Placental Malaria Induces Variant-Specific Antibodies of the Cytophilic Subtypes Immunoglobulin G1 (IgG1) and IgG3 That Correlate with Adhesion Inhibitory Activity

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Antibodies targeting variant antigens on the surfaces of chondroitin sulfate A (CSA)-binding malaria-infected erythrocytes have been linked to protection against the complications of malaria in pregnancy. We examined the isotype/subtype profiles of antibodies that bound to variant surface antigens expressed by CSA-adherent *Plasmodium falciparum* in pregnant Malawian women with and without histologically defined placental malaria. Women in their first pregnancy with placental malaria produced significantly greater amounts of immunoglobulin G1 (IgG1) and IgG3 reactive with surface antigens of malaria-infected erythrocytes than uninfected women of the same gravidity. IgG1 and IgG3 levels in infected and control women in later pregnancies were similar to those in infected women in their first pregnancy. Levels of IgG2 and IgG4 were similarly low in infected and uninfected women of all gravidities. IgM that bound to the surface of CSA-adherent *P. falciparum* occurred in all groups of women and malaria-naïve controls. There was a significant correlation between IgG1 and IgG3 levels, indicating that women usually produced both subtypes. Levels of IgG1 and IgG3 correlated with the ability of serum or plasma to inhibit parasite adhesion to CSA. Taken together, these data suggest that IgG1 and IgG3 dominate the IgG response to placental-type variant surface antigens. They may function by blocking parasite adhesion to placental CSA, but given their cytophilic nature, they might also opsonize malaria-infected erythrocytes for interaction with Fc receptors on phagocytic cells.

Malaria in pregnancy compromises the health of both mother and infant and is associated with accumulation of *Plasmodium falciparum*-infected erythrocytes (IEs) in the placenta (25). IEs isolated from the placenta have an unusual phenotype and bind to chondroitin sulfate A (CSA) and hyaluronic acid expressed on placental cells (1, 4, 12). Adhesion is probably mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a variant parasite protein expressed on the IE surface that binds different host receptors and has been shown to be a target of protective antibody responses in children (6).

Women in their first pregnancy (primigravidae [PG]) are more likely to be infected with malaria, and the consequences are more severe (5). This probably reflects their lack of pre-existing antibodies specific for the novel variant surface antigens (VSA) expressed by CSA-binding placental parasites (3, 13, 19). With successive pregnancies, malaria-exposed women develop antibodies that recognize surface antigens expressed by CSA-binding IEs (19) and inhibit parasite adhesion to CSA (13). These antibodies are associated with decreased prevalence of placental infection (13) and reduced risk of maternal anemia and infant low birth weight (10, 23), the major complications of malaria in pregnancy.

Recent evidence suggests that the relatively conserved PfEMP1, VAR2CSA, expressed on the surfaces of CSA-binding IEs is a key target of antibodies associated with protection against malaria in pregnancy (21). Recombinant proteins corresponding to *var2csa* domains are recognized by antibodies in plasma from malaria-exposed donors according to gravidity and gender, and antibodies to these domains are associated with reduced risk of infant low birth weight (21).

The isotype and subtype of an antibody confer specific functional activity. Binding of the Fc portions of cytophilic antibodies, immunoglobulin G1 (IgG1) and IgG3, to Fcγ receptors on phagocytic cells triggers a range of effector functions including phagocytosis, production of cytokines and chemokines, cytotoxicity, and generation of reactive oxygen and nitrogen species (17). Although antibodies to placental VSA are thought to inhibit parasite adhesion to CSA (13), they might also opsonize malaria-IEs for interaction with Fc receptors and so promote parasite clearance, release of inflammatory mediators, and presentation of malarial antigens to T cells. It is the cytophilic subtypes of antibodies targeting merozoite surface antigens that are associated with clinical and parasitological immunity (for a review, see reference 14), presumably because interaction of anti-merozoite antibodies with Fc receptors plays a critical role. The few studies which have examined the isotype profile of antibodies specific for VSA in nonpregnant individuals suggest that anti-VSA antibodies are also predominantly cytophilic (7, 16, 18, 26).

The isotype/IgG subtype profile of antibodies specific for placental VSA has not been characterized, so we do not know

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TABLE 1. Clinical features of women enrolled at delivery

Feature	Group			
	Primigravid		Multigravid	
	Malaria infected	Uninfected	Malaria infected	Uninfected
No. of women	23	23	10	10
Mean age (yr) $\pm$ SD	18 $\pm$ 2.3	18.7 $\pm$ 2.2	25.4 $\pm$ 4.8	26.5 $\pm$ 4.5
No. of antimalarial doses (median [range])	2 (1–4)	2 (1–3)	1 (1–3)	1 (1–2)
No. HIV infected/no. tested (%)	7/20 (32)	7/22 (35)	3/10 (30)	6/9 (67)

whether binding to Fc receptors is one of the mechanisms by which these antibodies mediate immunity to malaria in pregnancy. The isotype/subtype profile of antibodies inhibiting parasite adhesion to CSA is also unknown.

We have shown previously that placental malaria in primigravid Malawian women is associated with induction of antibodies that recognize CSA-adherent IEs of the It line CS2 and inhibit adhesion of CS2 IEs to CSA (3). CS2 is recognized by malaria-exposed sera in a gravidity- and gender-dependent manner (3), and it transcribes *var2csa* as the dominant *var* transcript (9). In sera or plasma from the same women, we have now examined the isotype/IgG subtype profile of antibodies reactive to CS2-IEs in relation to the ability to inhibit the adhesion of CS2 to CSA.

MATERIALS AND METHODS

Study population. Serum and plasma (in EDTA) were collected with informed consent from pregnant women attending The Queen Elizabeth Central Hospital, Blantyre, Malawi, for delivery (January 1998 to November 2000) (3). Clinical data were also collected (3). Negative-control sera were obtained from three Australian donors with no previous exposure to malaria (unexposed donors).

A case-control study design was used (3). Twenty-three PG and 10 multigravidae (MG) (third or later pregnancy) with placental malaria infection were each matched on the basis of gravidity, age (± 2 years), and delivery date (± 2 months) to one uninfected woman without evidence of current or recent malaria. Malaria status was established by peripheral and placental blood smears and placental histology. Case patients had evidence of active placental infection (visible parasites) together with positive peripheral and placental blood smears, while control patients had negative peripheral and placental blood smears and no evidence on placental histology of parasites, parasite pigment, or histopathological lesions often associated with malaria, such as fibrinoid necrosis of villi (20). All women had live births after 36 weeks of gestation. This study was approved by the College of Medicine Research Ethics Committee, Malawi, and the Human Research Ethics Committee, Melbourne Health Research Directorate, Australia.

***P. falciparum* culture.** The laboratory line CS2, obtained by selection of the It line FAF-EA8 on CSA, was maintained in continuous culture as described previously (3). Sorbitol synchronization and gelatin enrichment of knob-expressing parasites were performed regularly.

Flow cytometry and adhesion inhibition assays. Flow cytometry of antibody binding to *P. falciparum* CS2-infected erythrocytes was performed as described previously (3), using mouse anti-human IgG1, IgG2, IgG3, IgG4, or IgM (ZYMED, California), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (heavy plus light chains) (ZYMED). Mean fluorescence intensity (MFI) was taken to indicate the level of antibody bound to the IE surface. Sera from unexposed donors and pooled sera known to contain IgG specific for CS2-VSA were run in duplicate and used to standardize between assays. The adhesion inhibitory activities of these sera and plasma has been determined previously as described (3). Briefly, CS2 IEs at 1 to 4% parasitemia were incubated with RPMI-HEPES that contained test serum or plasma (1/10 dilution) for 30 min, then added to spots of CSA (linked to phosphatidylethanolamine) previously coated onto plastic petri dishes. After incubation for 20 min at 37°C, unbound cells were washed off and adhesion was expressed as a percentage of control adhesion (in the presence of pooled sera from unexposed Australian donors).

Statistical analysis. The statistical software packages STATA and GraphPad Prism were used. Subject ages were compared using unpaired *t* tests. The chi-square test was used for proportions. Mean fluorescence intensity values and adhesion inhibition data were analyzed using the Mann-Whitney rank sum test, the Kruskal-Wallis test, and Spearman's correlation coefficient.

RESULTS

To characterize antibodies to placental-type VSA induced by placental infection, IgM and IgG subtypes binding to the CSA-adherent *P. falciparum* line CS2 were measured by flow cytometry in women with placental malaria and in age- and gravidity-matched uninfected controls.

The ages of primigravid malaria-infected women and matched uninfected controls were not significantly different ($P = 0.90$) (Table 1). There were no significant differences between the two groups in the number of antimalarial (sulfadoxine-pyrimethamine) doses administered or in the proportion of women who were positive for human immunodeficiency virus (HIV) ($P = 0.83$). Multigravid malaria-infected women and matched uninfected control women were similar in age ($P = 0.48$) and had similar numbers of antimalarial doses ($P = 0.91$) (Table 1). HIV infection was more common in multigravid control women than in malaria-infected women, but this difference was not significant ($P = 0.11$). As a group, multigravid women were significantly older than primigravid women ($P < 0.0001$) and had significantly fewer antimalarial doses ($P = 0.02$), but similar proportions were HIV infected ($P = 0.30$).

Primigravid women with placental malaria infection had significantly higher levels of IgG1 ($P = 0.0002$) and IgG3 ($P = 0.0004$) specific for CS2-VSA than uninfected controls (Fig. 1). Levels of IgG2, IgG4, and IgM specific for CS2-VSA were not significantly different between infected and uninfected primigravid women and were similar to levels in unexposed Australian donors (Fig. 1). IgM that bound to the surfaces of CS2 parasites was detected in serum or plasma from malaria-infected and uninfected primigravid women and from unexposed individuals.

Levels of IgM and of each of the IgG subtypes targeting CS2-VSA were similar in multigravid women with placental malaria infection ($n = 10$) and matched uninfected women ($n = 10$) (for IgG1, $P = 0.97$; for IgG2, $P = 0.39$; for IgG3, $P = 0.91$; for IgG4, $P = 0.25$; for IgM, $P = 0.11$) (Fig. 1). In multigravid women with or without malaria infection, levels of antibodies to CS2-VSA were not significantly different from levels in malaria-infected primigravid women (Fig. 1) (for IgG1, $P = 0.50$; for IgG2, $P = 0.07$; for IgG3, $P = 0.36$; for IgG4, $P = 0.24$; for IgM, $P = 0.27$).

IgG1 and IgG3 correlated significantly with CS2-VSA-spe-

FIG. 1. Levels of IgG subtypes and IgM binding to VSA expressed by the chondroitin sulfate A-adherent *P. falciparum* line CS2. Flow cytometry was used to measure antibodies to CS2-VSA in serum/plasma from PG and MG women with histologically confirmed placental malaria (case) or from age- and gravidity-matched controls without evidence of current or previous infection on blood smears and placental histology. Sera from three unexposed Australian donors were also examined. Antibody binding is indicated by the MFI of FITC-labeled detection antibodies. Levels of IgG1 and IgG3 specific for CS2-VSA were significantly higher in PG cases ($n = 23$) than in PG controls ($n = 23$) ($P = 0.0002$ and $P = 0.0004$, respectively). Levels of IgG1 and IgG3 were similar in PG cases, MG controls ($n = 10$), and MG cases ($n = 10$). For IgM, IgG2, and IgG4, there were no significant differences between groups. Individual data points are shown, and horizontal bars indicate the medians.

cific total IgG (determined previously by using a secondary antibody specific for human IgG [3]), whereas there was no association between total IgG and IgG2 or IgG4 (results not shown), supporting the notion that IgG specific for CS2-VSA was predominantly composed of IgG1 and IgG3 subtypes.

There was a significant correlation between levels of IgG1 and IgG3 specific for CS2-VSA in primigravid women, in multigravid women, and when data from all women were combined (Table 2). IgG4 correlated significantly with levels of IgG2 in both groups of women. When data from all women were pooled, IgG4 correlated significantly with IgG2 and IgG3. Interestingly, there was a strong inverse correlation between IgG1 and IgM in primigravid women, in multigravid women, and among all women.

We examined the correlation between IgM and IgG subtypes and adhesion inhibitory activity (determined previously [3]). In primigravid women, there were significant inverse correlations between the levels of CS2-VSA-specific IgG1 ($r_s = -0.65$; $P < 0.0001$) and IgG3 ($r_s = -0.40$; $P = 0.007$) and adhesion of CS2 to CSA in the presence of serum/plasma.

TABLE 2. Correlation between IgM and IgG subtypes specific for CS2-VSA in pregnant Malawian women

Comparison	Result ^a for:					
	PG only		MG only		All women	
	r_s	P	r_s	P	r_s	P
IgG1 vs IgG2	0.16	0.30	-0.24	0.32	-0.04	0.77
IgG1 vs IgG3	0.50	0.0005	0.50	0.03	0.47	<0.0001
IgG1 vs IgG4	0.16	0.33	-0.13	0.58	0.15	0.25
IgG1 vs IgM	-0.58	<0.0001	-0.67	0.001	-0.60	<0.0001
IgG2 vs IgG3	0.22	0.15	0.18	0.45	0.19	0.14
IgG2 vs IgG4	0.36	0.02	0.54	0.01	0.35	0.007
IgG3 vs IgG4	0.30	0.06	0.31	0.18	0.32	0.01
IgG2 vs IgM	0.12	0.48	0.34	0.15	0.20	0.14
IgG3 vs IgM	-0.24	0.14	-0.10	0.68	-0.19	0.14
IgG4 vs IgM	0.18	0.25	0.17	0.46	0.13	0.32

^a r_s , Spearman's coefficient. Significant correlations are boldfaced.

Interestingly, although there was no association between IgM levels and placental infection status, the level of IgM that bound to CS2-VSA correlated directly with adhesion of CS2 to CSA ($r_s = 0.44$; $P = 0.006$) in primigravid women.

There was also a significant inverse correlation between adhesion to CSA and levels of CS2-VSA-specific IgG1 ($r_s = -0.56$; $P = 0.01$) in multigravid women. However, the associations seen in primigravid women between adhesion and levels of IgG3 and IgM were not significant in multigravid women, possibly due to the smaller sample size. When data were pooled from women of all gravidities, there were negative correlations between adhesion to CSA and levels of IgG1 ($r_s = -0.62$; $P < 0.0001$) and IgG3 ($r_s = -0.36$; $P = 0.004$) and a positive correlation between IgM levels and adhesion to CSA ($r_s = 0.39$; $P = 0.003$) (Fig. 2).

DISCUSSION

Using a case-control design, we showed that placental malaria in primigravid women was associated with induction of IgG1 and IgG3 specific for antigens expressed on the surface of the CSA-binding *P. falciparum* line CS2, in which *var2csa* is the dominant *var* gene transcribed. Uninfected primigravid women had significantly lower levels of IgG1 and IgG3 specific for CS2-VSA than infected primigravid women. These observations are consistent with published data suggesting that, during pregnancy, *P. falciparum* expresses unique variant surface antigens that bind CSA (2, 12). For primigravid women, these are novel antigens, so antibodies reactive with placental-type parasites are associated with exposure to placental infection in this group.

In multigravid women with or without placental malaria, levels of IgM and IgG subtypes were similar to those in infected primigravid women, and again, IgG1 and IgG3 were the dominant IgG subtypes detected. These observations reflect current infection or past exposure, probably in earlier pregnancies, and suggest that the isotype/subtype profile of anti-VSA antibodies does not alter with age, gravidity, or repeated infection. A larger prospective study of multigravid women would be necessary to demonstrate whether preexisting IgG1 and IgG3 specific for CSA-binding *P. falciparum* variant anti-

FIG. 2. Associations between binding of IgM and IgG subtypes to VSA expressed by the *P. falciparum* line CS2 and the ability of serum or plasma to inhibit adhesion of CS2-infected erythrocytes to chondroitin sulfate A. Levels of antibodies in serum or plasma from PG ($n = 46$) and MG ($n = 20$) women were measured by flow cytometry. Antibody binding is indicated by the MFI of FITC-labeled detection antibodies. Adhesion is expressed as a percentage of that observed in the presence of pooled sera from non-malaria-exposed donors. Linear regression lines are indicated for data from all women. Levels of IgG1 and IgG3 specific for CS2-VSA showed a significant inverse correlation with adhesion ($P < 0.0001$ and $P = 0.004$, respectively). Binding of IgM correlated positively with adhesion ($P = 0.003$).

gens can protect against placental malaria and its consequences.

IgG2 and IgG4 levels were not significantly higher in primigravida women infected with malaria than in uninfected controls and did not correlate significantly with total-IgG levels, suggesting that these IgG subtypes do not make up a major component of the IgG response to placental VSA.

Levels of CS2-VSA-specific IgG1 correlated strongly with IgG3 levels, indicating that these cytophilic subtypes are usually produced together. This association was significant in both primigravida and multigravida women, again suggesting that the profile of IgG subtypes specific for placental VSA does not vary with increasing gravidity. IgG4 levels correlated significantly with IgG2 in primigravida and multigravida women. Since exposure to placental malaria was not generally associated with induction of CS2-VSA-specific IgG2 or IgG4 subtypes, these observations suggest that in the subset of women who do produce these subtypes, both are generated.

We believe that IgM bound to the IE surface did not represent acquired antigen-specific antibodies, since high levels of IgM binding were also observed in sera from malaria-naïve controls. This phenomenon has been described previously for *P. falciparum* lines selected for adhesion to CSA (8) and may be mediated by PfEMP1. There was a highly significant negative correlation between levels of IgM and IgG1. We suggest

that high-affinity binding of specific IgG1 to placental VSA prevents nonspecific binding of IgM. The lack of a similar inverse correlation between IgM and IgG3 may reflect the generally lower levels of IgG3 antibodies. In light of these interactions, future studies could use purified immunoglobulin classes and subclasses to more precisely quantify the binding of each to the surfaces of CSA-adherent IEs.

We next examined whether levels of IgM and IgG subtypes specific for CS2-VSA were associated with the ability of serum or plasma to inhibit adhesion to CSA. Both IgG1 and IgG3 levels correlated inversely with adhesion, suggesting that IgG1 and IgG3 specific for placental-type VSA might be responsible for the adhesion inhibitory activity of acquired antibodies. Studies with purified IgG1 and IgG3 fractions are needed to confirm this. Levels of IgM showed a significant positive correlation with adhesion to CSA. This may be explained by the inverse relationship between IgM and IgG1 binding to the IE surface. The presence of IgG1 might inhibit adhesion to CSA and also prevent binding of nonspecific IgM, such that low levels of IgM binding are associated with reduced adhesion to CSA and vice versa. Alternatively, binding of nonspecific IgM may interfere with the adhesion inhibitory activity of IgG1 and IgG3 specific for VSA, or binding of nonspecific IgM could promote parasite adhesion to CSA. These observations warrant further investigation.

Only a few studies have examined the IgG subtypes of antibodies targeting VSA; however, our observations that anti-VSA antibody responses in pregnant women are predominantly cytophilic are consistent with data from previous studies of nonpregnant adults and children. In hyperimmune Papua New Guinean adults, IgG specific for VSA of a heterologous laboratory line was predominantly IgG1, with some individuals' plasma also containing VSA-specific IgG3 (18). In Kenyan children, antibodies to VSA expressed by autologous parasites were predominantly IgG3 (16). In Gabon, anti-VSA antibodies of the IgG3 subtype were prominent among healthy adults and children (7), and anti-VSA IgG1 and IgG3 were associated with acute malaria infection in children (26).

Although anti-VSA antibodies of all IgG subtypes bind to the surfaces of trophozoite-stage IEs, only IgG1 and IgG3 are able to mediate opsonization of trophozoites in vitro (15, 24). IgG2 and IgG4 may interfere with the protective activity of cytophilic anti-VSA antibodies by competing for target antigens (15). Further studies are required to establish whether the ability of sera or plasma to opsonize trophozoites in vitro is a correlate of protective immunity. However, our observations suggest that antibodies induced by placental malaria are theoretically capable of opsonizing IEs for interaction with Fc receptors on phagocytic cells, and this process may be critically important for control of placental malaria infection.

The major antigen on the surfaces of CS2-infected erythrocytes recognized by antibodies from malaria-exposed pregnant women is likely to be the variant protein PfEMP1. While the CD36-binding isogenic line FAF-EA8, from which CS2 is derived, is recognized by serum antibodies from women and men, CS2 shows a gender- and gravidity-dependent pattern of reactivity with sera from the same individuals by agglutination (3) and flow cytometry (S. R. Elliott, unpublished data), indicating that the major antigen recognized is variant. The probable target is PfEMP1 encoded by the *var2csa* gene (22). We have

shown that *var2csa* is the dominant *var* gene expressed by CS2 (9) and that selection of IEs based on reactivity with an anti-serum generated against CS2 trophozoites enriches for CSA-binding IEs that transcribe *var2csa* (11). It should be kept in mind that flow cytometry might also detect antibodies binding to other antigens on the surfaces of infected erythrocytes, both parasite proteins (e.g., rifins) and altered red cell proteins, but available data strongly suggest that PfEMP1 is the major target. Antibodies to recombinant proteins corresponding to *var2csa* domains have been associated with a reduced risk of infant low birth weight for malaria-exposed women (21), suggesting that the VAR2CSA protein is a potential vaccine candidate for malaria in pregnancy.

In conclusion, we have shown that placental infection is associated with induction of cytophilic antibodies IgG1 and IgG3 specific for VSA expressed by the CSA-binding *P. falciparum* line CS2, in which the dominant *var* gene transcribed is *var2csa* (9). Both subtypes correlated with the ability of serum or plasma to inhibit adhesion of CS2 IEs to CSA. Prospective functional studies are required both to establish whether pre-existing IgG1 and IgG3 antibodies specific for the VAR2CSA protein are associated with protection from placental malaria infection and its consequences and to determine the relative contributions of adhesion inhibitory activity and opsonization as mechanisms for this protection.

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