Brief Definitive Report

Evidence for the Involvement of VAR2CSA in Pregnancy-associated Malaria

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Abstract

In Plasmodium falciparum–endemic areas, pregnancy-associated malaria (PAM) is an important health problem. The condition is precipitated by accumulation of parasite-infected erythrocytes (IEs) in the placenta, and this process is mediated by parasite-encoded variant surface antigens (VSA) binding to chondroitin sulfate A (CSA). Parasites causing PAM express unique VSA types, VSA\textsubscript{PAM}, which can be serologically classified as sex specific and parity dependent. It is sex specific because men from malaria–endemic areas do not develop VSA\textsubscript{PAM} antibodies; it is parity dependent because women acquire anti-VSA\textsubscript{PAM} immunoglobulin (Ig) G as a function of parity. Previously, it was shown that transcription of var2csa is up-regulated in placental parasites and parasites selected for CSA binding. Here, we show the following: (a) that VAR2CSA is expressed on the surface of CSA-selected IEs; (b) that VAR2CSA is recognized by endemic plasma in a sex-specific and parity-dependent manner; (c) that high anti-VAR2CSA IgG levels can be found in pregnant women from both West and East Africa; and (d) that women with high plasma levels of anti-VAR2CSA IgG give birth to markedly heavier babies and have a much lower risk of delivering low birth weight children than women with low levels.

Key words: var gene • var2csa • Plasmodium falciparum • PfEMP1 • vaccine

Introduction

Individuals living in areas of intense Plasmodium falciparum transmission have acquired protective immunity to malaria by the time they reach sexual maturity. Pregnant women constitute an important exception to this rule, and pregnancy-associated malaria (PAM) is an important cause of maternal and perinatal morbidity and mortality in such areas (1). PAM is characterized by placental accumulation of parasite-infected erythrocytes (IEs), which are unusual in being able to bind to chondroitin sulfate A (CSA) in vitro (2) and in not being recognized by IgG in the plasma of malaria–exposed men who have IgG with specificity for IEs infesting non-pregnant individuals (3, 4).

These data suggest that the variant surface antigens (VSAs) responsible for placental adhesion to CSA are not only functionally and antigenically distinct from other molecules present at the iRBC surface, but also that they share relatively conserved antigenic determinants (2, 3, 5). Specific acquired immunity to PAM increases with increasing parity (3–5) and is mediated by IgG with specificity for PAM-type VSA (VSA\textsubscript{PAM}; 6, 7). Parasites that express VSA\textsubscript{PAM}-type antigens on the IE surface selectively transcribe an unusually structured var gene (var2csa) at high levels (8), but the presence of a short open reading frame upstream of the var2csa initiation codon (9, 10) has raised concern that var2csa mRNA may not be translated into protein despite high transcription rates (11). Here, we present evidence that VAR2CSA, which is a relatively conserved (8) member of the P. falciparum erythrocyte membrane protein 1 (PfEMP1) family (12) is expressed on the surface of CSA-selected IEs and that plasma levels of VAR2CSA-specific IgG correlate with protection from PAM.

Materials and Methods

Recombinant Proteins. A synthetic var2csa gene based on the P. falciparum clone 3D7 sequence (PlasmoDB accession no. 19879) was cloned into the expression vector pET28a (Novagen, Madison, WI) and overexpressed in Escherichia coli BL21 Star (DE3) cells, as described (13).

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**Results and Discussion**

*P. falciparum* parasites implicated in PAM express a serologically defined VSA subset (VSA<sub>PAM</sub>) on the IE surface. VSA<sub>PAM</sub> are not recognized by IgG in the plasma of malaria-exposed men (sex specificity), and levels of VSA<sub>PAM</sub>-specific IgG in the plasma of women depend on their number of pregnancies (parity dependency; references 3–5). To investigate whether var2csa-encoded proteins (VAR2CSA) are expressed on the surface of such IEs, we determined the IE surface reactivity of antisera raised against recombinant proteins corresponding to two var2csa domains.

We found that IgG in rabbit antisera raised against DBL5-e domain of var4 gene was recognized by plasma samples of Ghanaian individuals exposed to hyperendemic and seasonal *P. falciparum* transmission (Fig. 1, a and c). Furthermore, examination of IEs by confocal microscopy showed a punctate surface labeling characteristic of PfEMP1 surface localization (Fig. 1, e, g, and i). VAR2CSA-specific antisera did not label erythrocytes infected by isogenic parasite lines not expressing VSA<sub>PAM</sub> (Fig. 1, b, d, f, and h). Experiments using antisera raised against DBL1-X gave identical results (unpublished data). VSA<sub>PAM</sub>-expressing IEs have been shown to have a generally increased reactivity with IgG and IgM (17, 18). Therefore, we tested control rabbit antisera containing the same amount of total IgG and raised against a surface-exposed domain (DBL5-e) of a PfEMP1-associated with severe *P. falciparum* malaria in children (VAR4; references 13, 15). Neither VAR4-specific antisera (Fig. 1, a–d) nor rabbit prebleed control serum (not depicted) reacted with the surface of intact erythrocytes infected by any of the aforementioned parasite lines. The double peak in the flow diagram of anti-VAR2CSA–labeled CSA-selected NF54 (Figs. 1 a and 2 a) indicated that approximately half of these parasites expressed VAR2CSA, suggesting that the remaining parasites expressed other VSA<sub>PAM</sub> than VAR2CSA or that some of the parasites had changed VSA expression since CSA selection. To investigate this, we selected the NF54 CSA isolate on VAR2CSA antibodies using IgG-reactive Dynabeads and obtained a parasite line in which a markedly larger proportion of the IEs reacted with VAR2CSA IgG (Fig. 2, a and b). After IgG selection, the parasites were also better recognized by plasma from term-pregnant women and mostly unrecognized by plasma from sympatric men (Fig. 2, c and d).

**Figure 1.** Analysis by flow cytometry (a–d) and confocal microscopy (e–h) of intact erythrocytes infected by *P. falciparum* line NF54 (a, b, e, f, and i) or FCR3 (c, d, g, and h) and labeled by IgG in rabbit antisera raised against VAR2CSA DBL5-e (a–d, solid red, and e–h) or VAR4 DBL5-δ (a–d, solid gray) as a control serum. Infected erythrocytes subjected to several rounds of in vitro selection for adhesion to CSA (a, c, e, g, and i) or unsolicited (b, d, f, and h) are shown. Confocal microscopy images (e–h) were unstained (left), stained with ethidium bromide and labeled by anti-VAR2CSA IgG (middle), or stained by anti-VAR2CSA IgG only. Confocal panel i shows the double stained parasites. The left image (red) is IgG staining with pooled plasma from pregnant women; the middle image (green) is stained with anti-VAR2CSA IgG; and the right image is an overlay of the two first images. The staining of the central spot in the confocal images without EtBr is caused by autofluorescence of hemozoin.

**Statistical Analysis.** Sex specificity of IgG levels was evaluated by Mann-Whitney rank sum test (T), whereas parity dependency was evaluated by Spearman’s rank correlation coefficient (rs). Stat v. 7 (Stata Corporation), SigmaStat v. 3.0 (SPSS), and CIA v. 2.0 software were used.
To investigate the relationship between VAR2CSA and VSA\textsubscript{PAM} further, we used flow cytometry to analyze IEs double labeled with an antiserum to VAR2CSA DBL5-\textepsilon and plasma pools from \textit{P. falciparum}-exposed men and pregnant women, respectively. VSA\textsubscript{PAM}-expressing, CSA-selected IEs appeared as a double positive population (Fig. 3 a) when using the pool from pregnant women, but as a single (VAR2CSA DBL5-\textepsilon) positive population when using the male pool (Fig. 3 b). In contrast, unselected IEs expressing non-PAM-type VSA were not labeled by the DBL5-\textepsilon antiserum and were recognized to a similar degree by the plasma pools from men and pregnant women (Fig. 3, c and d). Examination of IEs by confocal microscopy showed that the targets of IgG in the DBL5-\textepsilon antiserum and the pooled plasma from pregnant women colocalized on the IEs surface (Fig. 1 i). Together, these results suggest that VAR2CSA is selectively expressed on the surface of IEs that have been selected for adhesion to CSA in vitro and that have a sex-specific and parity-dependent VSA\textsubscript{PAM}-type IgG recognition profile. The data also indicate that the targets of the VAR2CSA-specific and VSA\textsubscript{PAM}-specific IgG are identical or closely associated.

To examine whether human IgG recognition of VAR2CSA depended on plasma donor sex and parity, we used ELISA to measure levels of VAR2CSA-specific IgG in panels of individual plasma samples from \textit{P. falciparum}-exposed men and women. Median levels of IgG specific for recombinant proteins corresponding to the DBL1-X and DBL5-\textepsilon domains of VAR2CSA were significantly different (DBL1-X: P < 0.001 [T], median difference [95% CI] = 0.10 [0.054–0.20]; DBL5-\textepsilon: P < 0.001 [T], median difference [95% CI] = 0.14 [0.081–0.27]) in plasma from women than in plasma from men (Fig. 4 a). This pattern was even more pronounced when examining samples from a separate cohort of sympatric men and term-pregnant women (DBL1-X: P < 0.001 [T], median difference [95% CI] = 2.13 [1.54–2.45]; DBL5-\textepsilon: P < 0.001 [T], median difference [95% CI] = 2.34 [1.46–2.59]; Fig. 4 b). Negative control plasma from donors without \textit{P. falciparum} exposure did not contain VAR2CSA-specific IgG (unpublished data).

To exclude that the differences in VAR2CSA-specific IgG reactivity were simply due to a generally increased P. \textit{falciparum}-specific IgG recognition among the women, we also measured levels of IgG specificity for another, non-PAM-type PfEMP1 (VAR4; reference 13) and the non-PfEMP1 antigen, GLURP (16). We did not observe any significant sex-specific differences in the IgG levels for either of these antigens (VAR4 DBL5-\textepsilon: P = 0.096 [T], median difference [95% CI] = −0.047 [−0.22–0.16] [Fig. 4 a]; P = 0.78 [T], median difference [95% CI] = 0.00 [−0.18–0.13]; Fig. 4 b) or GLURP (P = 0.39 [T], median difference [95% CI] = −0.11 [−0.46–0.16] and P = 0.23 [T], median difference [95% CI] = 0.27 [−0.14–0.91]; Fig. 4 c). In addition to the sex specificity of VAR2CSA-specific IgG levels, we observed that these levels also depended on donor parity in individual plasma samples from term-pregnant women with parities ranging from 0 to 10 (rs = 0.38, P = 0.0027; Fig. 4 d). This correlation remained significant in a linear regression model, including both parity and age as explanatory variables (P = 0.03). In contrast, GLURP-specific IgG levels did not depend significantly on parity (P = 0.94; unpublished data).

Plasma levels of VSA\textsubscript{PAM}-specific IgG correlate with acquisition of protective immunity to PAM; in fact, there is strong recent evidence to suggest a causal relationship between
high levels of VSA\textsubscript{PAM}-specific antibodies and protection from maternal anemia, low birth weight, and prematurity due to PAM (6, 7). Adverse PAM-related pregnancy outcomes are concentrated among women with an ongoing placental infection of sufficient duration to allow detection of both IEs and hemozoin-laden phagocytes in the placenta at the time of delivery (active-chronic–type PAM; reference 14), and it is also among women with active-chronic PAM that the protective effect of VSA\textsubscript{PAM}-specific IgG is most readily detected (7).

To investigate whether a similar relationship exists between levels of IgG specific for VAR2CSA and birth weight of offspring, we studied the relationship between pregnancy outcome and VAR2CSA-specific plasma IgG levels at the time of delivery in a group of \textit{P. falciparum}-exposed women participating in a study of the impact of PAM on pregnancy outcome. The samples studied here belonged to the random subset of the original cohort used to study the relationship between VSA\textsubscript{PAM}-specific IgG levels and pregnancy outcome (7), and included all the 110 women in that subset who were found to suffer from active-chronic PAM. Logistic regression analysis showed that high (i.e., above median) plasma levels of VAR2CSA DBL5-\(e\)-specific plasma IgG delivered babies weighing 2.864 g (95% CI: 2.700–3.029 g) compared with 2.436 g (95% CI: 2.264–2.608 g) for women with low (i.e., below median) VAR2CSA DBL5-\(e\)-specific plasma IgG levels.

We have shown previously that the \textit{var2lsa} gene is remarkably conserved between parasite isolates (8). Here, we show that 3D7 VAR2CSA-specific antiserum labeled different CSA-selected parasite lines (Fig. 1), and that 3D7 VAR2CSA-specific IgG could be detected in plasma from pregnant women from both West and East Africa (Figs. 2–4). Together, these findings indicate substantial serological cross-reactivity between VAR2CSA from different parasites. The fact that levels of VAR2CSA-specific IgG correlated with sex and parity in plasma from \textit{P. falciparum}-exposed individuals (a characteristic shared with the VSA\textsubscript{PAM}-specific IgG mediating protection against adverse pregnancy outcome due to placental \textit{P. falciparum} infection; Fig. 4 and references 6, 7) is consistent with the paradigm that PAM develops in otherwise malaria-immune women when they are infected with parasites that rely on placental binding to CSA for survival and that express IE surface antigens to which the women do not have specific protective immunity.

Several groups have identified CSA-binding domains in PfEMP1 molecules (19–21). The most extensively studied of these genes (\textit{var1lsa}) does not show enhanced transcription in
In conclusion, we find that IEs with the VSAPAM phenotype implicated in PAM selectively express VAR2CSA and that high VAR2CSA-specific IgG levels are related to favorable birth outcome. This raises hope that VAR2CSA-specific IgG induced by vaccination of women in malaria-endemic areas before their first pregnancy can protect against adverse consequences of malaria in pregnancy.

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Table I. Multiple Logistic Regression Analysis of Risk of Low Birth Weight (<2.500 g) in 110 Delivering Women with Histological Evidence of Active-Chronic Placental P. falciparum Infection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio [95% CI]</th>
<th>P &gt; z</th>
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</thead>
<tbody>
<tr>
<td>VAR2CSA DBL5-e IgG &gt; median</td>
<td>0.25 [0.10–0.63]</td>
<td>0.003</td>
</tr>
<tr>
<td>Weight of mother</td>
<td>0.84 [0.75–0.94]</td>
<td>0.002</td>
</tr>
<tr>
<td>Middle arm circumference of mother</td>
<td>1.41 [1.02–1.93]</td>
<td>0.03</td>
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References