst stage parasites on the basal surface of the midgut epithelium (Baton and Ranford-Cartwright, 2005a). Ookinetes enter

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the midgut epithelium via an intracellular route and frequently migrate through multiple adjacent midgut epithelial cells (Becker-Feldman et al., 1985; Vernick et al., 1999; Han et al., 2000; Zieler and Dvorak, 2000; Vlachou et al., 2004; Baton and Ranford-Cartwright, 2004; Gupta et al., 2005). The midgut epithelial cells invaded by ookinetes exhibit a number of molecular and morphological changes indicative of significant pathology, undergo apoptosis/necrosis, and, eventually, are completely extruded from the midgut epithelium into the midgut lumen (Becker-Feldman et al., 1985; Han et al., 2000; Zieler and Dvorak, 2000; Kumar et al., 2004; Vlachou et al., 2004; Baton and Ranford-Cartwright, 2004; Danielli et al., 2005; Gupta et al., 2005). Ookinete invasion, therefore, results in the destruction and irreversible loss from the midgut epithelium of parasite-invaded midgut epithelial cells. Consequently, within individual mosquitoes, an appreciable proportion of the total number of midgut epithelial cells is presumably destroyed when many ookinetes simultaneously invade the midgut epithelium. There is, however, no direct evidence that ookinete invasion of the midgut epithelium *per se* is detrimental to the mosquito vector, and the mortality of very heavily infected mosquitoes is frequently no different from that of uninfected mosquitoes (Gad et al., 1979; Maier et al., 1987; Seitz et al., 1987; Meis and Ponnudurai, 1987; Han et al., 2000; Ferguson and Read, 2002; Baton and Ranford-Cartwright, 2004). This observation implies that adult female mosquitoes possess efficient mechanisms to restore the damaged midgut epithelium following malaria parasite infection. Indeed, two such mechanisms have been reported previously: actin-based mechanisms mediate extrusion of ookinete-invaded midgut epithelial cells from the midgut epithelium, while filopodia/lamellipodia extend from the surrounding healthy uninvaded midgut epithelium beneath the extruding midgut epithelial cells (Han et al., 2000; Vlachou et al., 2004; Gupta et al., 2005). These mechanisms account for the efficient removal of parasite-damaged midgut epithelial cells, and the maintenance of the integrity of the midgut epithelium during this process. However, neither mechanism explains how infected mosquitoes survive the loss of an appreciable proportion of the total number of midgut epithelial cells, bringing into question the claim that ookinete-invaded midgut epithelial cells undergo irreversible destruction (Shahabuddin, 2002). Possibly, malaria-infected mosquitoes do not replace the midgut epithelial cells lost as a result of ookinete invasion, and continue their remaining adult life with a significantly reduced number of midgut epithelial cells. Alternatively, restoration of the midgut epithelium of infected mosquitoes could be achieved through proliferation and differentiation of midgut epithelial cell precursors generating new midgut epithelial cells that replace those destroyed and lost as a consequence of ookinete invasion.

The mosquito midgut epithelium is a simple monolayer of polarized columnar epithelial cells throughout which other cell types are occasionally interspersed (Billingsley, 1990). Although the cellular composition of the midgut epithelium is currently controversial (Shahabuddin, 2002), probably only three morphologically distinct cell types are present: columnar epithelial cells, endocrine cells and regenerative cells (Baton and Ranford-Cartwright, 2004, 2005a). Regenerative cells are small pyramidally shaped cells sparsely scattered throughout the basal region of the midgut epithelium, between adjacent midgut epithelial cells, which terminate within the basal half of the midgut epithelium (Hecker et al., 1971; Hecker, 1977). By analogy to morphologically similar cells found in the midguts of other insects, regenerative cells are presumed to be a population of undifferentiated precursor stem cells capable of proliferating and developing into functionally mature midgut epithelial cells (Hecker et al., 1971). However, despite their nominal description, the function of so-called ''regenerative'' cells is unproven: no evidence has previously been published that these cells are mitotically active within the midgut epithelium of mature adult female mosquitoes (Hecker et al., 1971; Hecker, 1977; Weaver and Scott, 1990a). Consequently, some investigators have questioned whether, in mature adult mosquitoes, regenerative cells are precursor cells with a midgut epithelial cell replacement function (Billingsley, 1990; Weaver and Scott, 1990b). However, despite the lack of evidence for their function, unpublished studies have apparently shown that the damage resulting from ookinete invasion of the midgut epithelium ''can be repaired to a limited extent by proliferation of so-called regeneration [sic] cells or by neighbouring [midgut epithelial] cells approaching one another to close the gap'' (Becker-Feldman et al., 1985; Maier et al., 1987).

In the current paper, we present further observations made from examining histological sections prepared from the midguts of *Anopheles stephensi* mosquitoes infected with the human malaria parasite *Plasmodium falciparum* (Baton and Ranford-Cartwright, 2004). We present morphological evidence that regenerative cells within the midgut epithelium of adult female *A. stephensi* mosquitoes undergo cell division and subsequently differentiate into normal columnar midgut epithelial cells via intermediate morphological forms possessing apical microvilli-lined cavities. Furthermore, comparison of uninfected and *P. falciparum*-infected mosquitoes is used to demonstrate that the level of malaria parasite infection is positively associated with amount of putative regenerative cell division and/or differentiation, implying that the midgut epithelial cell destruction resulting from ookinete invasion triggers proliferative regeneration of the mosquito midgut epithelium.

2. Materials and methods

2.1. Malaria parasites and mosquitoes

The *P. falciparum* clone 3D7A, previously derived from isolate NF54 by limiting dilution (Walliker et al., 1987), was cultured *in vitro* under conditions permissive for the