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Functional Antibodies against VAR2CSA in Nonpregnant Populations from Colombia Exposed to *Plasmodium falciparum* and *Plasmodium vivax*

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In pregnancy, parity-dependent immunity is observed in response to placental infection with *Plasmodium falciparum*. Antibodies recognize the surface antigen, VAR2CSA, expressed on infected red blood cells and inhibit cytoadherence to the placental tissue. In most settings of malaria endemicity, antibodies against VAR2CSA are predominantly observed in multigravid women and infrequently in men, children, and nulligravid women. However, in Colombia, we detected antibodies against multiple constructs of VAR2CSA among men and children with acute *P. falciparum* and *Plasmodium vivax* infection. The majority of men and children (>60%) had high levels of IgGs against three recombinant domains of VAR2CSA: DBL5ε, DBL3X, and ID1-ID2. Surprisingly, these antibodies were observed only in pregnant women, men, and children exposed either to *P. falciparum* or to *P. vivax*. Moreover, the anti-VAR2CSA antibodies are of high avidity and efficiently inhibit adherence of infected red blood cells to chondroitin sulfate A *in vitro*, suggesting that they are specific and functional. These unexpected results suggest that there may be genotypic or phenotypic differences in the parasites of this region or in the host response to either *P. falciparum* or *P. vivax* infection outside pregnancy. These findings may hold significant clinical relevance to the pathophysiology and outcome of malaria infections in this region.

Malaria is a major public health problem that disproportionately affects young children and pregnant women. Malaria during pregnancy is associated with placental infection, maternal anemia, low-birth-weight (LBW) infants, and increased neonatal morbidity and mortality (1). Pregnant women, especially primigravidae, are highly susceptible to malaria infection, despite preexisting immunity, as they are exposed to a specific subpopulation of *Plasmodium falciparum* (*P. falciparum*) that accumulates in the placenta (2). Women living in areas of intense or stable *P. falciparum* transmission are mostly asymptomatic but at risk of severe maternal anemia, placental infection, and negative birth outcomes such as stillbirth and fetal growth retardation. In these areas, the pathogenic effects of pregnancy-associated malaria (PAM) decrease with increasing parity as women acquire PAM-specific protective immunity (3). In areas of lower transmission, malaria infection is often symptomatic in women of all parities and associated with substantial malaria-related fetal loss and maternal death (4).

During pregnancy, specific variants of *P. falciparum*-infected erythrocytes (IEs) sequester in the placenta. This mechanism is mediated by an interaction between chondroitin sulfate A (CSA) on the syncytiotrophoblasts and variant surface antigens (VSA), expressed by the parasite on the surface of IEs (2). The VSA family includes *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (5), repetitive interspersed family (RIFIN) proteins (6), subtelomeric variable open reading frame (STEVOR) proteins (7), and surface-associated interspersed gene family (SURFIN) proteins (8).

VAR2CSA belongs to the PfEMP1 family and is the main parasite ligand that mediates placental binding (9). Knockout studies demonstrated the pivotal role of the *var2csa* gene in parasite adhesion to placental CSA (10). *var2csa* is selectively transcribed in both *P. falciparum* parasites isolated from the placenta (11) and parasite strains selected *in vitro* for adhesion to CSA (12). The *var2csa* gene is relatively conserved between PAM strains. VAR2CSA is a large protein (350 kDa) that is structurally composed of six Duffy binding-like (DBL) domains (DBL1X, DBL2X, DBL3X, DBL4ε, DBL5ε, and DBL6ε), a cysteine-rich region between DBL2X and DBL3X, and several interdomains (13, 14). Each DBL domain contains conserved and polymorphic regions that can be targeted by surface-reactive antibodies (15–17). Conserved regions are predominant in DBL3X, DBL4ε, and DBL5ε domains. Interestingly, pregnant women exposed to malaria mainly recognize the DBL3X and DBL5ε domains, suggesting that specific immune memory to these VAR2CSA domains is naturally acquired with exposure. Several single domains from VAR2CSA bind to CSA *in vitro*, including DBL3X, DBL5ε, and ID1-ID2, which spans the DBL2 domain (14, 16–19). However, a specific and high binding affinity to CSA depends on the folded architec-

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ture of the full VAR2CSA protein (20). VAR2CSA is predominantly recognized in a parity-dependent manner by antibodies present in plasma of pregnant women exposed to malaria. Importantly, high levels of antibodies against VAR2CSA are associated with protection from delivering LBW infants (21). Furthermore, naturally acquired antibodies to VAR2CSA can efficiently block IE adhesion to CSA (22, 23). These antibodies are occasionally detected in nonpregnant patients (24, 25); however, the prevalence and levels of anti-VAR2CSA antibodies are much lower than in pregnant women from the same region (24).

Most studies on PAM focus on women in sub-Saharan Africa where malaria transmission is high. However, malaria is also endemic in many countries in Latin America. In Colombia, malaria transmission is low and unstable throughout the country. Over 100,000 cases are reported every year (26), and both *P. falciparum* and *Plasmodium vivax* (*P. vivax*) are prevalent (27). Intermittent preventive treatment in pregnancy (IPTp) using sulfadoxine/pyrimethamine has not been implemented in Colombia, and attendance at antenatal clinics is low. In a recent study, we demonstrated a high rate of submicroscopic malaria infections in asymptomatic pregnant women at delivery using sensitive molecular diagnostics. Despite a high frequency of infection (60%), no negative birth outcomes were observed (28).

Here, we investigated the levels, specificity, and antiadhesion activity of VAR2CSA antibodies among pregnant and nonpregnant populations from Colombia to determine exposure to placental parasite antigens in this region.

MATERIALS AND METHODS

Ethical approval. The study was approved by the Health Research Ethics Board of the University of Alberta (Canada), the Comité de Ética of Instituto de Investigaciones Médicas (Universidad de Antioquia, Colombia), the Comité Consultatif de Déontologie et d'Ethique of the Research Institute for Development (France), and the ethical committee of the Faculty of Health Science (University of Abomey-Calavi, Benin). All procedures complied with Canadian, European, Colombian, and Beninese national regulations.

Study region. Study participants exposed to malaria (pregnant women, children, and men) were recruited at the local obstetric facility or malaria clinic of the municipality of Puerto Libertador (07°54′N, 75°40′W) in the Department of Córdoba, northwest Colombia. The Department of Córdoba is within the malaria transmission region termed Urabá-Altos Sinú-San Jorge-Bajo Cauca. This region accounts for 60% of all malaria cases in Colombia (26). The entomological inoculation rate in this region ranges from 3.5 to 4.8 infective bites per person per year (29). *P. vivax* is reported in approximately 70% of cases, based on diagnosis by microscopy. The mean annual parasitic index (number of malaria cases/1,000 inhabitants) during 2000 to 2009 in Puerto Libertador was 23.4 (29).

Study design and sample collection. Pregnant women with acute malaria (quantitative PCR [qPCR] positive) and without malaria (qPCR negative) were enrolled either during their second or third trimester of pregnancy or at delivery. Children (≤15 years old) and men who presented to the malaria clinic in Puerto Libertador with acute, uncomplicated malaria were recruited. Twenty-five women and 25 men without a history of malaria or travel to an area of malaria endemicity (nonexposed) were recruited in the city of Medellín (6°13′55″N, 75°34′05″W), a municipality free of malaria transmission.

Blood samples (4 to 5 ml) were collected by venipuncture from each volunteer. Thick and thin blood smears were prepared for microscopy, and blood was spotted onto filter paper for DNA extraction. Malaria was diagnosed as described elsewhere (28). In brief, field-stained thick smears were read by an experienced microscopist in the local laboratory. Microscopy analysis was followed by genomic DNA extraction from the filter spots using the saponin-Chelex method (30). Real-time quantitative PCR (qPCR) was performed as described elsewhere (31). Serum was separated from the collected blood by centrifugation and stored at −20°C until processed.

Sera from Beninese women collected in a previous study (32) served as positive controls for VAR2CSA antibody levels and antiadhesion activity. Briefly, malaria transmission in Benin is high and peaks during two rainy seasons. *P. falciparum* is the predominant species, and the entomological inoculation rate ranges from 35 to 60 infective bites per person per year (33). Sera from 30 primigravid women and 30 multigravid women that had been previously characterized for their reactivity against VAR2CSA protein were used in this study. Controls included serum samples from 25 Beninese men and children exposed to malaria that were collected in a previous study (33) and sera from 20 Canadian adults without malaria exposure.

Antibody analysis assay. The ID1-ID2, DBL3X, and DBL5ε domains of VAR2CSA from *P. falciparum* strain FCR3 were produced in baculovirus-infected SF9 cells, as described previously (14, 16) (34). Optimal concentrations (0.5 μg/ml) of each protein were coated onto Maxisorb microtiter plates. The specific levels of IgG were measured in serum samples using an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (35). Briefly, the recombinant proteins were incubated with 100 μl of human sera at a dilution of 1:1,000 followed by horseradish peroxidase-conjugated anti-human IgG (1:6,000) to measure total IgG. Twenty sera from Canadian residents with no history of travel to areas of malaria endemicity served as negative controls. A pool of serum samples from multigravid women from Benin, previously demonstrated to have high levels of anti-VAR2CSA IgG against placental isolates, served as a positive control. Optical density (OD) values were converted into arbitrary units, as described previously (35). Antibody responders were defined as those having an antibody level (in arbitrary units [AU]) of >2 standard deviations (SD) above the mean absorbance of the negative controls.

All sera were tested for prior exposure to *P. falciparum* and *P. vivax* using recombinant *P. falciparum* merozoite surface protein 1 (PfMSP1; CTK-Biotech), *P. falciparum* glutamate-rich protein (PfGLURP) (36), *P. vivax* merozoite surface protein 1 (PvMSP1; CTK-Biotech), and *P. vivax* apical membrane antigen 1 (PvAMA1). ELISAs were used as described above except that sera were diluted at 1:8,000 for PfMSP1, 1:6,000 PvMSP1, and 1:1,000 for PvAMA1 and PfGLURP. A patient was regarded as exposed to a specific *Plasmodium* species if the serum reacted positively against at least one of the species-specific antigens. An ELISA endpoint titration assay was performed as described above, except that the DBL5ε recombinant protein was incubated with 100 μl of different pools of human sera at different dilutions (1:100, 1:200, 1:400, 1:500, 1:600, 1:1,000, 1:2,000, 1:4,000, 1:5,000, 1:10,000, 1:20,000, 1:40,000, 1:50,000, and 1:100,000). The antibody titer for each pool was determined based on the highest dilution at which the OD is 2 SD above the mean from the Canadian control at a dilution of 1/100 (0.793).

Competitive ELISA. Rabbits were genetically vaccinated with *var2csa* DNA. Antiserum against the full VAR2CSA protein was collected 75 days after the first immunization (day 75) as described in a previous study (23). Microtiter plates were coated with recombinant DBL5ε at a concentration of 0.5 μg/ml. Plates were blocked with 1× phosphate-buffered saline (PBS), 0.5 M NaCl, 1% Triton X-100, and 1% bovine serum albumin (BSA) for 4 h at room temperature (RT). Increasing dilutions (1:100, 1:1,000, 1:10,000, and 1:100,000) of the competing sera were added and incubated overnight at 4°C. The pool of sera from nonexposed Colombians served as the negative control. After samples were washed four times with PBS-Tween 20 at 0.1%, a fixed dilution (1:400) of noncompeting serum was added and incubated for 1 h at RT. A specific secondary antibody conjugated to horseradish peroxidase (either a goat anti-human [A0170, Sigma-Aldrich] or goat anti-rabbit [656120, Sigma-Aldrich]) directed against the noncompeting antibody diluted at 1:6,000 was added and incubated for 1 h at RT. After four washes, antibody reactivity of the

TABLE 1 General characteristics of the study population in Colombia

Category and pathogen	Sample size (no.)	Age (yr)[a]	No. with acute infection[b]	Parasite count/μl (range)[c]
Pregnant women	94[d]	21 ± 6 (13–38)		
P. vivax			38	3,513 (77–28,028)
P. falciparum			14	1,383 (160–30,109)
Men	57	33 ± 12 (19–70)		
P. vivax			28	4,732 (604–29,126)
P. falciparum			29	2,744 (554–82,880)
Boys	37	11 ± 2 (6–14)		
P. vivax			26	4,092 (118–14,700)
P. falciparum			11	2,616 (836–10,827)
Girls	20	9 ± 2 (5–14)		
P. vivax			10	3,608 (881–15,800)
P. falciparum			10	4,347 (769–19,670)

[a] Mean ± SD (range).
[b] Based on qPCR diagnosis.
[c] The geometric mean parasite density of patients with microscopic infections.
[d] Of these, 42 were not infected.

TABLE 2 Characteristics of the pregnant population

Category (*n*)	Infection profile (no. of women)			
	Not infected	Acute infection[a]	*P. vivax* infection	*P. falciparum* infection
Primigravid (15)	12	3	2	1
Multigravid (40)	28	12	6	6
Other (39)[b]	2	37	30	7

[a] Based on qPCR diagnosis.
[b] Gravidity not known.

noncompeting plasma/serum was visualized at 450 nm following the addition of tetramethylbenzidine (TMB; Sigma-Aldrich). The percent reduction in antibody reactivity in the presence of a competitor was calculated as follows: 100 × (OD with competitor antibody/OD without competitor antibody) (23).

Avidity assays. The avidity of anti-DBL5ε antibodies was assessed with a urea elution-based ELISA. Microtiter plates were coated with recombinant protein as described above. Sera (1:1,000) were incubated in quadruplicate wells and incubated overnight at 4°C and then washed three times with PBS-Tween 20 at 0.1%. Duplicate wells were incubated for 15 min with either urea (8 M) or 1× PBS. The plates were washed with PBS-Tween 20 at 0.1%. Incubation with the secondary antibody and developing enzyme reactions were performed as described above for ELISAs. The avidity index (AI) was calculated as the ratio of the OD value of urea-treated samples to that of the untreated samples, multiplied by 100. All AI values less than 30% were considered low-avidity antibodies, values between 30% and 50% were considered intermediate-avidity antibodies, and values greater than 50% were considered high-avidity antibodies (37).

IgG preparation. Total IgG was purified from human sera on a HiTrap protein G high pressure (HP) column according to the manufacturer's recommendations (GE Healthcare). In brief, 500 μl of sera was diluted in 20 mM sodium phosphate buffer (pH 7.4), applied to a preequilibrated column (17-0404-03; GE Healthcare), and incubated for 1 h at room temperature. After coupling, unbound proteins were washed through the column with 10 volumes of 20 mM sodium phosphate buffer (pH 7.4). Bound IgG was eluted with 0.1 M glycine-HCl (pH 2.7) and neutralized with 1 M Tris-HCl (pH 9). Eluted fractions were dialyzed against 1× PBS and concentrated using Amicon centrifugal filter devices (10,000 kDa; Millipore). Purified IgG was used for Western blot analysis and parasite adherence inhibition assays.

Inhibition of IE binding to CSPG by specific IgG. The static assay employed to evaluate the capacity of the antibodies to interfere with CSA-specific adhesion of IEs is described in detail elsewhere (38). *P. falciparum* parasite strains FCR3 and HB3 were repeatedly panned on the human choriocarcinoma cell line BeWo (FCR3-BeWo and HB3-BeWo, respectively), as described previously (39). In this assay, a petri dish (351029; Becton, Dickinson) was coated overnight at 4°C with 20 μl of ligand, 1% BSA, and 5 μg/ml chondroitin sulfate proteoglycan (CSPG) (decorine; Sigma) diluted in PBS. Each spot was subsequently blocked with 3% BSA in PBS for 30 min at RT. Enriched late-stage-infected IEs were also blocked in 3% BSA-RPMI medium for 30 min at RT. Parasite suspensions adjusted to 20% parasite density were incubated with serum (1:5 dilution), purified IgG (250 μg/ml final concentration), or 500 μg/ml soluble CSA for 30 min at RT before they were added to the ligand and incubated for 15 min at RT for binding. Nonadherent cells were removed by an automated washing system. Spots were fixed with 1.5% glutaraldehyde in PBS and stained with Giemsa. Adherent IEs were quantified by microscopy as the number of IEs bound per millimeter squared, estimated from 20 high-power fields (40).

Western blot assay. Recombinant VAR2CSA DBL5ε protein (2 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto 0.2-mm-pore-size Protan BA 83 nitrocellulose sheets (Invitrogen) for immunodetection. The membrane was blocked for 1 h with 5% nonfat dry milk in 1× PBS with Tween 20 at 0.1% and then incubated separately with a 1:2,000 dilution of purified IgG from Beninese multigravid women and a 1:1,000 dilution of purified IgG from Canadian controls or unexposed Colombian controls or from Colombian men, children, or pregnant women. After three washes, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (1:15,000 dilution) (A0170; Sigma-Aldrich). Bound antibody was detected with enhanced chemiluminescence (ECL) substrate (GE Healthcare Life Sciences).

Statistical analyses. Each sample was tested in duplicate and run in two independent experiments. Comparison of anti-VAR2CSA antibody levels between groups was performed using nonparametric Mann-Whitney/Wilcoxon and Kruskal-Wallis tests. A chi-square test was used to examine differences between categorical variables. Comparison of serological recognition of specific antigens by sera from different groups of patients was done similarly. Data were plotted using Prism software (version 6; GraphPad). EPI Info software (version 3.5.3) and Prism software (version 6; GraphPad) were used for statistical analyses. P values of <0.05 were considered to be statistically significant.

RESULTS

Characteristics of participants. The general characteristics of the different study populations are shown in Table 1. Fifty-seven men and 57 children from Colombia, all with acute malaria infection, were tested in the present study. Ninety-four pregnant women from Colombia were included in this study: 42 were not infected, 38 were positive for *P. vivax*, and 14 were positive for *P. falciparum* (Table 1). Among the 94 women, 15 were primigravid, and 40 were multigravid. The parity of the other 39 women was unknown. The detailed characteristics of the pregnant women are presented in Table 2.

Children, men, and pregnant women from Colombia have antibodies that recognize multiple domains of *P. falciparum* VAR2CSA. Based on studies primarily in Africa, it is well established that antibody levels against VAR2CSA increase with parity and correlate with acquired immunity to placental parasites. We first compared the levels of antibodies to the DBL5ε domain of VAR2CSA in pregnant women of different parities from Benin and Colombia (Fig. 1A). As expected, Beninese multigravid women had higher antibody levels against DBL5ε than primigravid women ($P < 0.0001$). In Colombia, the mean antibody

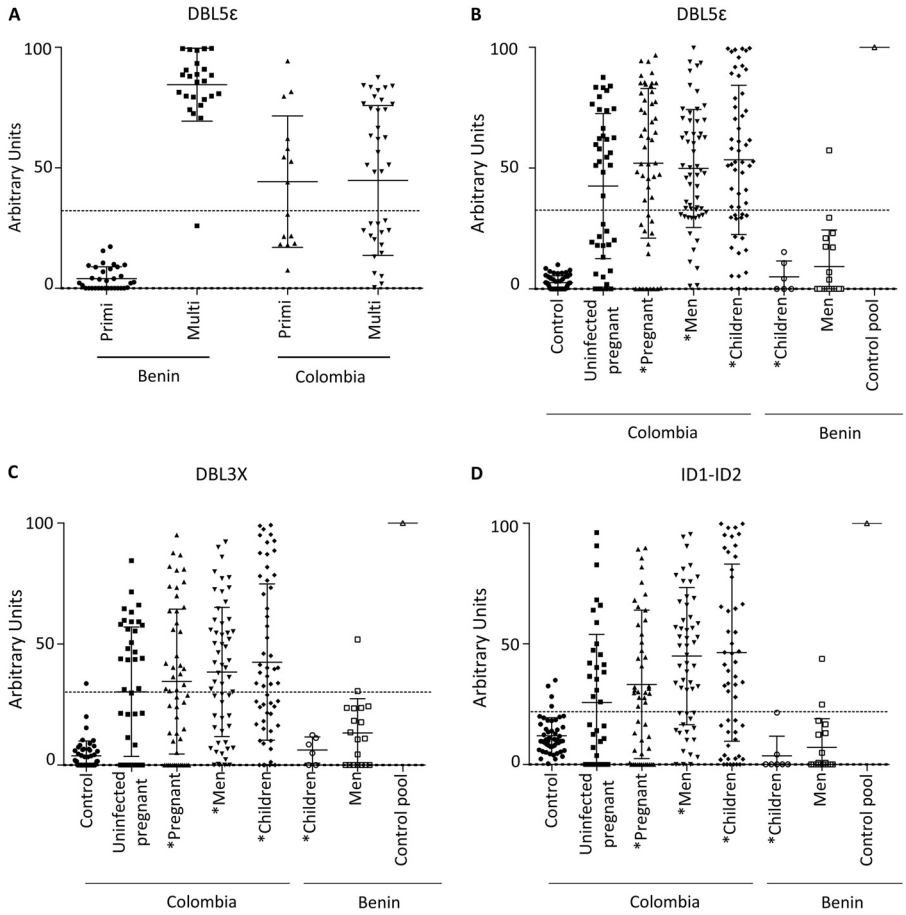

FIG 1 Sera from Colombian pregnant women, men, and children recognize several domains of VAR2CSA. DBL5ε antibody levels were quantified in primigravid (Primi) and multigravid (Multi) women from Benin and Colombia (A). Sera with specificity for DBL5ε (B), DBL3X (C), and ID1-ID2 (D) domains of VAR2CSA were measured in unexposed Colombians (Control), pregnant women with and without acute infection, men and children from Colombia, and men and children from Benin. A pool of sera from multigravid women from Benin (Control pool) served as a positive control. Sera were diluted 1:1,000. Data are expressed as arbitrary units. Bars indicate the means ± standard deviations. The experiment was performed twice with similar results. The cutoff level for classification as positive is represented by the horizontal stippled line. *, patients with acute malaria infection.

level was lower than in pregnant women from Benin, and there was no difference between primigravid and multigravid women ($P = 0.2272$). Moreover, the levels of anti-DBL5ε antibodies in Colombian pregnant women with or without a malaria infection were similar ($P = 0.1278$) (Fig. 1B).

Unexpectedly, men and children also had antibodies against the DBL5ε domain at similar levels ($P = 0.3455$) and frequencies (66%) to pregnant women (Fig. 1B). This is contrary to reports that antibodies to VAR2CSA are rarely observed in nonpregnant populations. Furthermore, men and children had antibodies that recognized two other domains of VAR2CSA, DBL3X and ID1-ID2, also with high frequencies (men, 58% DBL3X and 70% ID1-ID2; children, 56% DBL3X and 65% ID1-ID2) (Fig. 1C and D). The VAR2CSA antibody levels detected in the Colombian nonpregnant populations were higher than those quantified in Beninese nonpregnant populations (DBL5ε, $P < 0.0001$; DBL3X, $P < 0.0001$; ID1-ID2, $P < 0.0001$).

This antigen recognition is specific to malaria exposure since adults residing in the malaria-free city of Medellín did not have antibodies against these antigens, nor did the Canadian control group. These results were further confirmed by Western blot analysis that showed that total IgG purified from a pool of sera from either Colombian children or men recognized the purified, recombinant DBL5ε (Fig. 2A). The same product (37 kDa) was recognized by specific IgGs purified from Colombian pregnant women, as well as Beninese multigravid women. No band of the expected size was observed with purified IgG from either unexposed Canadians or Colombians.

Despite the pivotal involvement of VAR2CSA in PAM, men and children had antibodies against the VAR2CSA domains with comparable levels to those observed in pregnant women. All three populations had similar levels of anti-DBL5ε ($P = 0.6206$) and anti-DBL3X antibodies ($P = 0.1218$) (Fig. 1B and C). Interestingly, men and children had higher ID1-ID2 antibody levels than pregnant women ($P = 0.0007$) (Fig. 1D). We further compared VAR2CSA antibody levels in Colombian patients with those in Beninese multigravid women. Sera from all samples with positive reactivity against DBL5ε were pooled and serially diluted (Fig. 2B). A pool of sera from Canadian controls was used to define the endpoint titer for each patient group. Endpoint titers were similar for Colombian pregnant women and men (dilution of 1/1,000) while the endpoint titer of the sera from Colombian children was

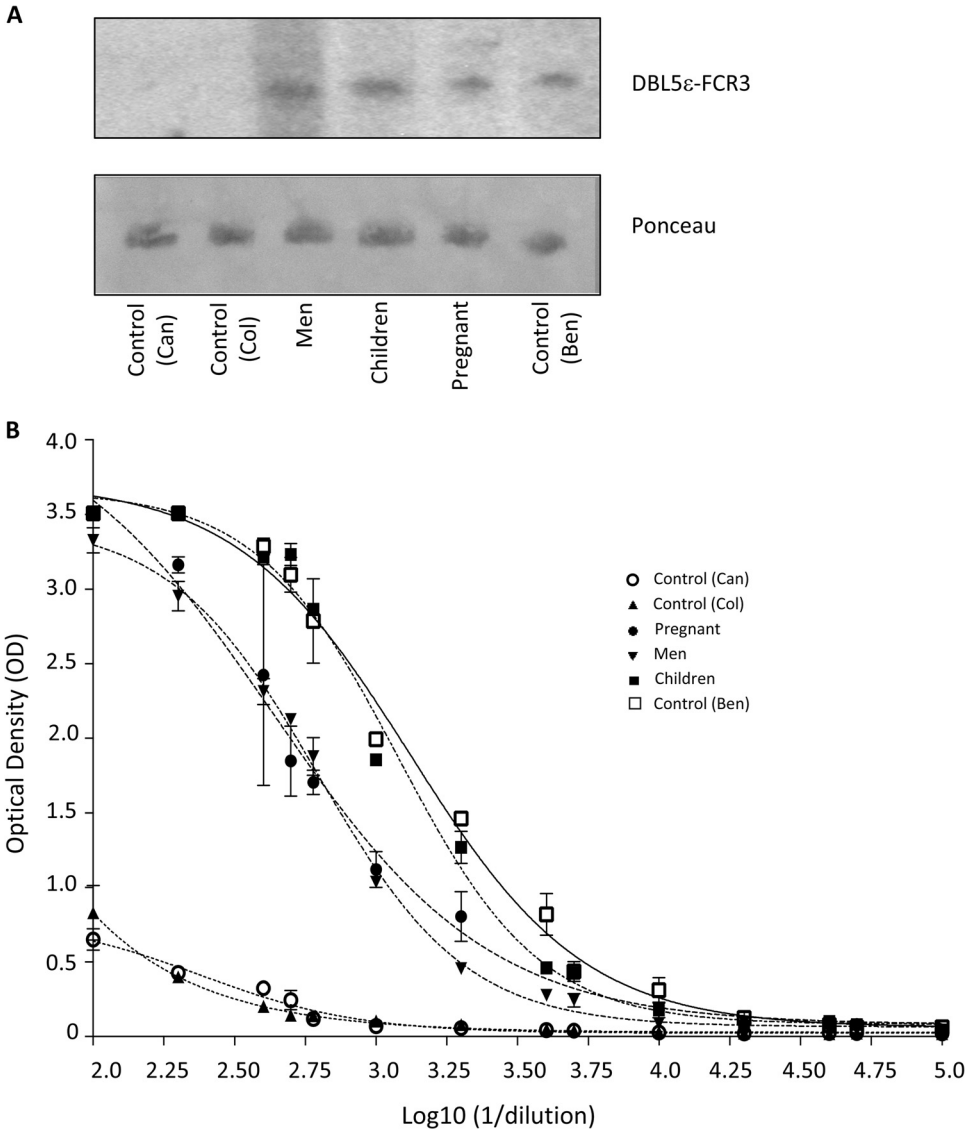

FIG 2 VAR2CSA antibody levels in Colombian populations. (A) SDS-PAGE with DBL5ε (2 μg) immunoblotted with purified IgG from pregnant women, men, and children from Colombia. IgG from Beninese multigravid women (Control Ben) served as a positive control, and unexposed adults from either Canada (Can) or Colombia (Col) served as negative controls. (B) Sera from Colombian pregnant women, men, and children with DBL5ε reactivity by ELISA were pooled and serially diluted. Endpoint titers were determined based on a 1/100 dilution of sera from unexposed Canadians. Assays were performed in duplicate, and values are means ± standard deviations. Experiments were performed twice with similar results.

lower (1/2,000). The titer from the Beninese multigravid women was 1/4,000.

Anti-VAR2CSA antibodies in patients with acute or prior exposure to *P. falciparum* and *P. vivax*. All of the men and children in our study had an acute malaria infection. A possible explanation for the high anti-VAR2CSA antibodies is that *P. falciparum* strains in Colombia indiscriminately express *var2csa* outside pregnancy. We therefore asked whether reactivity to the VAR2CSA domains correlated specifically with *P. falciparum* acute infection. While 76% of children, 79% of men, and 57% of pregnant women infected with *P. falciparum* had antibodies against VAR2CSA, a similar proportion had an acute infection with *P. vivax* (Table 3). Comparable results for the two species were observed for all three VAR2CSA domains ($P > 0.05$).

Alternatively, these antibodies may have been acquired during a prior exposure to *P. falciparum*. Exposure to *P. falciparum* or *P. vivax* was determined based on the levels of antibody to several surface antigens that are specific for each species (PfMSP1, PfGLURP, PvMSP1, and PvAMA1). More than 50% of pregnant women and 70% of men and children exposed to only *P. falciparum* reacted against DBL5ε (Table 4). Unexpectedly, the same reactivity was observed in patients with sera reactive only to antigens from *P. vivax*.

High-avidity antibodies against VAR2CSA among sera from Colombian men, children, and pregnant women. Although men and children exhibited high levels of antibodies to DBL5ε, the avidity of the antibodies for the antigen is an important indication of their specificity. The avidity of IgG antibody to DBL5ε was examined in positive sera ($n = 61$ for pregnant women, $n = 38$ for men, and $n = 40$ for children) using 8 M urea as a dissociation

TABLE 3 Relationship between Colombian samples that positively reacted against domains of VAR2CSA and acute infection

Category and pathogen (*n*)[a]	No. with acute infection	% of samples reactive to the indicated VAR2CSA domain		
		DBL5ε	DBL3X	ID1-ID2
Pregnant women (94)				
P. vivax	38	76	55	57
P. falciparum	14	57	36	50
Not infected	42	57	50	43
Men (57)				
P. vivax	28	53	50	61
P. falciparum	29	79	65	79
Children (57)				
P. vivax	36	67	58	64
P. falciparum	21	76	52	66

[a] *n*, number in group.

TABLE 5 Frequency of high-, intermediate-, and low-avidity antibodies to DBL5ε in Colombian patients with acute malaria infection

Patient group (*n*)	Antibody avidity (% of samples)[a]		
	HAI	IAI	LAI
Pregnant women (61)	80	20	0
Men (38)	74	13	13
Children (40)	75	17.5	7.5

[a] High avidity index (HAI), >50% of control; intermediate avidity index (IAI), 30 to 50% of control; low avidity index (LAI), <30% of control.

agent. High-avidity IgG was observed in 80% of pregnant women, 74% of men, and 75% of children. Twenty percent of pregnant women had intermediate-avidity antibodies compared to 13% of men and 18% of children (Table 5). Antibodies with low avidity were detected in 13% and 8% of men and children, respectively, but not in pregnant women. No significant correlation was observed between antibody avidity and acute infection (data not shown).

Antibodies from pregnant women, men, and children from Colombia as well as from Beninese multigravid women share common VAR2CSA epitopes. The ELISA and Western blot assays demonstrated that nonpregnant and pregnant subjects from Colombia had antibodies against recombinant domains of VAR2CSA. We further tested the specificity of these antibodies for epitopes within the full-length VAR2CSA protein using a competitive ELISA approach. The DBL5ε domain of VAR2CSA was used in a competitive ELISA to analyze the target epitopes of naturally acquired antibodies from patient sera and rabbit antiserum against the full VAR2CSA protein. Men and children as well as pregnant women from Colombia presented the same inhibition patterns in binding to the recombinant DBL5ε protein in the presence of increasing concentrations of the rabbit antiserum (Fig. 3A). Similar inhibition was observed when rabbit anti-VAR2CSA antiserum competed with antibodies from exposed Colombian subjects and multigravid women from Benin (Fig. 3B). No competition for binding was observed with the sera from unexposed Colombians.

IgG from Colombian populations inhibits adhesion of infected *P. falciparum* erythrocytes to CSA. One of the hallmarks of VAR2CSA antibodies in pregnancy is their ability to inhibit parasite adhesion to CSA. Using a static *in vitro* assay, we tested whether antibodies from the Colombian populations can inhibit parasite adhesion to CSA. As observed in studies of pregnant women from Africa, most sera from Colombian pregnant women (80%) inhibited adhesion to *P. falciparum* laboratory strains FCR3 repeatedly panned on BeWo cells (Fig. 4A). Surprisingly, a high proportion of sera from men (65%) and children (90%) also blocked adhesion to FCR3-BeWo (Fig. 4A). No inhibition was observed with the pool of sera from Canadians. As expected, the pool of sera from Beninese multigravid women demonstrated a strong inhibitory effect on both parasites strains.

Several studies demonstrated that nonspecific IgM antibodies can inhibit parasite adhesion (41, 42). To exclude this possibility, total IgG was purified from pools of sera from 6 men, 9 children, and 10 pregnant women. Samples with antiadhesion activity on CSA-binding parasites (Fig. 4A) were selected for each pool. Purified antibodies from all three groups demonstrated antiadhesion activity. This activity was observed on both FCR3-BeWo and HB3-BeWo parasite lines (Fig. 4B). This result confirms that the inhibition mechanism is mediated by naturally acquired IgG.

DISCUSSION

One of the key findings in this study is that pregnant women, men, and children from Colombia have high frequencies and levels of antibodies against *P. falciparum* VAR2CSA. Moreover, these antibodies can inhibit parasite adhesion to CSA *in vitro*. It was reported in previous studies that antibodies against VAR2CSA are not exclusively restricted to pregnancy (24, 25). However, the level and prevalence of the antibodies observed in those studies were lower in men and children than in exposed multigravid women (24). From proteomic studies, VAR2CSA was detected in parasites of nonplacental origin (43), and in another study, parasites from nonpregnant hosts could bind to CSA (24, 44). Consistent with our study, widespread DBL5ε reactivity was observed in plasma from Tanzanian children, suggesting that VAR2CSA in nonpregnant populations could share specific epitopes with VAR2CSA from placental parasites (25). The high proportion of anti-VAR2CSA antibodies detected in our Colombian populations in-

TABLE 4 Antibody reactivity to DBL5ε and malaria exposure in Colombian pregnant and nonpregnant populations

Pathogen exposure (surface antigen)	Pregnant women (*n* = 94)		Men (*n* = 57)		Children (*n* = 57)	
	No. infected	% DBL5ε^{+}[a]	No. infected	% DBL5ε^{+}	No. infected	% DBL5ε^{+}
P. vivax (PvMSP1 or PvAMA1)	59	69	25	56	30	70
P. falciparum (PfMSP1 or PfGLURP)	2	50	7	71	10	70
Both species	18	78	23	78	12	75
Not exposed	15	33	2	50	5	60

[a] Percentage indicates the proportion of samples that reacted against DBL5ε (DBL5ε^{+}) related to exposure to *P. vivax* and *P. falciparum*.

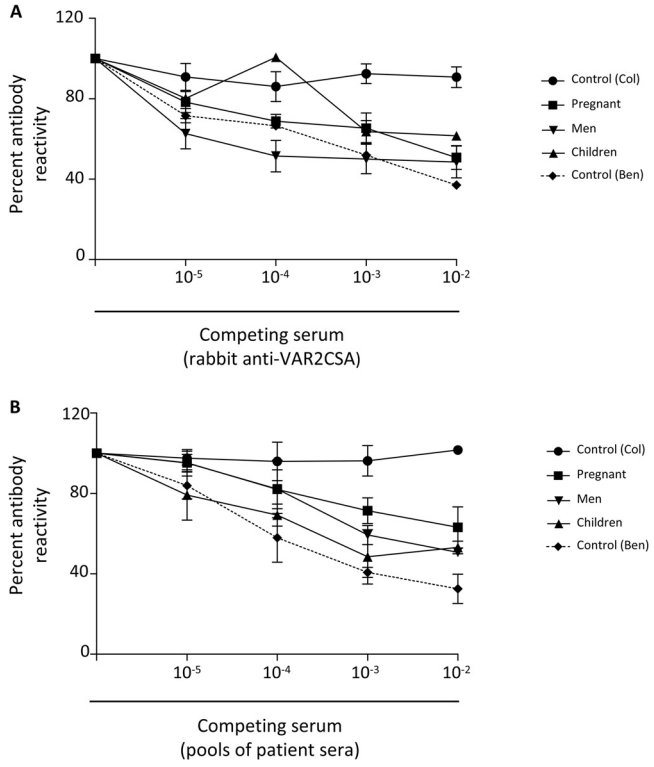

FIG 3 Competitive recognition of recombinant DBL5ε-VAR2CSA between rabbit antibodies against VAR2CSA and naturally acquired antibodies from Colombian and Beninese subjects. (A) Pools of sera (1:400) from Colombian unexposed controls, pregnant women, children, and men and from Beninese multigravid women were competed with increasing concentrations of day 75 VAR2CSA antiserum from a vaccinated rabbit (competing serum). (B) Day 75 VAR2CSA antiserum from a vaccinated rabbit (1:80,000) was competed with increasing concentrations of pooled sera from Colombian unexposed controls, pregnant women, children, and men and from Beninese multigravid women (competing sera). The error bars show the standard deviations of two independent wells. Experiments were performed twice with the same results. Antibody reactivity is expressed relative to the OD in the absence of competing serum as described in Materials and Methods.

dicates that low malaria transmission in this area is sufficient to induce high levels of VAR2CSA antibodies outside and during pregnancy.

The high frequency of VAR2CSA antibodies in Colombian populations suggests that exposure to VAR2CSA or to parasites expressing VAR2CSA-like antigens may be common in the general population. A plausible hypothesis is that exposure to these VAR2CSA-like antigens in Colombia may arise from the cocirculation of *P. falciparum* and *P. vivax* (26). In our study region, patients exposed to either *P. falciparum*, *P. vivax*, or both species had VAR2CSA antibodies. Other DBL-containing proteins from *P. falciparum* (other PfEMP1s) or from *P. vivax* may generate cross-reactive antibodies. Ours is the first study to suggest that epitopes from *P. vivax* antigens may cross-react with VAR2CSA from *P. falciparum*. Study of VAR2CSA expression and function in other countries where *P. vivax* or both *Plasmodium* species circulate will further characterize the *var2csa* gene and protein function in regions outside Africa.

It is also possible that the *var2csa*-expressing strains in Colombia are genetically or phenotypically different from parasites encountered in Africa. A study of the evolution of *P. falciparum* strains from their African origins to their introduction in South America revealed highly differentiated parasite populations in northwestern Colombia (45). These parasites may be characterized by sequence variation within the *var2csa* gene or have altered mechanisms of VAR2CSA protein expression, regulation, and immunogenicity resulting in expression of *var2csa* outside pregnancy. DNA sequence and expression analysis of *var2csa* in parasites isolated from this region may reveal important differences in genetic control at this locus compared with African isolates. Other contributing factors may relate to the geography of our study region. Many infectious diseases are prevalent in this population, and other pathogens could potentially express antigens that induce cross-reactive antibodies to VAR2CSA. Consistent with this hypothesis, we identified a few patients exposed neither to *P. falciparum* nor *P. vivax* who nevertheless had antibodies to DBL5ε. Alternatively, the genetics of the host population, which is largely of indigenous descent, could also play a role in modulating the immune response to malaria infection.

Our data suggest that populations from Colombia are exposed

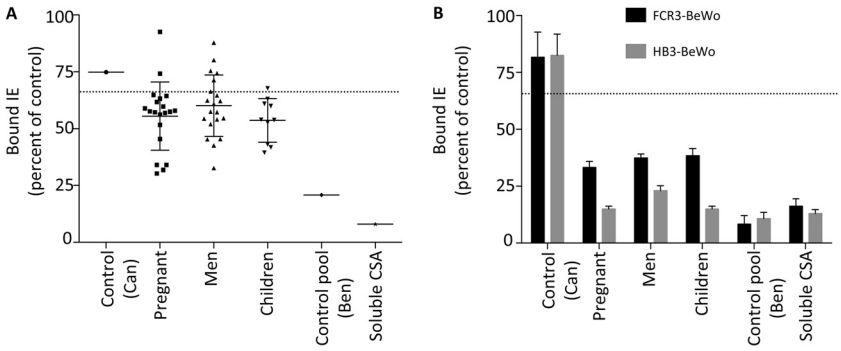

FIG 4 Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes to CSA by sera from pregnant women, men, and children from Colombia. (A) Individual serum samples from pregnant women ($n = 20$), men ($n = 20$), and children ($n = 10$) from Colombia with positive reactivity to DBL5ε inhibited binding to CSA of FCR3-BeWo-infected erythrocytes (IE) expressing VAR2CSA. A pool of Canadian nonimmune sera served as a negative control (Control Can). A pool of sera from Beninese multigravid women served as a positive control (Control Ben). Soluble CSA was used as the competitor. (B) Purified IgGs from Colombian pregnant women, children, and men specifically inhibited binding of FCR3-BeWo and HB3-BeWo IEs to CSA. Purified IgGs from unexposed Canadians (Control Can), Beninese multigravid women (Control Ben), and soluble CSA served as controls. Each point represents the mean of duplicate wells ± standard deviations. Each experiment was performed twice with similar results.

to parasite variants, possibly expressing particular *P. falciparum* proteins, such as PfEMP1 alleles or *P. vivax* proteins that share similar or cross-reactive epitopes with VAR2CSA. An important consideration is whether these antibodies are protective. The current thinking is that antibodies against VAR2CSA are acquired in a parity-dependent manner, and high levels of antibodies are associated with improved birth outcomes (46). We were unable to observe parity-dependent effects on anti-VAR2CSA IgG levels in our pregnant population given the small sample size of pregnant women with known parity. Instead, we observed that pregnant women, men, and children all had high levels of antibodies. These data suggest that high levels of antibodies against VAR2CSA do not lead to subsequent protection against PAM. Moreover, we did not observe an association between the levels of VAR2CSA antibodies and parasite densities (data not shown). Infections in Colombia are generally characterized by low parasitemia, but whether VAR2CSA antibodies contribute to this remains to be explored. Even if these antibodies are not fully protective, they may curb parasite density and limit anemia, which may explain the benign clinical outcomes of malaria observed previously in this region (28). This hypothesis is consistent with a recent study in Papua New Guinea in which high levels of malaria-specific IgG did not prevent infection but controlled acute parasitemia (47).

Nearly all of the studies on VAR2CSA have focused on sub-Saharan Africa, which bears the largest burden of pregnancy-associated malaria (11, 17, 21, 23). This study provides unique insight into acquired antibodies to VAR2CSA in pregnant and nonpregnant populations in a Latin American setting. These data prompt further studies on *var2csa* gene expression and function in areas of malaria endemicity outside Africa and suggest additional roles for this antigen in malaria immunity beyond pregnancy.

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