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Neonatal and Maternal Immunological Responses to Conserved Epitopes within the DBL-γ3 Chondroitin Sulfate A-Binding Domain of *Plasmodium falciparum* Erythrocyte Membrane Protein 1

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*Plasmodium falciparum* erythrocyte membrane protein 1 (PIEMP1) mediates the adherence of *P. falciparum*-infected erythrocytes to placental syncytiotrophoblasts via interactions with chondroitin sulfate A (CSA), a characteristic of pregnancy-associated malaria. Pregnancy-associated malaria predicts increased susceptibility of newborns to malaria, and it is postulated that transplacental passage of parasite antigen induces immune regulatory activity in the neonate. We wished to examine the immune responsiveness to a CSA-binding domain of PIEMP1, the DBL-γ3 domain, in cord and maternal venous blood obtained from pregnancies with various histories of *P. falciparum* infection. We assessed in vitro T-cell cytokine and plasma immunoglobulin G (IgG) and IgM responses to four peptides corresponding to highly conserved regions of a DBL-γ3 domain common to central African parasite isolates. The presence of placental *P. falciparum* infection at delivery was associated with elevated frequencies of DBL-γ3 peptide-specific CD3+ interleukin-10-positive T cells in cord blood, while treatment and clearance of infection prior to delivery was associated with elevated frequencies of CD3+ gamma interferon-positive T cells. DBL-γ3 peptide-specific IgM antibodies were detected in 12 of 60 (20%) cord plasma samples from those born to mothers with *P. falciparum* infection during pregnancy. Consistent with polyclonal anti-PIEMP1 antibody responses that are associated with protection against pregnancy-associated malaria, the presence of maternal IgG antibodies with specificity for one of the DBL-γ3 peptides showed a parity-dependent profile. These data demonstrate that peptides corresponding to conserved regions of the DBL-γ3 domain of PIEMP1 are immunogenic in *P. falciparum*-infected mothers and their offspring.

In malaria-endemic regions, pregnancy is associated with increased risk of *Plasmodium falciparum* infection that has deleterious consequences for both maternal and neonatal health (32). Susceptibility to pregnancy-associated malaria is related to the abundant expression of chondroitin sulfate A (CSA) on placental syncytiotrophoblasts. Chondroitin sulfate A is a proteoglycan that acts as a receptor for *P. falciparum* erythrocyte membrane protein 1 (PIEMP1) (24) expressed on the surface of infected erythrocytes (26). Infected erythrocytes accumulate in the intervillous spaces of the placenta (8), and naturally acquired antibodies that interfere with CSA-mediated adherence of infected erythrocytes are associated with protection against pregnancy-associated malaria (9) and increase with parity (25).

Variants of PIEMP1 are encoded by individual members of the *var* multigene family and most comprise at least one cysteine-rich interdomain region with a variable number of Duffy binding-like (DBL) domains (29). The repertoire of PIEMP1 variants expressed on infected erythrocytes found in association with pregnancy-associated malaria is narrower than that expressed on infected erythrocytes of non-pregnancy-associated malaria parasites, perhaps due to constraints imposed by receptor specificity, which may help to explain the relatively rapid acquisition of immunity to pregnancy-associated malaria (12). The DBL-γ3 domain of PIEMP1 expressed by placental parasite isolates binds to CSA (4, 10), and antibodies directed against recombinant DBL-γ3 block infected erythrocyte adhesion to CSA (5). Monoclonal antibodies raised against DBL-γ3 bind to the surface of CSA-adhering parasites obtained from different geographic areas (19), which is itself probably a reflection of the relatively conserved nature of the DBL-γ3 domain that supports the feasibility of a vaccine against pregnancy-associated malaria.

The knowledge of B- and T-cell activity directed to specific epitopes of PIEMP1 in naturally exposed humans is very limited (1), and no studies have reported PIEMP1-specific immune responses in the cord blood from neonates born to mothers with malaria. Epidemiological studies suggest that pregnancy-associated malaria increases the likelihood of early infection in the newborn (6, 18), possibly as a result of antigen exposure inducing immunosuppressive pathways during fetal development (2, 3). In this study, we wished to determine whether DBL-γ3 domain-specific antibody and T-cell responses are present in cord blood and maternal venous blood. We tested a panel of peptides corresponding to conserved regions of the DBL-γ3 domain present in closely related PIEMP1 variants expressed by placental parasites isolated...
from Cameroon and Gabon (15, 16). For comparative purposes, we also used recombinant glutamate-rich protein, a 
P. falciparum antigen shown to be present in cord blood (14).

DBL-γ3 domain sequence-specific peptide selection was based both on amino acid conservation and HLA-DR allele-binding agiretope prediction (23). Our results show that maternal 
P. falciparum infection during pregnancy is associated with increased frequencies of DBL-γ3 peptide-specific T cells and IgM in cord blood.

MATERIALS AND METHODS

Study population. The study was carried out at the Albert Schweitzer Hospital in Lambaréné, Gabon, a site with perennial transmission of 
P. falciparum (33). Informed consent for participation was obtained from mothers prior to inclusion in the study. From May to December 2003, 85 maternal venous and umbilical cord blood samples were collected into heparinized Vacutainer tubes (BD Biosciences, Heidelberg, Germany). The presence of 
P. falciparum parasites in the maternal, placental, and cord blood at the time of delivery was determined through microscopic examination of Giemsa-stained thick and thin smears. The medical records of uninfected mothers were examined to verify those who had been appropriately diagnosed and treated for 
P. falciparum malaria episodes during their pregnancy. The majority of those with such a history received chemotheraphy with quinine, a drug with 100% efficacy for the treatment of uncomplicated 
P. falciparum malaria in the study area (22), at least 2 weeks prior to delivery.

Based on these criteria the following distinct groups were defined: (i) negative: no evidence of 
P. falciparum parasitism or active infection in any compartment at delivery and no record of malaria during pregnancy; (ii) placenta positive: 
P. falciparum asexueal stage parasites present in placental blood; and (iii) treated: no evidence of 
P. falciparum parasitism or active infection in any compartment at delivery but recorded history of a diagnosed and treated malaria episode during pregnancy. A significant higher proportion of primiparous mothers had placa- cental 
P. falciparum malaria episodes during their pregnancy. The majority of those with such a history received chemotherapy with quinine, a drug with 100% efficacy for the treatment of uncomplicated 
P. falciparum malaria in the study area (22), at least 2 weeks prior to delivery.

In order to calculate the relative antibody concentrations of the test plasma samples, standard curves using serial dilutions of pooled plasma samples from five Gabonese adults who tested positive for DBL-γ3-specific antibodies were determined through ELISA. In order to determine the cutoff value for a positive result, we calculated the mean optical density plus three standard deviations of plasma samples from negative controls (10 Europeans with no known exposure to 
P. falciparum).

Peptides. Synthetic peptides corresponded to a DBL-γ3 sequence from a placentally derived 
P. falciparum isolate obtained in Cameroon in 1996 (isolate 732, GenBank accession number AF334807) (16) that showed >98% amino acid sequence identity to two isolates obtained in Lambaréné, Gabon in 2000 (15). The SYFPEITHI algorithm (23) was used to predict motifs most likely to bind to HLA-DR molecules present in the Gabonese population (21), and belonging to the main HLA-DR super type (30). Figure 1 shows the amino acid sequences with the algorithm scores of the four peptides used in this study. The DBL-γ3 peptides were synthesized at >98% purity by Thermohybaid (Ulm, Germany), and resuspended in sterile PBS.

Statistical analyses. All figures were drawn using StatView. The significance of differences in continuous variables between groups was assessed using the non--parametric Kruskal-Wallis test and the Mann-Whitney U test (two groups), for associations between two continuous variables the nonparametric Spearman rank correlation test, and for differences in proportions the Fisher exact test.

RESULTS

In vitro T-cell cytokine responses to DBL-γ3 peptides in cord and maternal blood. Flow cytometric analysis was used to measure intracellular IFN-γ, IL-13, and IL-10 in CD3+ cells after overnight stimulation with the four DBL-γ3 peptides of cord blood mononuclear cells, maternal peripheral blood mononuclear cells, and malaria-unexposed European peripheral blood mononuclear cells. The proportion of cord blood mononuclear cell samples that produced cytokine in response to peptide stimulation ranged from 0.09 to 0.23, with DBL120 inducing the greatest response (Fig. 2A), while the proportion of maternal peripheral blood mononuclear cell samples with positive responses ranged from 0.19 to 0.29 (Fig. 2A). Cord blood mononuclear cells samples from the treated group of neonates contained significantly higher percentages of DBL-γ3 peptide-specific CD3+ IFN-γ+ cells compared to the negative group, while cord blood mononuclear cells samples from the placenta-positive group contained significantly higher percentages of DBL-γ3 peptide-specific CD3+ IL-10+ cells (Fig. 2B).

Cord blood mononuclear cells samples from both the pla- centa-positive and treated groups of neonates contained ele-
IgM and IgG antibodies with specificity for DBL-γ3 peptides are present in cord and maternal plasma. Plasma samples collected from cord blood and maternal venous blood were analyzed for IgM and IgG antibodies with specificity for the four DBL-γ3 peptides, and for comparative purposes recombinant glutamate-rich protein and purified protein derivative protein preparations. Maternally derived IgM antibodies do not cross the placental barrier and P. falciparum-specific IgM detected in cord plasma is therefore assumed to reflect stimulation of fetal B cells by transplacentally transferred antigen. With the exception of DBL78, the highest proportions of cord blood samples that contained IgM antibodies specific for the DBL-γ3 peptides or glutamate-rich protein were found in the placenta-positive group (Fig. 3A).

The proportions of positive responses for each peptide among all neonates were DBL25 (8%), DBL78 (8%), DBL120 (10%) and DBL132 (4%). One-third of cord plasma samples with IgM specific for DBL120 contained IgM antibodies specific for DBL132. In the treated group of neonates, the proportions of plasma samples with IgM responses to DBL25, DBL78, DBL120, and glutamate-rich protein were more than 1.5-fold higher than found in plasma of the negative group (Fig. 3A). Overall, IgM with specificity for any DBL-γ3 peptide was detected in 7 of 32 (22%) cord plasma samples from neonates born to mothers with placental P. falciparum infection at delivery, and 5 of 28 (18%) cord samples from neonates whose mothers were treated for P. falciparum infection during pregnancy, compared with only 1 of 25 (4%) cord samples from neonates whose mothers did not have P. falciparum infection during pregnancy (Fig. 3B).

The mean ± 3 standard deviations of IgM antibody levels detected in plasma samples of malaria-naïve (unexposed) Europeans was used to establish the cutoff value for a positive response (illustrated for DBL120-specific IgM in Fig. 3C). The proportion of mothers’ plasma samples from all groups with IgM specific for at least one peptide was 77 of 85 (90%). No correlations were observed between cord and maternal IgM antibody levels for any of the DBL-γ3 peptides, or for glutamate-rich protein- or purified protein derivative-specific IgG antibodies (Fig. 5).

The mean + 3 standard deviations of IgM antibody levels in plasma samples of malaria-naïve (unexposed) Europeans was used to establish the cutoff value for a positive response (illustrated for DBL120-specific IgM in Fig. 3C). The proportion of mothers’ plasma samples from all groups with IgM specific for at least one peptide was 77 of 85 (90%). No correlations were observed between cord and maternal IgM antibody levels for any of the DBL-γ3 peptides, or for glutamate-rich protein- or purified protein derivative-specific IgG antibodies (Fig. 5A). The mean + 3 standard deviations of IgM antibody levels detected in plasma samples of malaria-naïve (unexposed) Europeans was used to establish the cutoff value for a positive response (illustrated for DBL120-specific IgM in Fig. 3C). The proportion of mothers’ plasma samples from all groups with IgM specific for at least one peptide was 77 of 85 (90%). No correlations were observed between cord and maternal IgM antibody levels for any of the DBL-γ3 peptides, or for glutamate-rich protein- or purified protein derivative-specific IgG antibodies (Fig. 5A). The mean + 3 standard deviations of IgM antibody levels detected in plasma samples of malaria-naïve (unexposed) Europeans was used to establish the cutoff value for a positive response (illustrated for DBL120-specific IgM in Fig. 3C). The proportion of mothers’ plasma samples from all groups with IgM specific for at least one peptide was 77 of 85 (90%). No correlations were observed between cord and maternal IgM antibody levels for any of the DBL-γ3 peptides, or for glutamate-rich protein- or purified protein derivative-specific IgG antibodies (Fig. 5A). The mean + 3 standard deviations of IgM antibody levels detected in plasma samples of malaria-naïve (unexposed) Europeans was used to establish the cutoff value for a positive response (illustrated for DBL120-specific IgM in Fig. 3C). The proportion of mothers’ plasma samples from all groups with IgM specific for at least one peptide was 77 of 85 (90%). No correlations were observed between cord and maternal IgM antibody levels for any of the DBL-γ3 peptides, or for glutamate-rich protein- or purified protein derivative-specific IgG antibodies (Fig. 5A).
DISCUSSION

This study had as its foundation the evidence-based premise that conserved regions of a domain of a P. falciparum protein antigen implicated in parasite persistence during pregnancy would be immunogenic, and furthermore, that the presence of antigen in the placental compartment could lead to transplacental transfer and in utero sensitization of the fetal immune system. Thus, we selected four peptides from conserved regions of the PfEMP1 CSA-binding domain, DBL-γ3, which displays a very high degree of spatial and temporal conservation within Central African P. falciparum placental isolates (15, 16). This level of conservation in PfEMP1 suggests that these regions may be fundamental to the parasite’s survival in the placental compartment, perhaps via participation in CSA binding. B-cell epitopes that also bind CSA have been identified in other DBL-γ3 domain variants, and a CSA-binding PfEMP1 variant associated with pregnancy-associated malaria that contains no DBL-γ3 domain has been described (10, 27, 28). The latter findings clearly imply the existence of a degree of diversity in the receptor-ligand interactions that are thought to be integral to the development and persistence of pregnancy-associated malaria.

The algorithm-based method we used to identify potential agretopes, and by extension T-cell epitopes, within the DBL-γ3 domain revealed peptides that are recognized by both B and T cells from the mother and fetus. We found that antibody responses with specificity for at least one of the peptides (DBL120) were significantly enhanced in the peripheral blood of mothers with pregnancy-associated malaria, and that they showed the pattern of parity-dependence commonly associated

**FIG. 2.** Intracellular cytokine activity of cord and maternal T cells following stimulation of cord blood mononuclear cells and peripheral blood mononuclear cells with DBL-γ3 peptides. A) Percentage of samples containing CD3⁺ cells with a cytokine response to DBL-γ3 peptides in cord and maternal blood. The mean net percent gated CD3⁺ cells in cord and maternal blood that produce IFN-γ, IL-13, or IL-10 in response to B) DBL-γ3 peptides or C) phytohemagglutinin (PHA) in groups segregated by maternal P. falciparum infection history during pregnancy. Bar graphs illustrate mean percentages with standard deviations. Sample sizes: European control, 7; Cords: negative, 19; placenta positive, 14; treated, 17; Mothers: negative, 8; placenta positive, 7; treated, 7. *, P < 0.05 by either Fisher exact test or the nonparametric Mann-Whitney test.
with protection against pregnancy-associated malaria (25, 31). In the same context it is noteworthy that similar although mostly statistically nonsignificant patterns were observed for the antibody responses to the DBL78 peptide, which was predicted to bind most strongly to HLA-DRB1*1501, which is a particularly common allele among the Gabonese.

We interpret the presence of DBLγ3 peptide- as well as glutamate-rich protein-specific IgM in cord blood plasma samples primarily of those born to mothers with past or present \textit{P. falciparum} infection as confirmation of our premise concerning in utero sensitization. The proportions of cord samples we found with IgM specific for DBLγ3 peptides (4 to 10%) are comparable to the proportion of Kenyan cord samples containing IgM antibodies specific for recombinant \textit{P. falciparum} MSP-119 (6%) (17), and to the proportion of Cameroonian cord samples with IgM specific for crude parasite lysate preparations (14%) (36). These findings, coupled with our observation of DBLγ3 peptide-specific cytokine activity, identify \textit{PfEMP1}, or at least components thereof, as a parasite antigen that is transferred transplacentally and that sensitizes fetal T and B cells.

There is mounting evidence that prolonged placental \textit{P. falciparum} infection, as reflected by high titers of anti-pregnancy-associated malaria antibodies in cord blood, leads to greater...
susceptibility to malaria during infancy (6, 18). Untreated placental *P. falciparum* infection appears to further reduce already poor nonspecific neonatal T-cell responsiveness (13), while our own work has shown that the presence of placental *P. falciparum* infection at delivery is associated with reduced major histocompatibility complex class I and II expression on monocytes, and IL-10-mediated suppression of *P. falciparum* antigen-specific Th1-type responses (2, 3). In the study presented here, cord blood samples of those born to mothers with placental *P. falciparum* infection at delivery generally had the highest anti-DBL-γ3/F9253 IgG antibody titers and the highest DBL-γ3 peptide-specific IL-10 responses, findings that are consistent with those of the studies cited above.

Although no statistically significant associations in cytokine activity and *P. falciparum* infection during pregnancy were found in mothers, peripheral blood mononuclear cells from negative mothers contained elevated frequencies of major histocompatibility complex class I and II expression on monocytes, and IL-10-mediated suppression of *P. falciparum* antigen-specific Th1-type responses (2, 3). In the study presented here, cord blood samples of those born to mothers with placental *P. falciparum* infection at delivery generally had the highest anti-DBL-γ3 IgG antibody titers and the highest DBL-γ3 peptide-specific IL-10 responses, findings that are consistent with those of the studies cited above.

Although no statistically significant associations in cytokine activity and *P. falciparum* infection during pregnancy were found in mothers, peripheral blood mononuclear cells from negative mothers contained elevated frequencies of Th2-type cells (35), as well as Th1-type 1 cells (20), are likely required for protection against *P. falciparum* malaria in semi-immune adults. We did not see significant enhancement of DBL-γ3 peptide-specific IgG or IgM in plasma from negative mothers, although a trend toward enhanced DBL78-specific IgG antibodies was observed. An evaluation of IgG subtypes may reveal a different profile indicative of protective antibodies in this group.

Although short peptides do not have conformations representative of the native protein displayed on the surface of the infected red blood cell, the controls that we used in this study were intended to confirm sensitivity and to differentiate nonspecific responses. For example, the absence of strong antibody responses to the DBL-γ3 peptides in nonexposed Europeans validates an association of these response to our cohort, while the parity dependency of maternal plasma antibody levels specific for peptides DBL78 and DBL120, but not glutamate-rich protein, supports the idea that these two DBL-γ3 peptides are specifically associated with pregnancy-associated malaria. Additional studies are needed to determine the degree of specificity of the antibodies interacting with these DBL-γ3 peptides, for example whether these antibodies are present in malaria exposed adults who have not been pregnant, whether the addition of recombinant DBL-γ3 to the plasma prior to detection of antibodies by ELISA reduces peptide-specific responses, and whether the addition of the DBL-γ3 peptides would block antibody binding to infected erythrocytes.

In summary, this study is the first to analyze maternal and neonatal immune responses directed to peptides corresponding to conserved regions of the DBL-γ3 domain of PfEMP1.
Two of the four peptides were particularly immunogenic, associated with both B- and T-cell responses in pregnant women and their offspring. On the maternal side, higher levels of antibodies recognizing one of these peptides were associated with pregnancy-associated malaria and parity. On the fetal side, the presence of both cytotoxic- and IgM peptide-specific responses demonstrates that components of the DBL-y3 domain of PIEMPI cross the placental barrier and sensitize the fetal immune system.

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