Selective Accumulation of Mature Asexual Stages of *Plasmodium falciparum*-Infected Erythrocytes in the Placenta

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A feature of malaria in pregnancy is accumulation of *P. falciparum*-infected erythrocytes (IEs) in the placenta, which is associated with adverse outcomes for mothers and infants. Infection appears to involve parasite adhesion to molecules such as chondroitin sulfate A, hyaluronic acid, and immunoglobulins. In vitro, adhesion is predominantly a property of mature asexual forms of IEs; however, adhesion of immature or ring forms has recently been reported. We have assessed the parasitemia and developmental stages of IEs in the placenta by examination of placental blood and histological sections with comparison to parasites in the peripheral blood from the same individuals. Approximately 90% of IEs in the placenta were mature forms. Compared to peripheral blood, the placental parasitemia was 10-fold higher and the density of mature IEs was over 200-fold higher. By contrast, the average peripheral and placental ring-stage parasitemias were not significantly different. In 2 of 14 cases, the density of ring forms was higher in placental than in peripheral blood. These findings demonstrate prominent selective accumulation of mature asexual-stage IEs but infrequent accumulation of ring stages in the placental blood spaces, consistent with an important role for mature-stage IE adhesion.

Infection with *Plasmodium falciparum* during pregnancy is an important cause of morbidity and mortality in infants and mothers and predisposes those infected to maternal anemia and low birth weight through intrauterine growth retardation and premature delivery (5). A key feature of malaria in pregnancy is the accumulation of *P. falciparum*-infected erythrocytes (IEs) in the maternal blood spaces of the placenta (22). Events mediating IE sequestration in various organs are not fully understood, and a number of mechanisms have been proposed, including IE adhesion to host cells and rheological changes in IEs (reviewed in reference 1). Histological examination of infected placental tissue suggests some IEs are adherent to syncytiotrophoblasts, which line the placental blood space, whereas other IEs are not (22) and appear to be retained by other means (3).

Studies in Africa have implicated parasite adhesion to chondroitin sulfate A (CSA) and hyaluronic acid (HA), present on syncytiotrophoblasts, in parasite sequestration in the placenta (reviewed in reference 3). IEs isolated from infected placentas typically adhere to CSA and HA in vitro rather than to other known host adhesion molecules (2, 4, 9). Recently, parasite binding to immunoglobulins has been suggested as an additional mechanism involved in placental infection (8).

Cell adhesion is predominantly a feature of mature-stage IEs, pigmented trophozoites, and schizonts and is mediated by the expression of parasite-derived proteins on the surface of the erythrocyte (11). Of these, the variant protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates adhesion to CSA (16) and other receptors (1) and is expressed on the IE surface of late-ring-stage and mature pigmented IEs, from approximately 16 to 48 h of the 48-h life cycle (10, 23). The expression of PfEMP1 corresponds with the disappearance from the peripheral circulation of mature IEs, which are thought to sequester in deep vascular beds to avoid splenic clearance (17).

The hypothesis that parasites sequester in the placenta due to cell adhesive processes, largely mediated by PfEMP1, implies that only mature stages of IEs would specifically accumulate. However, to our knowledge this has not been formally assessed. Although some studies have reported a predominance of mature IEs in the placenta (6), it has also been proposed that parasites might replicate locally within the placenta (22), and recent studies have suggested that early developmental stages, or ring forms, can adhere and sequester in the placenta and brain (14, 20).

To address these issues in placental malaria, we have assessed and compared the parasitemias and developmental stages of IEs present in the placenta and peripheral blood of matched samples from the same individuals.

**MATERIALS AND METHODS**

Pregnant women attending for normal delivery at the Queen Elizabeth Central Hospital, Blantyre, Malawi, were enrolled into a larger study of the epidemiology, pathology, and pathogenesis of malaria during pregnancy (2, 4, 19), following informed consent. From these women, 17 cases with moderate to heavy plasmodial infection were selected for the present study.

Immediately following delivery, several biopsies of placental tissue (approximately 1.5 to 2 cm in each dimension, up to 6 cm3) were cut from different areas of the maternal side of the placenta that appeared grossly normal. For preparation of placental histology sections, biopsy tissue was fixed in neutral buffered formalin and paraffin embedded. Sections were made and stained with Giemsa using standard methods. Placental blood containing parasites was washed from...
placental tissue by incubating several biopsy samples together in a 50-ml tube containing phosphate-buffered saline (pH 7.2) with 50 mM EDTA (the placental tissue occupied no more than one-third of the volume) on a tube roller for 60 min at room temperature. This method was previously found to be an effective way of isolating viable parasites from infected placentas (2). After removal of placental tissue and supernatant, cells harvested were examined by microscopy of thin smears of blood washed from placental tissue sections. Parasite forms present in all cases (Fig. 1), and mature-stage parasites were observed in only 7 of 14 cases examined. Overall, 96.5% of all IEs examined were mature forms.

RESULTS AND DISCUSSION

Comparison of 17 matched placental and peripheral blood parasitemias demonstrated a marked concentration of IEs in the placenta (Table 1). Overall, the mean placental parasitemia, calculated from placental washings, was around 10-fold higher (P < 0.01; Wilcoxon’s test) than the mean peripheral blood parasitemia (mean ± standard error of the mean [SEM] for placental samples, 14.2% ± 3.5% [range, 2.0 to 51.4%]; mean ± SEM for peripheral samples, 1.36% ± 0.4% [range, 0.07 to 7.0%]). Additionally, in 16 of 17 cases the placental parasitemia was substantially higher than the corresponding peripheral blood parasitemia.

The vast majority of IEs from placental samples comprised mature-stage parasites (pigmented trophozoites or schizonts). Developmental stages were easier to define in smears of placental washings than in placental tissue sections, and a greater proportion of ring forms and schizonts was identified in placental washings. Overall, 73.8% ± 5.9% (mean ± SEM; range, 26.6 to 100%) of IEs were mature pigmented trophozoites, 13.1% ± 3.2% (range, 0 to 41.1%) were schizonts, and 13.1% ± 3.4% (range, 0 to 40.9%) were ring forms by examination of placental washings (Fig. 1). By comparison, examination of placental tissue sections suggested 96.3% ± 1.9% of IEs were mature pigmented trophozoites, 2.3% ± 0.9% were schizonts, and 1.4% ± 0.5% were ring forms. In all cases, the proportion of mature-stage IEs was greater than that of ring-stage parasites.

Peripheral blood smears were available for staging from 14 of 17 cases. Ring forms comprised the majority of parasite types observed in all cases (Fig. 1), and mature-stage parasites were observed in only 7 of 14 cases examined. Overall, 96.5% ± 1.9% (range, 79.4 to 100%) of all IEs were ring forms (Fig. 1), and no schizonts were seen. The mean parasitemia of pigmented trophozoites in placental washings was over 200 times higher than that of peripheral blood (11.2% versus 0.048%).

These findings indicate that IEs selectively accumulate in the blood spaces of the placenta. This is predominantly a feature of pigmented trophozoites, which express PfEMP1 on their surface (11) and may adhere to CSA and HA or bind immunoglobulins (4, 8, 14, 18). The intraerythrocytic parasite life cycle lasts approximately 48 h, with 24 h in the ring stage, 12 h as pigmented trophozoites, and 12 h as schizonts (20). Therefore, in the absence of selective processes, random sampling of parasites should yield approximately 50% ring-stage IEs, 25% pigmented trophozoites, and 25% schizonts. However, in the placenta around 90% of all IEs examined were mature forms.
In contrast to the accumulation of mature asexual-stage parasites, the sexual forms of *P. falciparum*, gametocytes, do not appear to sequester in the placenta (7).

To assess the possibility that ring-stage parasites specifically accumulate in the placenta (14), we calculated ring-stage parasitemia based on the parasitemia and proportion of ring forms present in smears of placental washings and peripheral blood for 14 matched cases (Table 1). The average ring-stage parasitemia among placental samples was not significantly different (*P* = 0.535; Wilcoxon’s test) from that of matched peripheral blood samples (mean ± SEM, 0.73% ± 0.19%, and median, 0.58% for placental samples; for peripheral blood samples, mean ± SEM, 1.23% ± 0.47%, and median, 0.58%).

Overall, these findings do not support a major role for the sequestration of ring-stage IEs or local parasite replication in the placenta. A high proportion (36 to 41%) of ring forms in three placenta (Table 1, cases F, J, and K) and a substantially higher ring parasitemia in placental than peripheral blood of one case (Table 1, case B) might reflect a role for ring-stage adhesion and sequestration in some instances. Although case B had a higher ring parasitemia in placental blood, 97% of placental parasites were mature forms. In another study, early ring forms adhered to placental tissue and endothelial cells in vitro, but the level of adhesion at 8 h of development was only around 11% of that for mature forms (14). A separate study reported no adhesion of ring forms to cultured syncytiotrophoblasts (12). Our data suggest that the majority of ring-stage IEs circulate and only sequester when they mature to pigmented trophozoites and express specific adhesion molecules. In vitro, maximal IE adhesion (10) and expression of PfEMP1 on the IE surface (23) commences among late ring forms. This may also account for the high proportion of rings observed in three placenta. Among these placenta, the majority (>80%) of rings were mid- or late stages rather than early forms. However, we note that reliably identifying the different stages of rings in clinical samples was difficult.

Examination of brain tissue collected post mortem revealed that the parasitemia of ring-stage IEs in cerebral vessels was around 10-fold higher than in peripheral blood and constituted on average 27% of all IEs identified in cerebral vessels (20). Schizonts were underrepresented, with the ratio of trophozoites to schizonts being 7:1, whereas it would be expected to be roughly equal. We found the ratio of trophozoites to schizonts in the placenta was around 4:1 (from analysis of placental washings). Adhesion of IEs to CSA, HA, and cultured syncytiotrophoblasts is reduced among schizonts compared to pigmented trophozoites (12; 15; J. G. Beeson, unpublished data).

Mature-stage *P. falciparum* IEs have markedly reduced deformability compared to uninfected erythrocytes, which may contribute to their accumulation in various organs (13). If rheological changes are a principal determinant of parasite sequestration, equal numbers of trophozoites and schizonts should sequester, which was not the case. Multiple factors, such as adhesion, changes in IE rheology, and other processes, may combine to augment IE sequestration in the placenta.

It is possible that the method of washing parasitized blood from placental tissue removes a greater proportion of nonadherent or weakly adherent IEs, such as ring forms and schizonts, leading to an overestimation of the proportion of these parasite stages present in the placenta. If this were the case, the placental ring-stage parasitemia may have been lower than what we calculated here, further suggesting a limited role for ring-stage adhesion in placental parasite sequestration. Previously, we compared the present technique for harvesting parasites from the placenta with a more vigorous extraction method. Few differences between the methods were seen when assessing IE adhesion in vitro (2). Although histology has the potential advantage of examining sequestered parasites in situ, we found it more difficult to readily determine developmental stages by this approach than by examination of thin smears of blood washed from placental tissue. Changes in placental malaria appear to be diffuse rather than regional (22); however, a detailed evaluation is needed. Here, we sampled parasites from several different locations of each placenta in order to calculate the parasitemia and assess the developmental forms present.

To our knowledge this is the first report in which the developmental stages of IEs present in the placenta have been quantified, and the findings have significant implications for understanding the pathogenesis and immunology of placental malaria. The preferential accumulation of mature asexual-stage IEs in the blood space of the placenta is consistent with an important role for parasite-host cell adhesion, mediated by PfEMP1, in placental infection by *P. falciparum*.

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