BRIEF REPORT

The etiology of placental *Plasmodium falciparum* malaria in African women

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Short title: Etiology of placental *falciparum* malaria

Abstract

Plasmodium falciparum parasites causing placental malaria (PM) express the VAR2CSA type of the clonally variant antigen family PfEMP1. This enables evasion of pre-existing immunity and results in placental accumulation of infected erythrocytes (IEs). We present data on seasonal variation in levels of VAR2CSA-specific IgG and IgG specific for a PM-unrelated PfEMP1 protein among Ghanaian women at first antenatal visit. Our results indicate that PM does not require recent exposure to infected mosquitoes, in contrast to malaria in general. This has implications for the impact of insecticide-treated bed nets on PM incidence, and for antenatal care in woman with pre-existing immunity.

Key words

Acquired immunity, IgG; PfEMP1; Placental malaria; *Plasmodium falciparum*; Pregnancy, Seasonal variation; Mosquito transmission; VAR2CSA; Variant-specific immunity

Background

People living in areas with stable transmission of *P. falciparum* parasites acquire substantial immunity to malaria during childhood [reviewed in ref. 1]. This protection is largely mediated by IgG antibodies targeting the asexual blood-stages of the infection, which are responsible for all the clinical symptoms of malaria [2]. IgG targeting parasite antigens on the IE surface, in particular members of the clonally variant antigen family *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), appear to be of particular importance [reviewed in ref. 3]. PfEMP1 proteins mediate adhesion of mature IEs to receptors in the vasculature of the infected individual. This tissue sequestration facilitates parasite evasion of phagocytic destruction of IEs in the spleen. However, it can also lead to severe disease precipitated by inflammation, circulatory disturbances, and high parasite loads. PfEMP1-specific IgG is thought to be protective by interfering with IE sequestration and by opsonizing IEs for improved phagocytosis.

Although *P. falciparum* parasites have evolved to subvert and delay acquisition of immunity by switching among the approximately 60 different PfEMP1 proteins encoded in the parasite genome [reviewed in ref. 3], girls living in stable transmission areas have acquired substantial protection from malaria (apart from placental infection) well before the time they reach childbearing age. Clinical disease episodes are rare by then, but carriage of low-density parasitemia, suppressed by immunity to densities often too low to be detected by rapid diagnostic tests or standard examination of blood films, remains common. This notwithstanding, pregnancy is accompanied by a sudden reappearance of susceptibility to malaria, particularly among primigravidae. It is now well-established that this is due to parasites expressing a particular type of PfEMP1. It is called VAR2CSA, and it enables sequestration of IEs in the placenta by binding to an adhesion receptor (oncofetal chondroitin sulfate) available there, but not elsewhere in the body. Expression of VAR2CSA is therefore incompatible with parasite survival in a non-pregnant person. Because VAR2CSA is not only functionally, but also antigenically distinct from all other PfEMP1 types, PfEMP1-specific protective immunity acquired prior to the first pregnancy, does not protect against IEs expressing VAR2CSA on their surface. However, once the immune system is exposed to VAR2CSA during the first placental infection, protective immunity to this antigen type develops. Placental malaria is therefore mainly a problem during the first few pregnancies. Once acquired, protection is maintained between pregnancies by specific memory B cells rather than by VAR2CSA-specific IgG, which normally declines to relatively low levels shortly after delivery. This low durability of the specific IgG response once the antigen is gone, is not restricted to VAR2CSA. It is rather the rule for most PfEMP1-specific IgG responses, and indeed for IgG responses to many other malaria antigens as well. With these facts in mind, we assessed the relative importance in the etiology of placental malaria of infections acquired from mosquitoes during pregnancy versus sub-clinical parasitemia established before conception.

Methods

Study site and participants

The study was conducted in the town of Asutsuare, located about 85 km northeast of Accra. *P. falciparum* transmission intensity in this area is seasonally variable, peaking during and immediately after the rainy season [4]. Pregnant women, who consented to participate in the study after receiving oral and written information about its aims at their first or second antenatal visit, were recruited consecutively from July 2006 to September 2008. Women with any form of pregnancy complication were excluded from participation. Data regarding age, gestational age, pregnancy history, number of remembered previous malaria episodes, as well as clinical data (temperature, hemoglobin, blood type, parasitemia) were collected from all participants, in addition to a venous anti-coagulated (EDTA) blood sample (4 mL). Some plasma samples were inadvertently lost during storage prior to antibody analysis. Plasma samples from 30 healthy men and 30 non-pregnant women from the same community were included for comparison, and 10 Danish healthy adults without previous exposure to *P. falciparum* were included as negative controls.

Ethics Statement

This study was reviewed and approved by the ethics committee of the Noguchi Memorial Institute for Medical Research, University of Ghana (Permission no. NMIMR- IRB CPN #014/05/-06).

Malaria-specific plasma antibody levels

The plasma levels of IgG antibodies to two recombinant PfEMP1 proteins were measured by ELISA as described in detail previously [5]. One protein (FV2) represented the full-length ectodomain of the VAR2CSA-type antigen IT4VAR04. The other protein (FV6), represented the full-length ectodomain of another PfEMP1 protein (HB3VAR06), which is a commonly recognized PfEMP1 antigen that is not associated with placental malaria.

Statistical analysis

Monthly variation (Jan-Dec) in rainfall was analyzed by one-way analysis of variance (test statistic: F). The difference in antibody levels in women with and without parasitemia was analyzed by Mann-Whitney's test (T). Intergroup differences (men, pregnant women and non-pregnant women; women of different gestational age) were evaluated by Kruskal-Wallis one-way analysis of variance on ranks (H), followed by Dunn's post-hoc test to isolate statistically significant pairwise differences. The association between FV2- and FV6-specific IgG responses was evaluated by Spearman's rank-order correlation analysis (r_s). Associations between antibody levels and donor parity were evaluated by Cuzik's test for trend (z). P-values below 0.05 were considered significant.

Results

Baseline characteristics of the study participants and study site

A total of 807 pregnant women were recruited into the study. Recruitment was fairly even over the study period (Supplementary Fig. 1). Samples from 644 women were available for antibody measurements. The characteristics of the latter women are summarized in Table 1. The temporal distribution (calendar month) of recruitment was similar for women with and without detectable peripheral parasitemia (P(T)>0.15).

As proxy marker for seasonal variation in *P. falciparum* transmission intensity in our study area at the time the study was conducted, we collected and analyzed monthly rainfall data 2006-2008 from the meteorological records of the nearby town of Akuse. The data supported our assumption of marked seasonal variation, as significant overall and annual seasonal variation in rainfall was evident during the study period (P(F)<0.001), with peaks in April-May during the long rains and in September during the short rains (Supplementary Fig. 2).

Plasma levels of PfEMP1-specific IgG among study participants

Plasma levels of FV2-specific (Fig. 1A) and FV6-specific IgG (Fig. 1B) differed significantly among the study groups (P(H)<0.001). Post-hoc analysis (Dunn's test) showed that the pregnant women had significantly higher plasma levels of FV2- and FV6-specific IgG than the men and the non-pregnant women from the same area (P<0.05). In contrast, the levels did not differ significantly between men and non-pregnant women for either antigen. Whereas about 40% of the men and non-pregnant had FV6-specific IgG levels above cut-off, only about 15% had levels of FV2-specific IgG above negative cut-off. There was a moderate positive correlation between FV2-specific and FV6-specific IgG plasma levels ($P(r_s=0.60)$ <0.0001, N=634).

Levels of FV2-specific IgG depended significantly and positively on parity (P(z)<0.0001) (Fig. 1C), but did not depend significantly on either gestational age at recruitment (P(H)=0.7), or on the calendar month of recruitment (P(H)=0.1). In contrast, FV6-specific IgG levels tended to decrease with increasing parity (P(z)=0.01) (Fig. 1D), did not vary with gestational age at recruitment (P(H)=0.06), but showed marked variation with calendar month of recruitment (P(H)<0.001). Essentially identical results were obtained when these analyses were restricted to primigravidae. Levels of FV2- and FV6-specific IgG were lower in women with self-reported prophylaxis (median=3.78 and 4.04, respectively) than in women who reported not to use prophylaxis (median=3.91 and 4.22). The difference was highly significant for FV6-specific IgG (P(T)<0.001) and borderline significant for FV2-specific IgG (P(T)=0.05).

Discussion

The incidence of clinical episodes of malaria varies markedly with seasonal changes in the intensity of parasite transmission, whereas the prevalence of infection (whether symptomatic or not) generally shows much less seasonal variation [6, 7]. These findings suggest that chronic parasitemia in semi-immune individuals is often suppressed by immunity to low levels that do not cause symptoms, whereas clinical disease is associated with mosquito-mediated acquisition of parasites capable of expressing new antigenic types not controlled by pre-existing immunity [8]. However, recent reports indicate that placental *P. falciparum* malaria may be an important (partial) exception to this rule [9, 10]. Their clinical, parasitological, and histological evidence suggest that placental malaria often follows when already infected women become pregnant, rather than being the result of already pregnant women becoming infected by mosquito bites. This scenario is supported by occasional case reports [e.g., ref. 11].

The PfEMP1-based interpretation of the above is that placental malaria may be caused by asymptomatic infections carried before conception (in addition to newly acquired infections), as the parasites seize the opportunity to escape pre-existing immunity when the placenta develops, effectuated by switching to expression of VAR2CSA-type PfEMP1. To test this hypothesis directly, we selected a study area with marked seasonal variation in rainfall (and hence likely also in transmission intensity) (Supplementary Fig. 2), and two PfEMP1 antigens, IT4VAR04 (FV2) and HB3VAR06 (FV6). The former antigen is a VAR2CSA-type PfEMP1, whereas the latter is a PfEMP1 protein associated with rosetting and severe malaria in children, but not with placental malaria.

VAR2CSA-specific IgG levels increase during pregnancy, but decline again within months of delivery, as maintenance of acquired protection against placental malaria appears to rely primarily on memory B cells [5]. Thus, only limited systematic variation with seasonal fluctuations in transmission intensity would be expected, if indeed placental infection often arises from pre-existing, non-placental parasitemia switching to expression of VAR2CSA-type PfEMP1 without the need for acquisition of new infection. Indeed, we did not detect much seasonal variation in plasma levels of VAR2CSA-specific IgG among the pregnant women we studied. In contrast, FV6-specific IgG levels would be expected to show some seasonal variation, as boosting should generally require new infection. Again, this is what we observed. Nevertheless, we also found that FV2- and FV6-specific IgG levels correlated to some extent. Although this limits the power of our investigative approach, it is not surprising. Thus, some of the parasites in a rapidly expanding population expressing VAR2CSA-type PfEMP1 during placental malaria are likely to switch to other PfEMP1 types, thereby boosting any pre-existing immunological memory to those variants. This was indeed observed in a previous longitudinal study [5]. Furthermore, new infections acquired from mosquito bites undoubtedly also contributes to placental malaria [10].

Our study has several limitations. The complex rainfall pattern in the study area makes it difficult to analyze in detail the causal relationship between temporal variation in transmission pattern and antibody levels. This problem is complicated further by the many variables other than transmission intensity that potentially contribute to the observed variation in antibody levels. All this notwithstanding, our study supports the notion that placental malaria often arises from parasites already present at the time of conception, rather than being caused by new infection of women already pregnant. This mode of developing placental malaria has not often been considered in the literature, but should not be unexpected, as low-grade, asymptomatic parasitemia is common among African adults [12]. It might explain, at least partially, the negative impact on

pregnancy outcome of low parasitemia early in pregnancy, even before a fully functional placenta has developed [13]. Perhaps even more importantly, this etiology might also contribute to the modest protective efficacy of insecticide-treated bed nets against PM compared to their efficacy against malaria in children [14, 15].

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Figure legends

Fig 1. Plasma levels of PfEMP1-specific IgG

A: Levels of FV2-specific IgG in plasma from Ghanaian pregnant women, non-pregnant women, and men. Medians (center line), central 50% (boxes), central 80% (bars), and outliers (points) are shown. The number of donors in each group is indicated along the bottom of the panel. Statistically significant differences ($P<0.05$) are indicated by horizontal bars along the top of the panel. B: Levels of FV6-specific IgG in plasma from Ghanaian pregnant women, non-pregnant women, and men. Presentation as in A. C: Relationship between plasma levels of FV2-specific IgG and parity in the pregnant women. D: Relationship between plasma levels of FV2-specific IgG and parity in the pregnant women. The cut-off for positivity (calculated as the mean plus two standard deviations of levels measured in 10 non-exposed Danish control donors) is indicated by gray shading in all the panels.

Supplementary Fig. 1. Temporal distribution of donor recruitment

A: The recruitment pattern of study participants during the study period. B: The pattern of recruitment according to calendar month (Jan-Dec.).

Supplementary Fig. 2. Rainfall pattern in the study area

A: The actual precipitation pattern during the study period, according to meteorological records of rainfall for the town of Akuse, near the Atutsuare study site. B: Seasonal variation in rainfall during the study period, calculated as the median of the data shown in A. Statistically significant ($P<0.05$) pairwise differences between months, evaluated by post-hoc analysis of the overall highly significant ($P<0.001$) seasonal variation, are indicated by horizontal bars along the top of the panel.

Tables

Table 1: Characteristics of study participants

Parameter	Median	[Interquartile range] or distribution	N
Age (years)	25	[20; 30]	634
Parity	2	[1; 3]	634
0	–	–	175
1	–	–	129
2	–	–	119
>2	–	–	211
First antenatal visit	–	Yes: 580 /No: 51	631
Self-reported prophylaxis	–	Yes: 245 / No: 382	627
Gestational age (months) [a]	5	[4; 6]	632
Peripheral parasitemia detected	–	Yes: 104 [b] / No: 540	644
Axillary temperature (°C)	36.9	[36.5; 37.1]	622
Hemoglobin (g/L)	11.0	[9.9; 11.8]	632
HbAS	–	Yes: 60 /No: 573	633

[a] Based on time since reported last menstruation.

[b] Range: 80 to 21,000 asexual parasites/µL.

Figure 1.