Enhanced Gametocyte Formation in Erythrocyte Progenitor Cells: A Site-Specific Adaptation by *Plasmodium falciparum*

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Gametocytogenesis by *Plasmodium falciparum* is essential for transmission of the parasite from human to mosquito, yet developing gametocytes lack expression of surface proteins required for cytoadherence. Therefore, elimination from the circulation should occur unless they are sequestered in regions of low blood flow such as the extracellular spaces of the bone marrow. Our data indicate that gametocytogenesis is enhanced in the presence of erythroid progenitors found within the bone marrow. Furthermore, atomic force microscopy indicates that developing gametocytes undergo remarkable shifts in their erythrocyte membrane elasticity, which may allow them to be retained within the bone marrow until maturation.

**Keywords.** *Plasmodium falciparum*; gametocytes; erythroid progenitor cells; atomic force microscopy.

Malaria, caused by *Plasmodium falciparum* infection, remains a devastating disease. Transmission between the human host and the mosquito vector relies on the production of gametocytes within the human host which are then ingested by the Anopheles mosquito during a blood meal. *P. falciparum* gametocytes undergo a long maturation period of between 10 and 14 days during which they develop from stage I to stage V gametocytes. Although stage I–III gametocytes are difficult to distinguish morphologically from asexual parasites, stage IV and V gametocytes adopt the classical falciform (crescent) shape. Mature *P. falciparum* gametocytes (stage V), unlike the mature asexual stages of the parasite, appear to circulate within the host’s blood stream, while immature gametocytes sequester. However, evidence suggests that unlike the asexual stages (ASX) the sexual stages of the parasite (stages II–IV) do not express either the knob associated histidine rich protein (KAHRP) or *P. falciparum* Erythrocyte Membrane Protein 1 (PFEMP1) on their surface both of which are essential for cytoadherence of the parasite under the physiological flow conditions found within the microcirculation [1, 2], although other surface-expressed proteins may play a role, possibly via mechanical retention [3]. It has been postulated that immature gametocytes are sequestered in areas of reduced circulatory flow such as the extracellular spaces of the bone marrow [4], which may provide protection from the host immune system despite the lack of the cytoadherence complex. The question remains, however—why do gametocytes appear to accumulate in these specific areas? It may involve a purely stochastic process whereby the noncytoadherent stage I–IV gametocytes generated in the vascular circulation are removed by passage through the spleen unless they become lodged within the bone marrow or, alternatively, there is a site specific adaption by *P. falciparum* to enhance gametocyte production in these areas that may underlie the accumulation.

Using erythroid precursor cells from umbilical cord blood derived hemopoetic progenitor cells, we investigated the effect of invasion of the parasite into erythroid precursor cells. Our results demonstrate that when ASX parasites are exposed to erythroid precursor cells, there is a concomitant increase in gametocytogenesis. This observation suggests that gametocytes may preferentially form within these cells. Additionally, atomic force microscopy (AFM) indicates that early stage gametocytes have reduced membrane elasticity compared to uninfected erythrocytes, while mature gametocytes are as deformable as uninfected erythrocytes. This may indicate that early gametocytes can be retained within the bone marrow without the necessity of the cytoadherence complex, while the deformable mature gametocytes are released into the circulation to be taken up with the mosquito blood meal.
EXPERIMENTAL PROCEDURES

Parasite Culture and Erythoid Precursor Cells
The transgenic parasite clone 3D7-16 was maintained in culture as described elsewhere [5]. 3D7–16 parasites express a green fluorescent protein (GFP) chimera of Pfs16. Late-stage erythroid precursor cells (reticulocytes and orthochromatic normoblasts) were produced using a published methodology [6].

Gametocytogenesis Assay
Trophozoite-stage parasites were cultured in either the presence of late-stage erythroid precursor cells or mature erythrocytes. The cultures were sorted using a FACSCalibur flow cytometer to determine the number of GFP positive cells.

Stage-Specific Parasite Preparations
Gametocyte production was induced in 3D7-16 cultures as described elsewhere [5] and early stage gametocytes sorted and collected using a BD FACSAria II cell sorter (BD Biosciences). The gametocytes are sorted based on expression of the GFP reporter gene to give pure early stage gametocytes (EG1). EG1 sorted gametocytes were returned to culture and then matured in vitro for a further 2 days (EG2) or 7 days (LG). EG1 parasites were morphologically indistinguishable from ASX parasites. Synchronous ASX parasites were grown to trophozoite stage and enriched by gelatine flotation to give a culture of approximately 50% parasitized erythrocytes.

Parasitized and nonparasitized erythrocytes were attached to glass coverslips previously coated with poly L lysine as described elsewhere [7]. All slides were processed within 48 hours.

Atomic Force Microscopy
To determine the surface mechanical properties of all the cells, a TopoMetrix Explorer TMX-2000 AFM (Bruker) was used in the force-vs-distance (f-d) mode of operation.

RESULTS AND DISCUSSION

It has previously been reported [8] that P. falciparum parasites have the ability to invade late-stage erythroid precursor cells efficiently and undergo asexual replication. Our results confirm that P. falciparum parasites can efficiently invade both orthochromatic normoblasts and reticulocytes derived from ex vivo culture of hemopoietic stem cells (Figure 1A), indicating that not only can asexual replication of the parasite occur within the bone marrow but parasites can also undergo sexual differentiation within these cells (Figure 1B).

Previous evidence indicated that there was a marked increase in gametocytogenesis in blood containing increased numbers of reticulocytes [9]. The data presented here support the hypothesis that in the presence of late-stage erythroid precursor cells there is a significant increase in the number gametocytes generated. When P. falciparum trophozoites were cultured in the presence of only late-stage erythroid precursor cells there was a statistically significant increase in gametocyte formation by at least 2-fold (Figure 1C) when compared to mature
Given the previous observations, that gametocytes unlike ASX parasites, do not express either KAHRP or PfEMP1 on their surface [2] (a prerequisite for cytoadherence in the deep vasculature and micro-circulation), we investigated the changes in cellular elastic properties from early gametocytes to stage V gametocytes using AFM.

Previous studies have indicated that ASX parasites are much more rigid than uninfected erythrocytes, even when they do not display KAHRP [1, 10]. Studies on gametocytes using a variety of methodologies generally showed a reduction in gametocyte deformability between early stage I–II gametocytes when compared to stages IV and V [3, 11]. However, Aingaran et al. [12] reported an increase in membrane shear modulus from stage II to stage IV gametocytes followed by a subsequent decrease at stage V. However this reduction only reached levels comparable to that of stage II gametocytes [12]. Our study shows (based on Young's modulus values) a closer deformable value for stage V gametocytes and uninfected cells.

A range of biomechanical quantities are often used as a measure of cell deformity such as spring constant (force/distance), shear stress (force/area), traction force, dynamic viscosity (force/area), Young’s/shear modulus (force/area), and in-plane shear modulus. Cell deformation, whether elastic or viscoelastic, can be studied using tools that generate compressive or tensile forces, shear forces, bending forces, twisting forces, or a combination of some of these methods. Current methods include micropipette aspiration, magnetic/optical tweezers, flow chambers, elastic substratum methods, flexible sheets with
micropatterned dots or grids and atomic force microscopy (AFM). Importantly, AFM is now becoming an emerging complementary technique to aid in cell motility studies based on cell compliance. Indentations larger than 10% of the sample thickness can result in a large overestimation of the elastic modulus. In order to minimize such substrate effects, we have limited our study to indentations no larger than 300 nm. AFM investigations can provide information about the viscoelastic properties of the cell membrane and the cell cytoskeleton organization and provide an overall cell compliance value. Measurement of the cellular stiffness can be employed as a direct method for characterizing the physical properties of the erythrocyte cell membrane and cytoskeleton [7]. Young’s modulus data (Figure 2A) indicate that EG1 parasites, even though they have been reported to lack both KAHRP and PfEMP1, display similar cellular stiffness to ASX parasites. This indicates that changes in the red cell membrane and cytoskeleton are independent of the major cytoadherence complex. As the gametocytes age, an increase in membrane elasticity (ie, E values decrease) between EG1 and EG2 parasites is seen, although EG2 parasites are still significantly more rigid than uninfected erythrocytes. However, as these parasites transition to late-stage gametocytes (stage V), their membrane elasticity increases dramatically, until they have a similar elastic profile to that of uninfected erythrocytes. This transition to lower rigidity and increased membrane elasticity is concomitant with their appearance within the circulation of the host. This may indicate that there are significant changes to both the erythrocyte membrane and cytoskeleton of the stage V gametocyte when compared to immature gametocytes. This result is in contrast to that observed with other methodologies where shear stress analysis of stage V gametocytes by ektocytometry did not show a return to levels seen in uninfected erythrocytes, which may be due to the confounding fact of having the cell infected with a parasite, whereas AFM measurements were predominately at the cell surface.

Two intriguing unanswered questions in *P. falciparum* biology are: Where do *P. falciparum* gametocytes sequester during their long development phase of up to 10 days? And why do they not express the major cytoadherence complex containing KAHRP and PfEMP1? It may be that the parasite lacks the ability to remove this complex once inserted within the erythrocyte membrane and therefore has evolved a different mechanism by which to sequester the immature gametocyte. Given the evidence that PfEMP1 and KAHRP are an absolute requirement for cytoadherence within the microvasculature under physiological flow conditions [1] only anatomical locations that have reduced flow (eg, the bone marrow and spleen) seem likely to fulfill these requirements. Nonetheless, as the role of the spleen is to remove nondeformable erythrocytes from the circulation, this seems an inhospitable environment for the developing gametocyte. Thus, the bone marrow appears the obvious choice for gametocytes to develop. Our results demonstrate that not only can parasites effectively invade late-stage erythroid precursors such as orthochromatic normoblasts but that when this occurs there is a concomitant increase in gametocytophagia. The rapid decrease in membrane and cytoskeleton elasticity seen in developing gametocytes possibly due to insertions of *P. falciparum* proteins [3] may prevent their egress from the bone marrow. Once mature, and when their membrane elasticity returns to that approximating an uninfected erythrocyte, they then can egress the bone marrow and return to the peripheral circulation to be ingested by the mosquito during a blood meal.

The data presented here indicate that there may be a site-specific adaptation by *P. falciparum* leading to increased gametocyte formation in human locations that are most likely to result in attainment of maturity and transmission of the parasite to the mosquito. Nonetheless, given the multifactorial processes leading to gametocytophagia, gametocytes will likely form throughout the host’s circulatory system. Whether or not these gametocytes have a reduced survival compared to those generated within the bone marrow cannot be as yet ascertained. Additionally, the invasion of erythroid precursor cells allows the gametocytes to develop in young erythrocytes with a median life expectancy much longer than that of circulating erythrocytes that become increasingly rigid with age and thus allow these long-lived gametocytes to circulate for the maximum time possible.

Notes

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