Antibodies to Variant Surface Antigens of *Plasmodium falciparum*-Infected Erythrocytes Are Associated with Protection from Treatment Failure and the Development of Anemia in Pregnancy

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Background. In pregnancy-associated malaria (PAM), *Plasmodium falciparum*-infected erythrocytes (IEs) express variant surface antigens (VSA-PAM) that evade existing immunity and mediate placental sequestration. Antibodies to VSA-PAM develop with gravidity and block placental adhesion or opsonize IEs for phagocytic clearance, helping to prevent maternal anemia and low birth weight in infants.

Methods. Using serum samples from 141 pregnant Malawian women with parasitemia enrolled in a randomized trial of antimalarials and VSA-PAM-expressing CS2 IEs, we quantified levels of immunoglobulin (Ig) G to VSA-PAM by flow cytometry and levels of opsonizing antibodies by measuring uptake of IEs by THP1 promonocytes.

Results. After controlling for gravidity and antimalarial treatment, higher levels of IgG to VSA-PAM were associated with decreased anemia at delivery (odds ratio [OR], 0.66 [95% confidence interval {CI}, 0.46–0.93]; *P* = .018) and were weakly associated with decreased parasitological failure (OR, 0.78 [95% CI, 0.60–1.03]; *P* = .075), especially reinfection (OR, 0.73 [95% CI, 0.53–1.01]; *P* = .057). Higher levels of opsonizing antibodies to CS2 IEs were associated with less maternal anemia (OR, 0.31 [95% CI, 0.13–0.74]; *P* = .008) and treatment failure (OR, 0.48 [95% CI, 0.25–0.90]; *P* = .023), primarily because of recrudescent infection (OR, 0.49 [95% CI, 0.21–1.12]; *P* = .089).

Conclusion. Higher levels of both IgG antibodies to VSA-PAM and opsonizing antibodies, a functional measure of immunity, correlate with parasite clearance and less anemia in pregnancy malaria.

Globally, 247 million people become ill with malaria each year [1], of whom 881,000 die. Pregnant women have an increased risk of *Plasmodium falciparum* infection, especially in their first and second pregnancies [2]. Maternal malarial infection occurs partly because infected erythrocytes (IEs) accumulate in the placenta [3]. Studies suggest that the *var2csa* variant of *P. falciparum* membrane protein 1 (PfEMP1) is the key protein that mediates this accumulation [4].

Women acquire immunity to pregnancy-associated malaria (PAM) by generating antibodies to PAM variant surface antigens (VSA-PAM) in a gravidity-dependent manner [5–8]. The level of PAM-specific antibodies remains low before first and even second pregnancies, increasing significantly with increased gravidity. These antibodies have been associated with protection from maternal malaria and its consequences in subgroups of pregnant women [5, 9, 10]. The protection may result from blocking the binding of IEs to chondroitin sulfate A (CSA) on syncytiotrophoblasts in the placenta [5, 8, 11] or from promoting clearance by opsonic phago-
cytosis of IEs in the peripheral blood and the placenta [12–14]. Levels of opsonizing antibodies are correlated with levels of PAM-specific immunoglobulin (Ig) G [12], but their relationship to clinical outcomes is unknown.

Host immunity against malaria is believed to be an important factor in malaria treatment success [15], and studies in children or nonimmune adults have demonstrated associations between specific measures of immunity to malaria (most commonly levels or titers of IgG to defined antigens as measured by enzyme-linked immunosorbent assay) and treatment outcome [16–21]. Such studies are lacking in pregnant women.

Prevention of malaria in pregnancy in Africa still relies on sulfadoxine-pyrimethamine (SP), but parasite resistance leads to treatment failures in children [22]. Beneficial effects of SP are seen in pregnant women, even where there are moderate levels of pediatric treatment failure [23]. We hypothesized that immunity to VSA-PAM, particularly levels of antibodies that opsonize IEs for phagocytic clearance, could be an important component of the acquired maternal immune response involved in clearing infection and protecting pregnant women from treatment failure and adverse pregnancy outcomes.

In the present study, we compared a recently developed assay for VSA-PAM–specific opsonic activity with flow cytometry measurements of total IgG to VSA-PAM in serum samples collected from Malawian women with parasitemia in midpregnancy. Antibody levels with each assay were examined as predictors of clinical outcomes, including treatment success, maternal anemia at delivery, and birth weight.

METHODS

Study population. During a randomized clinical trial of antimalarials for the treatment of parasitemia in pregnancy, 141 serum samples were collected; the trial was conducted at the Mphemba and Madziabango Health Centers in Blantyre District, Malawi, from September 2003 through September 2004 [24]. Women who were 14–26 weeks pregnant and had parasitemia demonstrated on peripheral blood film examination were eligible to participate, whether or not they had symptoms. Participants were randomly assigned to 1 of 3 treatment groups: SP (3 tablets; 500 mg of sulfadoxine and 25 mg of pyrimethamine per tablet), SP plus azithromycin (1 g/day for 2 days), or SP plus artesunate (200 mg/day for 3 days). All participants were followed up until delivery. At delivery, infant birth weight and maternal and infant hemoglobin concentrations were recorded. Anemia was defined as maternal hemoglobin level ≤11 g/dL, and low birth weight was defined as infant birth weight <2500 g. Parasitological treatment failure was defined as a further episode of parasitemia from day 7 after treatment until the end of study, and heteroduplex tracking assays were performed to distinguish recrudescence (isolation of genetically identical parasites at a subsequent time point) from reinfection (isolation of novel parasite types not seen at enrollment), on the basis of polymorphisms in the gene for merozoite surface protein 1 [25].

Culture of P. falciparum. The laboratory-adapted P. falciparum line CS2, which expresses var2csa and binds to CSA, was cultured in Roswell Park Memorial Institute (RPMI) 1640–HEPES medium with 0.5% Albumax (Gibco Invitrogen) and 0.18% sodium bicarbonate (NaHCO₃). A second P. falciparum line, E8B-ICAM, which binds to intercellular adhesion molecule (ICAM)–1, was cultured in RPMI 1640–HEPES medium with 0.25% Albumax, 5% heat-inactivated human serum, and 0.18% NaHCO₃. All parasites were synchronized by gelatin selection weekly [26].

Culture of THP1 cells. THP1 cells were maintained in RPMI 1640 medium with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 10 mmol/L HEPES, 0.05 mmol/L 2-mercaptoethanol, and 10% fetal bovine serum. Cell density was monitored closely between 1 and 10⁶ cells/mL. Cells were passaged every 6 days, when cell density approached 1×10⁶ cells/mL.

Flow cytometry. In vitro–cultured IEs were used at 4%–8% parasitemia to quantify the level of total IgG to VSAs expressed on the surface of erythrocytes infected with CS2 or E8B-ICAM parasite lines. After being washed with 0.1% newborn calf serum (NCS) in phosphate-buffered saline (PBS), IEs were resuspended at 0.1% hematocrit in 0.1% NCS in PBS and incubated with individual serum samples at a 1:20 dilution (vol/vol) for 30 min. IEs were then washed 3 times with 0.1% NCS in PBS and incubated with polyclonal rabbit anti–human IgG (Dako A0424) at a 1:100 dilution for 30 min, followed by 3 washes and incubation with Alexa Fluor 488 donkey anti–rabbit IgG (Molecular Probes A-21206; 1:500 dilution) plus 10 μg/mL ethidium bromide (EtBr) for 30 min. By means of a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software (version 5.2.1), 2000 IEs were counted, and the geometric mean fluorescence intensity (MFI) was recorded. Pooled serum samples from Malawian hyperimmune multigravid women were used as a positive control, and pooled serum samples from malaria-naïve Melbourne donors were used as a negative control. Both positive and negative controls were included in each assay. The adjusted MFI was calculated by subtracting the MFI in channel FL1 of the EtBr-negative cell population from that of the EtBr-positive cell population. Finally, we used the relative level of IgG compared with the positive control (relative MFI) as the indicator of total IgG level.

Opsonic phagocytosis. We adapted methods published elsewhere to examine phagocytic uptake of opsonized IEs [13,
Table 1. Summary of the study population, by gravidity and treatment group.

<table>
<thead>
<tr>
<th>Variable^a</th>
<th>Gravidity</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Primigravid</td>
<td>Secundigravid</td>
</tr>
<tr>
<td></td>
<td>women (n = 80)</td>
<td>women (n = 16)</td>
</tr>
<tr>
<td>Parasitological failure</td>
<td>26 (32.5)</td>
<td>5 (31.2)</td>
</tr>
<tr>
<td>Recrudescence</td>
<td>17 (21.2)</td>
<td>3 (18.8)</td>
</tr>
<tr>
<td>Reinfection</td>
<td>14 (17.5)</td>
<td>2 (12.5)</td>
</tr>
<tr>
<td>Age, mean ± SD, years</td>
<td>18.3 ± 1.8</td>
<td>22.1 ± 3.0</td>
</tr>
<tr>
<td>Maternal anemia</td>
<td>15 (19.8)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>Birth weight, mean ± SD, g</td>
<td>2705 ± 528</td>
<td>3088 ± 289</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of women, unless otherwise indicated. SD, standard deviation; SP, sulfadoxine-pyrimethamine.

^a Participants who experienced both recrudescence and reinfection were counted only once for parasitological failure.

THP1 cells were plated into 96-well plates at 5 × 10^3 cells/well in THP1 culture medium plus 100 nmol/L phorbol myristate acetate (PMA) for 12–24 h, to allow differentiation to adherent macrophage-like cells. This was replaced with culture medium without PMA on the second day, and cells were incubated for another 2–3 days before use.

Trophozoite-stage CS2 IEs at ≥8% parasitemia were purified by Percoll gradient centrifugation to 90%–95% parasitemia,
washed 3 times in tubes coated with fetal calf serum, and re-suspended in cold PBS. After that, 2.25 μL of heat-inactivated patient serum samples were incubated with 8 × 10^6 IEs for 1 h at room temperature for opsonization. Unbound serum components were removed by 3 washes with PBS, and 1 × 10^6 IEs were added to wells containing THP1 cells in quadruplicate and incubated for 2 h to allow phagocytosis. After incubation, 100 μL of cold PBS was added to each well to stop phagocytosis, followed by incubation for 3 min in 100 μL of 0.2% sodium chloride to lyse nonphagocytosed IEs. The wells were washed 4 times with warm THP1 culture medium to remove lysed IEs. THP1 cells and phagocytosed IEs were lysed by adding 100 μL of 0.2 mol/L Tris–hydrochloric acid and 6 mol/L urea for 30 min to release hemoglobin. After lysing of the cells, 100 μL of 1 mg/mL 2,7-diaminofluorene (Sigma; D17106–1G) with 0.3% hydrogen peroxide was added to each well, and hemoglobin release was measured spectrophotometrically at 620 nm.

Standard curves were constructed in triplicate using unopsonized IEs from the same culture. Eleven 2-fold serial dilutions were made from a starting concentration of 2.5 × 10^5 unopsonized cells. The standard curve was used to estimate the number of ingested erythrocytes from the amount of hemoglobin released, which was then converted to a phagocytosis index (PI) of ingested erythrocytes per 100 macrophages. The same pooled serum from multigravid Malawian pregnant women already described was used as a positive control. The same pooled serum from primigravid Malawian pregnant women was used as a reference. The percentage of the PI obtained using the positive control women already described was used as a positive control. The relative MFI against CS2 and E8B-ICAM IEs was measured spectrophotometrically at 620 nm.

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**Statistical analysis.** Data were entered into Microsoft Excel spreadsheets and analyzed using Stata software (version 9.2; StataCorp). The total IgG level (relative MFI) and the total opsonizing antibody level (relative PI) were log-transformed. The associations between relative MFI or relative PI and gravidity were estimated using linear regression. To investigate the association between immunity against PAM and malaria treatment outcome, maternal anemia, and infant birth weight, relative MFI against CS2 and E8B-ICAM and relative PI at enrollment were defined as exposure variables. Recrudescence, reinfection, parasitological failure, maternal anemia at delivery, and low birth weight were defined as outcome variables. Maternal gravidity, classified as first or second/subsequent pregnancy, was considered to be a confounding factor, as was the participant’s treatment group. Multiple logistic regression was performed to investigate the association between exposure and outcome variables, with adjustment for gravidity and treatment group.

**Ethics.** Ethical approval was obtained from the College of Medicine Research Ethics Committee, University of Malawi, and from the Melbourne Health Human Research Ethics Committee.

**RESULTS**

**Summary of study population.** Serum samples were tested from 141 pregnant women with a mean age of 21.2 years (standard deviation, 4.7 years). Characteristics of the study population are listed in table 1. Forty-seven participants were assigned to each treatment group—SP monotherapy, SP plus azithromycin, and SP plus artesunate. The relative MFI against CS2 IEs increased with gravidity. Linear regression showed that the relative MFI against CS2 IEs was higher in secundigravid (ratio of geometric means, 2.01 [95% confidence interval [CI], 1.17–3.43]; P = .012) and multigravid (ratio of geometric means, 2.62 [95% CI, 1.82–3.78]; P < .001) women than in primigravid women. In contrast, the relative MFI against E8B-ICAM IEs did not differ between the gravidity groups (figure 1A and 1B).

Relative PIs were similar in primigravid and secundigravid women (ratio of geometric means, 1.05 [95% CI, 0.84–1.32]);
Immunity against PAM: association with malaria treatment outcome. Both relative MFI against CS2 IEs and relative PI were associated with measures of treatment outcome. The associations between relative MFI against CS2 IEs or relative PI and parasitological failure are shown in table 2, table 3, and figure 2. Every 2-fold increase in relative MFI against CS2 IEs, which indicates the level of total IgG against VSA-PAM, was associated with a 22% reduction in the odds of parasitological failure after malaria treatment (odds ratio [OR], 0.78 [95% CI, 0.60–1.03]; P = .075). A similar but stronger association was observed between relative PI and parasitological failure (OR, 0.48 [95% CI, 0.25–0.90]; P = .023).

When parasitological failures were classified as recrudescence or reinfection [25], relative MFI against CS2 IEs was associated with both recrudescence and reinfection at a marginally significant level (table 2). Every 2-fold increase in relative MFI against CS2 IEs was associated with a 25% decrease in the odds of recrudescence (OR, 0.75 [95% CI, 0.56–1.01]; P = .057) and a 23% decrease in the odds of reinfection (OR, 0.77 [95% CI, 0.57–1.03]; P = .083). After adjustment for gravidity and treatment group, the relationship with recrudescence was no longer significant, but there remained a marginally significant relationship with risk of reinfection (OR, 0.73 [95% CI, 0.53–1.01]; P = .057). Relative PI was more strongly associated with the risk of recrudescence (table 3). For each 2-fold increase in relative PI, there was a 59% decrease in the odds of recrudescence (OR, 0.41 [95% CI, 0.20–0.85]; P = .016). After adjustment for gravidity and treatment, the magnitude of the effect attenuated slightly (OR, 0.49 [95% CI, 0.21–1.12]; P = .089).

Immunity against PAM: association with maternal anemia and low birth weight in infants. Host immunity against PAM was associated with risk of maternal anemia at delivery. Both relative MFI against CS2 IEs and relative PI were significantly associated with a decrease in the risk of maternal anemia. As shown in table 2 and figure 3, a 2-fold increase in relative MFI against CS2 IEs was associated with a 36% decrease in the odds of maternal anemia.
Both opsonizing antibody levels and levels of total IgG to VSA-PAM were associated with treatment outcome and with a lower prevalence of maternal anemia at delivery, but the relationship between opsonizing antibody levels and treatment outcome or maternal anemia was stronger than that between levels of total IgG to VSA-PAM and outcome, suggesting that measurement of the opsonizing function of antibodies may be a more specific assay of the protective effect of IgG in patient serum samples. These data provide the first evidence that antibodies to VSA-PAM expressed on the surface of CSA-binding IEs may be important determinants of malaria treatment outcome in pregnant women; moreover, they may be useful predictors of anemia as a consequence of such infection.

The outcome of malaria treatment varies with age [15], and relatively more pregnant women than children clear infection when treated with a partially effective drug [30]. Drug treatment is more likely to fail in primigravid women than in multigravid women [31], and the former are at highest risk of malaria in pregnancy. Our data suggest that this difference may be attributable, at least in part, to the lower levels of antibodies to VSA-PAM found in primigravid women [8, 12].

Antibodies of the cytophilic subclasses IgG1 and IgG3 can opsonize infectious agents for phagocytosis, and IgG1 and IgG3 responses to VSA-PAM are sex and parity dependent [28, 29]. The fragment crystallizable (Fc) domains of cytophilic antibodies bind to Fc receptors expressed on the surface of macrophages followed by phagocytic clearance. Decreased levels of antibodies to VSA-PAM may partly explain the susceptibility of human immunodeficiency virus (HIV)–infected pregnant women to malaria [32], and Keen et al. [12] recently reported that HIV infection significantly decreases opsonic activity and levels of IgG1 and IgG3 to VSA-PAM in pregnant Kenyan women. Together with our observations that opsonizing antibodies are important predictors of treatment outcome and are associated with protection from anemia, this suggests that opsonic activity is a biologically important function of PAM-specific antibodies, in addition to their ability to prevent placental sequestration.

Maternal antibodies to P. falciparum could block IEs from adhering to CSA, preventing placental sequestration [5, 9, 10], or they may opsonize IEs and promote phagocytic clearance [12, 13]. We chose not to test serum samples for adhesion-blocking antibodies in this study, because in a separate cohort we found weaker correlations between assays of these antibodies and either HIV serostatus or pregnancy outcomes [33].
It would nevertheless be of interest to compare different assays—including assays directly measuring levels of antibody to VAR2CSA protein, the dominant VSA-PAM (reviewed in [6] and [34])—in longitudinal studies such as this one.

The optimal format for assays of opsonic activity has yet to be resolved. Keen et al. [12] assayed opsonizing antibody by means of thioglycollate-elicited mouse macrophages or human monocyte–derived macrophages and direct counting of ingested cells by microscopy. We used PMA-primed THP1 human promonocytic cells and adapted a published protocol [13] for spectrophotometric measurement of ingested erythrocytes that used a 96-well plate format [27, 35], allowing the increased throughput that is necessary for clinical studies. Primary human macrophages are most relevant to the in vivo situation, but interhost variability in phagocytic activity [36] makes them less useful for large sample sets, because of the need to use cells from multiple donors. We are examining protocol modifications that might allow sample sets of 100–200 serum samples to be tested using the same cells. In each case, it is unlikely that other host serum factors influence assay outcome, because opsonization is followed by extensive washing before opsonized IEs are added to macrophages.

Malaria in pregnancy may cause anemia, a risk factor for maternal mortality and morbidity as well as for low birth weight in infants [37]. In Malawi, the risk of anemia at delivery among pregnant women with malarial infection decreases with increased gravidity [38]. Maternal anemia is caused by a variety of factors [6, 34], but, as our data show, higher opsonizing antibody levels at study entry were associated with a significantly lower risk of anemia at delivery. Every 10-U increase in relative PI against CS2 IEs resulted in a decrease of ~50% in the prevalence of anemia at delivery. A similar but weaker relationship was seen between relative MFI against CS2 IEs and anemia. We previously demonstrated a strong epidemiological association between malaria and mild or moderate degrees of anemia in pregnancy in Malawi [38]. Opsonizing antibodies may facilitate clearance of malarial infection and remove its effects on red blood cell destruction and suppression of erythropoiesis [39].

Our data suggest that opsonizing antibodies may also protect pregnant women from recurrence of parasitemia after intermittent preventive treatment, which is recommended by the World Health Organization as an important prevention approach against PAM. Moreover, this increased immunity decreases the risk of maternal anemia. Our sample size precluded us from restricting analysis to women of a particular gravidity, as others have done [5, 9, 10], and we did not find any relationship between either antibody measure and birth weight.

Because our study had a relatively small sample size, we could not investigate whether the associations between antibody response and the outcome measures were modified by gravidity or treatment. The levels of antibodies to VSA-PAM (especially opsonizing antibodies) remained significantly associated with treatment outcome and protection from anemia after we controlled for these variables, except for relative MFI in association with parasitological failure (tables 2 and 3). When treatment failures were divided into recrudescences and reinfections, levels of total IgG to VSA-PAM were particularly associated with a decrease in new infections, whereas opsonizing antibodies were associated with protection from recrudescence, although this association was of borderline significance (OR, 0.49 [95% CI, 0.21–1.12]; P = .089). Further studies are required to confirm these findings, which suggest that opsonizing antibodies may play a particularly important role in eliminating infection in pregnant women.

HIV is known to be an important factor in malarial infection [40, 41], but only 78 participants (55%) were tested for HIV infection. Among this subgroup, HIV infection was not correlated with either measurement of immunity against PAM (data not shown). Thus, we did not adjust our results for HIV infection.

In conclusion, opsonic phagocytosis was particularly strongly associated with decreased risk of further episodes of parasitemia and of maternal anemia at delivery, suggesting that opsonizing antibodies form a key component of immunity against malaria in pregnant women. These potential protective effects suggest that active immunization with vaccines against VSA-PAM that elicit opsonizing antibodies may improve the effectiveness of preventive treatment and pregnancy outcomes. Development of simple assays for antibodies to VSA-PAM may allow identification of women at particular risk of complications of PAM.

Acknowledgments

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References