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Multilaboratory Approach to Preclinical Evaluation of Vaccine Immunogens for Placental Malaria

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Pregnancy malaria is caused by *Plasmodium falciparum*-infected erythrocytes that adhere to the placental receptor chondroitin sulfate A (CSA) and sequester in the placenta; women become resistant to pregnancy malaria as they acquire antiadhesion antibodies that target surface proteins of placental parasites. VAR2CSA, a member of the *P. falciparum* EMP1 variant surface antigen family, is the leading candidate for a pregnancy malaria vaccine. Because VAR2CSA is a high-molecular-weight protein, a vaccine based on the full-length protein may not be feasible. An alternative approach has been to develop a vaccine targeting individual Duffy binding-like (DBL) domains. In this study, a consortium of laboratories under the Pregnancy Malaria Initiative compared the functional activity of antiadhesion antibodies elicited by different VAR2CSA domains and variants produced in prokaryotic and eukaryotic expression systems. Antisera were initially tested against laboratory lines of maternal parasites, and the most promising reagents were evaluated in the field against fresh placental parasite samples. Recombinant proteins expressed in *Escherichia coli* elicited antibody levels similar to those expressed in eukaryotic systems, as did the two allelic forms of the DBL4 and DBL5 domains. The procedures developed for this head-to-head comparison will be useful for future evaluation and down-selection of malaria vaccine immunogens.

Malaria in pregnant mothers is a major public health problem. Women in areas where malaria is endemic acquire resistance to malaria after years of exposure, but their susceptibility increases significantly during pregnancy, particularly during the first pregnancy. In high-transmission areas, pregnancy malaria (PM) due to *Plasmodium falciparum* is estimated to cause 40% of the cases of severe anemia in first-time mothers (1). The greatest impact is on newborns who are born with a low birth weight, and this effect of pregnancy malaria is estimated to cause 62,000 to 363,000 infant deaths in Africa each year (2, 3).

The hallmark of pregnancy malaria is sequestration of parasites in the placenta. Placental isolates of *P. falciparum* uniformly bind to chondroitin sulfate A (CSA), expressed on the surface of syncytiotrophoblasts (4). Over successive pregnancies, women develop antibodies that inhibit parasite adhesion to CSA (5, 6). Immune women have a reduced risk of infection and improved control of parasitemia during infection, resulting in increased birth weight and reduced maternal anemia risk (7, 8). Naturally acquired antibodies that inhibit parasite adhesion to CSA are broadly reactive: sera donated by mothers in Asia and Africa cross-react with placental parasites collected on either continent, indicating that the antigen(s) or epitope(s) targeted by these protective antibodies is conserved.

VAR2CSA is a member of the *P. falciparum* EMP1 family preferentially expressed by placental parasites and laboratory isolates selected for adhesion to CSA (9, 10). Several VAR2CSA domains have been shown to bind to CSA in *in vitro* binding assays (11–13). However, *in vitro* binding assays are complicated, especially when the binding interaction involves a highly charged molecule such as CSA (14, 15).

VAR2CSA specifically appears on the surface of CSA-binding infected erythrocytes (IEs) (16–20), and levels of antibody to VAR2CSA domains increase over successive pregnancies (21–24), as women become resistant to pregnancy malaria. These properties have positioned VAR2CSA as the leading candidate for a PM vaccine.

However, the protein encoded by *var2csa* has a high molecular mass (300 kDa) consisting of 6 Duffy binding-like (DBL) domains, making manufacture of the full-length protein as a vaccine impractical. Therefore, a goal of pregnancy malaria vaccine development has been to identify the best domain or domain combination as an alternative to the full-length protein to manufacture as an immunogen. To achieve this goal, a consortium of laboratories formed the Pregnancy Malaria Initiative (PMI) to assess multiple VAR2CSA domains expressed in a variety of expression platforms, including prokaryotic and eukaryotic systems, as vaccine candidates. Leading candidates produced by each PMI lab

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TABLE 1 Recombinant VAR2CSA domains produced by the PMI consortium laboratories

Expression system	Recombinant proteins	Laboratory[a]
P. pastoris	DBL4-7G8, DBL5-7G8	Joseph Smith, Seattle Biomed; David Narum, LMIV NIH
E. coli::pET28a	DBL4-7G8, DBL5-7G8, DBL4-FCR3, DBL5-FCR3	Artur Scherf, Institut Pasteur
E. coli::pET28b	DBL4-7G8, DBL5-7G8, DBL4-FCR3, DBL5-FCR3	Patrick Duffy, Andrew Oleinikov, Seattle Biomed
Baculovirus	DBL4-7G8, DBL5-7G8, DBL4-FCR3, DBL5-FCR3	Thor Theander, Ali Salanti, CMP Copenhagen

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were compared head-to-head at central facilities for immunizations and assays that determined the levels of functional (antiadhesion) antibodies that target these antigens.

MATERIALS AND METHODS

Parasite samples. Maternal parasites adapted to *in vitro* culture were originally collected from pregnant women who were enrolled between September 2002 and October 2005 in a longitudinal cohort conducted by the Mother-Offspring Malaria Studies (MOMS) Project in Muheza District, Tanzania. Binding-inhibition assays were also performed on fresh parasite samples collected from pregnant women who were enrolled in 2011 and 2012 in a longitudinal cohort conducted in Ouelessebougou, Mali. Pregnant women age 16 years or older without clinical evidence of chronic or debilitating illness were asked to participate in the study and gave signed informed consent after receiving a study explanation form and oral explanation from a nurse in their native language. Ethical clearance for the study in Muheza, Tanzania, was obtained from the institutional review boards of the Seattle Biomedical Research Institute and the National Institute for Medical Research in Tanzania. Ethical clearance for the study in Ouelessebougou, Mali, was obtained from the National Institute of Allergy and Infectious Diseases (NIAID) at the U.S. National Institutes of Health (NIH) and the Faculty of Medicine, Pharmacy and Dentistry (FMPOS), University of Bamako.

Recombinant protein expression. DBL domains were cloned and expressed in *Pichia pastoris*, *Escherichia coli* (using two vectors, pET28a and pET28b), and baculovirus as previously described (25–28). In parallel, each laboratory expressed a control protein using the same procedure applied for the test immunogens (Fig. S1 in the supplemental material). The recombinant proteins expressed by the consortium laboratories are listed in Table 1. The recombinant proteins were assessed for integrity and purity by reverse-phase high-pressure liquid chromatography (HPLC), mass spectrometry, and SDS-PAGE. Briefly, for reverse-phase HPLC, samples were analyzed on a C_4 column (2 mm by 250 mm; particle size, 5 μm; Phenomenex). The column was equilibrated in 95% mobile phase A (0.1% [wt/vol] trifluoroacetic acid [TFA] in water) and 5% mobile phase B (0.1% [wt/vol] TFA in acetonitrile), and elution was performed by increasing mobile phase B from 20% to 70% over 50 min. Mass spectrometry (MS) was performed on the major peaks eluted from the reverse-phase HPLC. Samples were mixed in a 1:1 ratio with a saturated solution of 2,5-dihydroxybenzoic acid in 0.1% TFA–H_2O–50% acetonitrile, and 1.5 μl was spotted on a ground-steel target plate. The plate was loaded onto an UltrafleXtreme matrix-assisted laser desorption ionization–time of flight/time of flight mass spectrometer (Bruker Daltonics) equipped with a proprietary Smartbeam-II laser with a 1,000-Hz repetition rate. For SDS-PAGE, samples were separated under nonreducing and reducing conditions on 4 to 12% bis-Tris gels. The total amount of protein loaded per well was 4.2 μg for both conditions, and the reducing agent used was 5% dithiothreitol (DTT).

Animal immunization. To standardize the animal immunization studies, equal amounts of proteins from all contributing laboratories were sent to an outside animal facility, Antibodies Inc., and 5 rats were immunized with each of the test and control immunogens. Each dose included 30 μg recombinant protein. Initial immunization was performed using Freund's adjuvant, followed by 3 boost immunizations with incomplete Freund's adjuvant 3 weeks apart. The final bleed was performed 2 weeks after the last immunization. Antibody titer determinations, standardized comparative binding-inhibition assays, and flow cytometry assays were performed at the central assay laboratory in Seattle, WA. The protocol for animal immunization was approved by the Institutional Animal Care and Use Committee of the Seattle Biomedical Research Institute.

IgG purification. IgG was purified from rat sera using GammaBind Plus Sepharose (GE Healthcare) according to the manufacturer's instructions, followed by dialysis over 24 h with 3 buffer changes.

ELISA. Antibody titers in sera collected from immunized rats were measured by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates (Immulon 2HB) were coated with 1 μg/ml recombinant protein overnight at 4°C. After blocking the plates, serially dilutions of plasma from 1/1,000 to 1/125,000 were added and the plates were incubated for 2 h at room temperature. After the plates were washed, horseradish peroxidase (HRP)-conjugated goat anti-rat antibody (Sigma-Aldrich, St. Louis, MO) was added at a dilution of 1/1,000 for 1 h at room temperature. After additional washes, the HRP substrate *o*-phenylenediamine dihydrochloride (OPD) was added and the absorbance was measured in a VersaMax microplate reader.

Blinding procedure. All rat sera were aliquoted and coded by a technician that was not involved in the assays. Each set of immunogens provided by the different labs was assigned a series number (100, 200, etc.), while control sera received corresponding labels (101, 201, etc.), and then individual samples were indicated by an animal number. Control IgG was compared to IgG purified from nonimmune human sera to confirm the absence of antiadhesion activity. Upon completion of the assays, a staff member that was not involved with performing the assays received all the results, unblinded the data, and distributed the results to all groups in the consortium simultaneously.

Parasite binding-inhibition assay. We optimized several assay parameters as part of the process of developing a standardized assay. As in the original technique (29), CSA from bovine trachea (Sigma-Aldrich, St. Louis, MO) was immobilized by adsorption on petri dishes in 20-μl spots to form numerous wells to support IE binding. The CSA wells were sited on a petri dish using a template, so that each well was equidistant from the outer edge of the dish. CSA was adsorbed on each site overnight at 4°C and under 100% humidity during all steps to prevent unequal evaporation effects. The wells were blocked with bovine serum albumin (BSA) to prevent nonspecific cell attachment to the plastic dish.

Parasites were grown to a minimum 4% parasitemia in 4% hematocrit. When IEs reached the trophozoite stage, they were harvested and enriched by floating on 0.5% porcine gelatin. The enriched parasites were suspended with uninfected red blood cells (RBCs) to achieve 20% parasitemia and hematocrit of 0.5% in medium containing BSA (Probumin) as a blocking reagent. Parasite suspensions were preincubated with test or control sera for 30 min at 37°C and then allowed to settle on individual CSA wells (blocked with BSA) for 15 min at room temperature in the humidity chamber. Binding-inhibition plates were washed by placing them on a rotating platform set to a uniform speed and delivering phosphate-buffered saline (PBS) wash solution through a peristaltic pump to control inflow and outflow (see Fig. 3 in reference 30). In particular, the peristaltic pump delivered PBS through the inlet tubing at a fixed rate without disturbing the settled layer of parasitized red cells, and upon reaching the level of the outlet tubing, PBS was removed from the plate at

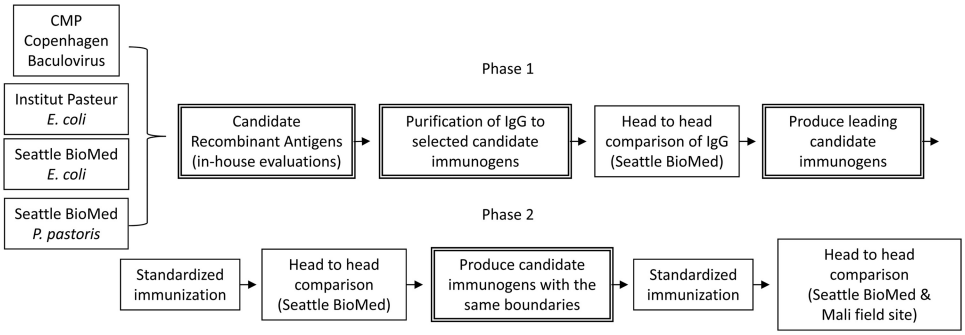

FIG 1 Consortium work flow. Down-selection of vaccine candidates was conducted through several head-to-head comparisons. During phase 1, individual centers assessed their candidates internally, and purified IgG antibodies from sera with the highest functional activity were subsequently assessed at the core assay center. In phase 2, selected recombinant antigens were sent to a common animal facility for immunization using similar amounts of proteins, similar adjuvants, and a similar immunization schedule. Subsequent evaluation included expression of the same domains (using the same alleles and boundaries) by the different consortium labs, followed by standardized immunization and head-to-head analyses of the immunogens' activity at core facilities. Activities conducted in each of the consortium laboratories are indicated in the boxes framed with double lines. CMP, Centre for Medical Parasitology, University of Copenhagen.

the same rate that it was added. Plate rotation continued for 2 min until the blood pellet of unbound infected and uninfected erythrocytes reached the center of the plate, the plates were removed from the platform, and all PBS was removed. The plates with adherent cells were fixed in 0.5% glutaraldehyde, stained with 10% Giemsa for 2 min, and then interpreted by microscopy using an automated counting system.

For assays performed in the central laboratory, the petri dish was immobilized on a stage that automatically moved in a fixed pattern, and images of 20 randomly selected fields were acquired and analyzed using customized software. Cell density was used to distinguish between IEs and uninfected RBCs. The numbers of infected red blood cells (IRBCs) and RBCs in each field were recorded; the number of RBCs reflected background cell binding. Average binding was calculated as the mean number of IRBCs obtained from the counting of 20 fields at a ×400 magnification. Binding-inhibition assays of fresh maternal isolates were performed as described above, except that the binding-inhibition plates were washed and cells were counted manually as previously described (29).

Flow cytometry. IRBCs were incubated with rat sera at a 1:10 dilution, followed by Alexa Fluor 488-labeled secondary anti-rat IgG (Invitrogen) at a 1:500 dilution. To distinguish between infected and uninfected cells, samples were stained with ethidium bromide at a 20-μg/ml concentration. The signal from labeled samples was quantified on an LSRII flow cytometer (BD Biosciences), and the data were analyzed with the FlowJo (Tree Star, Inc.) software program. The mean fluorescence intensity (MFI) was calculated as the (geometric mean of test IRBCs − geometric mean of test RBCs) − (geometric mean of control IRBCs − geometric mean of control RBCs).

RESULTS

Establishing systematic assessment of immunogens from different laboratories. Although *var2csa* is less polymorphic than other *P. falciparum* EMP1 genes, the basis for the selection of VAR2CSA and the boundaries of the VAR2CSA domain(s) that would elicit antisera with broad reactivity to the different placental isolates were unknown and therefore were initially empirical. At the time that the consortium was initiated, published studies had described variable results when antibodies raised against VAR2CSA domains were tested for surface recognition of CSA-binding parasites and functional antiadhesion activity. Numerous factors might influence the activity of antibodies raised against VAR2CSA domains, including allelic forms, domain boundaries, expression system, and the parasite isolates used in assays.

The PMI consortium was established to identify the VAR2CSA domains and variants that could be expressed in recombinant form as immunogens that elicit broadly reactive functional antibodies and to develop a standardized assay platform that could be used to evaluate immunogens prepared by different groups. Our approach included expression of VAR2CSA in a variety of expression platforms, including prokaryotic and eukaryotic systems. Antisera against these proteins raised in animals were assessed for functional activity in standardized binding-inhibition assays to down-select superior immunogens.

The process of down-selecting immunogens was carried out through several rounds of head-to-head comparisons of potential candidates (Fig. 1). In phase 1, participating laboratories assessed a series of antisera prepared in-house against their own immunogens, to identify those with the greatest functional activity against homologous and/or heterologous isolates. Based on the in-house determination of the best targets, individual laboratories provided the corresponding purified IgG or recombinant immunogens to the coordinating team at the central facility. IgG was used directly in assays, after standardization for purity and concentration, in a head-to-head assay comparison. Subsequently, to control for variability in immunization regimens, lead immunogens were submitted for simultaneous immunization of animals using standard procedures, including standard adjuvant formulations, immunogen quantity, and timing of immunization, in a combined immunization and assay head-to-head comparison. For phase 2, animal immunizations were simultaneously performed with all leading immunogens at a commercial facility, and antisera raised in this fashion were compared head-to-head in a standardized inhibition assay of maternal IEs binding to CSA at the central PMI laboratory. Based on results obtained in the initial head-to-head comparisons (see Fig. S2 and S3 in the supplemental material) and data collected by individual PMI laboratories, the consortium focused on the DBL4 and DBL5 domains of VAR2CSA as the most promising targets for developing immunogens.

Recombinant expression of candidate immunogens. In order to determine the superior or optimal expression platforms for producing VAR2CSA immunogens, the consortium aligned on the same domain boundaries for two allelic forms (FCR3 and 7G8) of both DBL4 and DBL5 (Table 2). DBL4-FCR3 is 90% identical to DBL4-7G8, and DBL5-FCR3 is 79% identical to DBL5-7G8. Recombinant domains were expressed in the scalable expression systems used by PMI groups: *Pichia pastoris*, baculovirus, and

TABLE 2 VAR2CSA domain boundaries

Allele (GenBank accession no.)	Amino acids	
	DBL4	DBL5
FCR3 (AY372123)	1583–1989	2003–2281
7G8 (EF614233)	1593–2005	2019–2311

TABLE 3 Main elution peaks obtained by reverse-phase HPLC

Recombinant protein	Elution time (min)	% main peak
P. pastoris DBL4-7G8	33.2	52
P. pastoris DBL5-7G8	30.5	97
pET28a DBL4-7G8	33.6	62
pET28a DBL4-FCR3	33.7	68
pET28a DBL5-7G8	33.0	90
pET28a DBL5-FCR3	32.2	99.2
pET28b DBL4-7G8	33.5	38
pET28b DBL4-FCR3	34.0 and 35.1	36 and 28
pET28b DBL5-7G8	30.6	98
pET28b DBL5-FCR3	33.7	73
Baculovirus DBL4-7G8	32.7 and 33.0	52 and 31
Baculovirus DBL4-FCR3	33.1	57
Baculovirus DBL5-7G8	31.7	93
Baculovirus DBL5-FCR3	32.9	86

E. coli (using both pET28a and pET28b as expression vectors). All of the recombinant proteins contained a His tag to facilitate protein expression, but no attempts were made to standardize the location of the fusion tag at the N or C termini because of the complexity of the different plasmid vectors used for prokaryotic and eukaryotic expression. All four targeted VAR2CSA domains could be expressed in all of the expression platforms, except that only two of the four candidates could be expressed at sufficient purity and yield in *P. pastoris* (Fig. 2).

Prior to animal immunizations, the recombinant proteins were assessed for quality, purity, and integrity by SDS-PAGE (Fig. 2) and reverse-phase HPLC, and the major peaks obtained from the reverse-phase HPLC (Table 3) were analyzed by electrospray ionization (ESI)-MS. According to the ESI-MS analyses, the observed masses of the recombinant proteins were similar to the predicted masses (Table 4), and most of the recombinant proteins consisted of a single major species (Table 3). Reverse-phase HPLC chromatographs for all the recombinant proteins are shown in Fig. S4 in the supplemental material.

Titers of antibodies against the corresponding recombinant proteins in rat antisera were determined by ELISA. In general, antibody titers did not differ significantly between rats within a group. ELISA endpoint dilutions at an optical density (OD) of 0.1 of sera reactive against the specific immunogens are shown in Fig. 3.

Antiadhesion activity induced by immunogens. IgG antibodies purified from sera from rats immunized with the FCR3 and 7G8 allelic forms of recombinant DBL4 and DBL5 were tested in binding-inhibition assays as the primary measure of functional activity. Unrelated control proteins were expressed and purified using the same procedures used for the test immunogens. IgG elicited by control proteins had activity similar to that of IgG purified from the nonimmune human serum pool, and this level of activity was defined as 0% inhibition (see Fig. S1 in the supplemental material).

Antisera raised against both homologous and heterologous proteins were initially assessed on the CS2 parasite line, which is derived from ItG2 parasites selected for CSA binding and is isogenic with FCR3. Compared to the forms expressed in *E. coli* and baculovirus, DBL4-7G8 and DBL5-7G8 produced in *P. pastoris* elicited lower inhibition activity against the heterologous CS2 parasite line (Fig. 4). Antibodies to DBL4 and DBL5 had similar levels of antiadhesion activity, and DBL4 expressed in *E. coli* and DBL5 produced in *E. coli* and baculovirus did not differ according to antiadhesion activity (Fig. 4). Although the CS2 parasite line is isogenic with FCR3, the antiadhesion activity of antibodies raised to the FCR3 allelic form was similar to that of antibodies raised against the 7G8 allelic form (Fig. 4). Of interest, the level of antiadhesion activity induced by some *E. coli*- and baculovirus-expressed immunogens was similar to that observed in pooled multigravid plasma (Fig. 4).

Our overall goal is to develop an antiadhesion vaccine with broad heterologous reactivity to multiple parasites. We therefore expanded the analysis to assay IgG for functional activity against

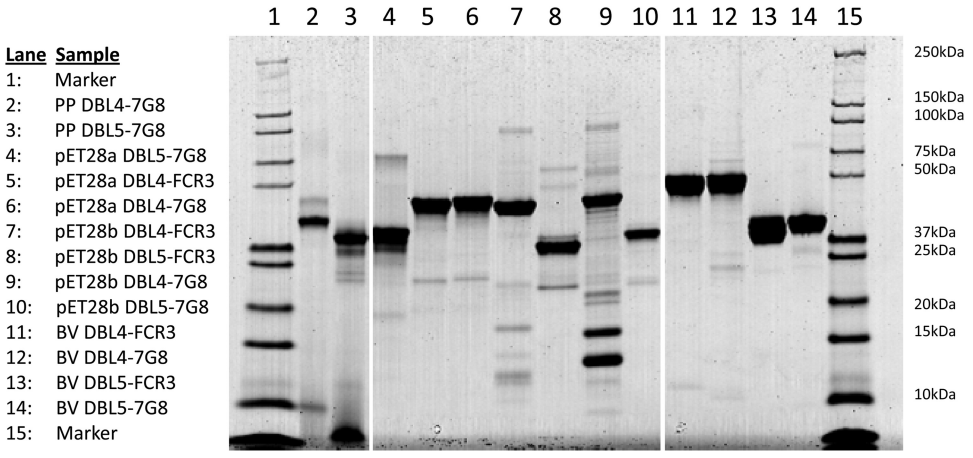

FIG 2 Analysis of VAR2CSA DBL domains. Purified DBL domains (4.2 mg) expressed in *Pichia pastoris* (PP), *E. coli* vectors pET28a and pET28b, and baculovirus (BV) were analyzed by Coomassie blue-stained SDS-PAGE under reduced conditions.

TABLE 4 Expected molecular mass versus molecular mass observed by MS analysis

Recombinant protein	Molecular mass (Da)	
	Expected	Observed by MS
P. pastoris DBL4-7G8	48,700	49,330
P. pastoris DBL5-7G8	35,700	36,842
pET28a DBL4-7G8	50,766	51,346
pET28a DBL4-FCR3	50,058	50,554 and 50,779
pET28a DBL5-7G8	35,183	37,935 and 75,197 (dimer)
pET28a DBL5-FCR3	35,000	35,760
pET28b DBL4-7G8	48,500	Peak 1, 25,146; peak 2, 50,135
pET28b DBL4-FCR3	47,700	Peak 1, 49,511; peak 2, 49,680
pET28b DBL5-7G8	34,800	36,933
pET28b DBL5-FCR3	32,900	34,905
Baculovirus DBL4-7G8	50,601	54,390
Baculovirus DBL4-FCR3	49,893	54,295
Baculovirus DBL5-7G8	36,978	38,636
Baculovirus DBL5-FCR3	34,324	35,926

five maternal parasite isolates adapted to *in vitro* culture. The maternal isolates were tested before assays to confirm that they had maintained their polyclonality, defined by the number and size of MSP2 allelic fragments amplified by PCR (31). *var2CSA* gene transcription by the parasite isolates used in the assays was determined by quantitative reverse transcription-PCR with primers corresponding to sequences in the DBL4 domain (32). *var2csa* was transcribed at similar levels among the maternal isolates (data not shown). A CD36-binding child's isolate that was adapted to *in vitro* culture was used as a control parasite to assess nonspecific binding-inhibition activity. Similar to the analysis using the CS2 laboratory isolate, IgG antibodies were tested for inhibitory activity against field isolates at concentrations of 0.5 mg/ml and 1 mg/ml. Assays were repeated three times against each isolate. The binding-inhibition activity observed at a concentration of 0.5 mg/ml was much lower than the activity observed at 1 mg/ml (data not shown). Analysis of isolate-specific antiadhesion activity demonstrated that measurements of functional antiadhesion activity against any individual isolate were reproducible between

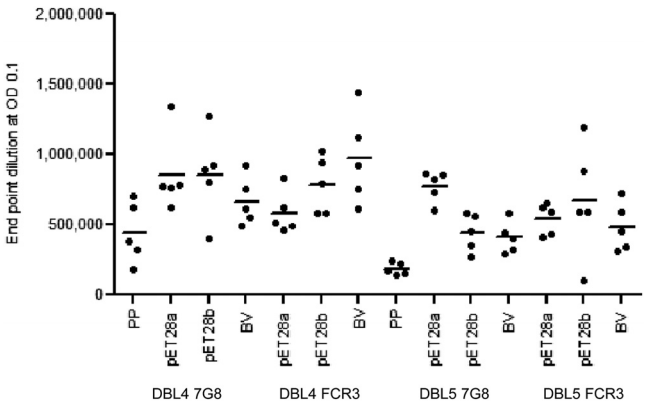

FIG 3 Endpoint ELISA titers at an OD of 0.1 of sera from rats immunized with VAR2CSA DBL4 and DBL5. Results for individual rats are indicated by the dots, and the bars indicate the mean values.

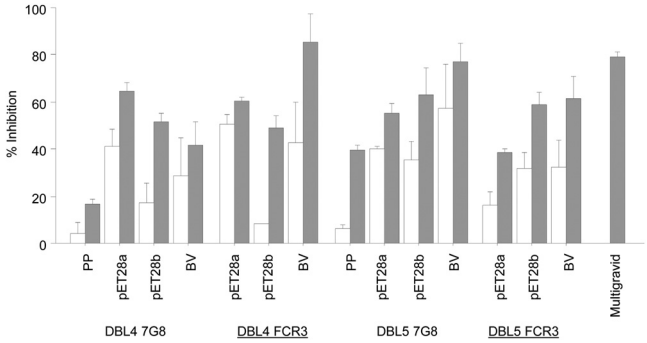

FIG 4 Binding inhibition to CSA of CSA-selected line CS2 by purified IgG directed to VAR2CSA domains DBL4 and DBL5. Binding inhibition was performed in the presence of 0.5 mg/ml (open bars) and 1 mg/ml (gray bars) IgG. The results are presented as the means and SEs obtained from two independent binding-inhibition assays. DBL domains of the homologous FCR3 allele are underlined.

assays (Fig. 5). The wide range of binding-inhibitory activity was due to differences in functional activity against the different isolates (Fig. 5). In most cases, inhibitory activity with 3 out of 5 maternal isolates was significantly higher than background activity, and in particular, maternal isolate M466 was inhibited to a similar degree as laboratory isolate CS2. These results indicate that the recombinant proteins elicited antibodies with heterologous antiadhesion activity. The different expression systems yielded immunogens with similar levels of functional activity in their antisera. In particular, recombinant proteins expressed in *E. coli* elicited antibodies at levels similar to those expressed in eukaryotic systems. Similarly, the two allelic forms of the DBL4 and DBL5 domains elicited similar levels of antiadhesion activity.

We subsequently analyzed the antisera raised against the two allelic forms of the DBL4 and DBL5 domains for activity against fresh maternal parasites that were not adapted to *in vitro* culture at a field site in Mali. Due to the limited amount of infected blood, the functional activity of individual IgG preparations was tested once on each of the isolates except for isolate 5210, in which a partial set of IgG was included (IgG to DBL4 and DBL5 of FCR3 expressed in pET28a and baculovirus and DBL5 FCR3 expressed in pET28b). Among the IgG antibodies raised against the four immunogens, the IgG antibody to DBL5 of the FCR3 allelic form had the broadest antiadhesion activity (Fig. 6). IgG to DBL5-FCR3 raised against recombinant protein expressed in *E. coli*::pET28a, *E. coli*::pET28b, and baculovirus inhibited the binding of 3/5, 3/5, and 2/3 isolates, respectively, at a level of more than 60% inhibition, and functional activity was dose dependent. IgG antibodies to the other recombinant proteins showed a dose-dependent inhibitory activity with only one or none of the isolates.

Surface recognition of maternal parasites. We tested IgG for surface recognition of the homologous and heterologous isolates that were used in the functional assays, including CS2, three maternal isolates, and a child's isolate (negative control). Surface recognition was measured by flow cytometry. High homologous reactivity against laboratory isolate CS2 was obtained with antibodies raised against DBL4-FCR3 expressed by baculovirus (Fig. 7), and the high surface reactivity corresponded to the high degree of binding-inhibition activity. However, the relationship between surface recognition and binding inhibition of CS2 parasites was not observed with antibodies raised against DBL4-FCR3

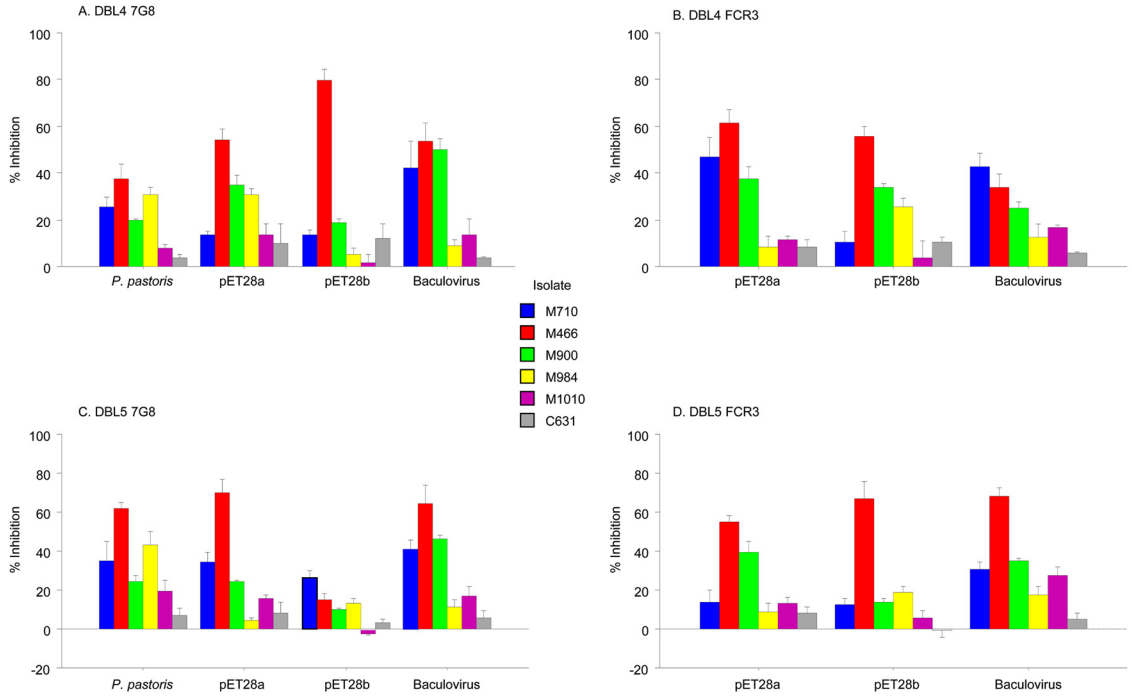

FIG 5 CSA binding inhibition of maternal isolates adapted to *in vitro* culture, using purified IgG directed to VAR2CSA domains DBL4 (A and B) and DBL5 (C and D) at a concentration of 1 mg/ml. The results are presented as means and SEs obtained from three independent binding-inhibition assays. Isolates M710, M466, M900, M984, and M1010 were collected from pregnant women; a control child isolate (C631) was used in inhibition of binding to CD36 to determine the level of nonspecific inhibition.

in other expression systems or DBL5-FCR3 regardless of the expression system that produced the immunogen. Similarly, surface recognition of maternal parasites was not consistently related to binding-inhibition activity (Fig. 8). For example, antibodies to DBL4 and DBL5 of the 7G8 allele had the highest heterologous surface recognition of maternal isolate M984, an isolate for which binding to CSA was inhibited less than 50%. The poor correlation between the level of antiadhesion activity and surface recognition

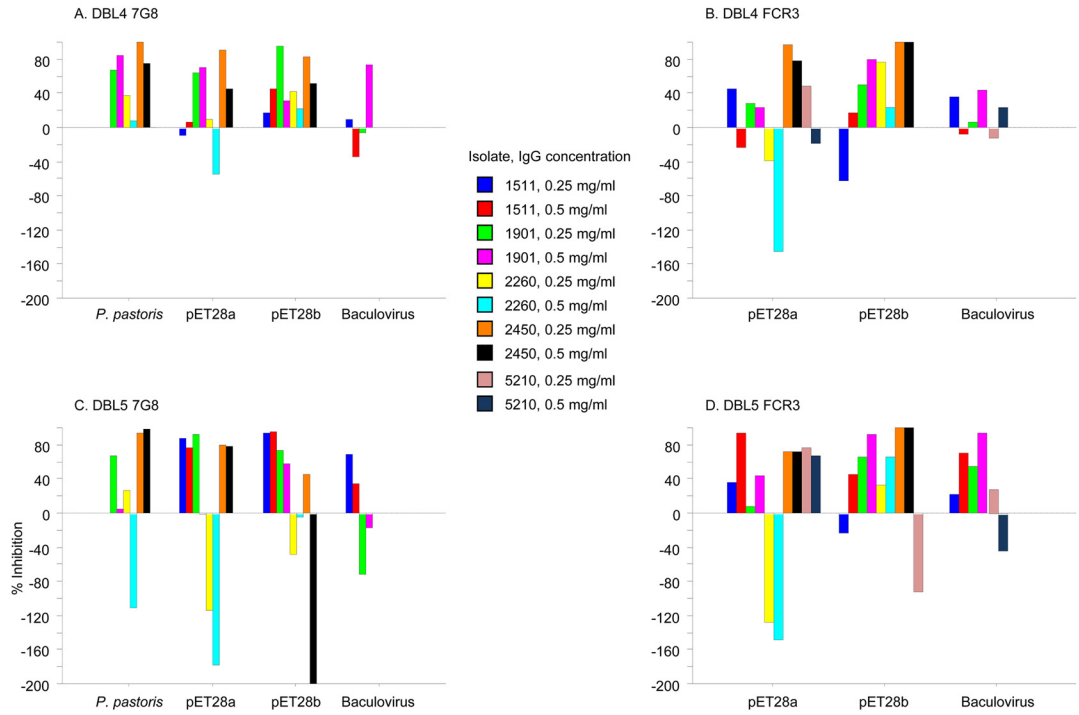

FIG 6 CSA binding inhibition of maternal field isolates not adapted to *in vitro* culture, using purified IgG raised against VAR2CSA domains DBL4 (A and B) and DBL5 (C and D).

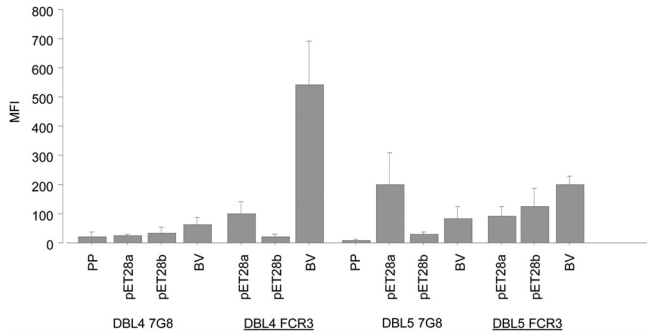

FIG 7 Surface recognition of CSA-selected line CS2 by purified IgG directed to VAR2CSA domains DBL4 and DBL5. The results are presented as means and SEs obtained from three independent repeats of flow cytometry assays. DBL domains of the homologous FCR3 allele are underlined.

by flow cytometry is consistent with a previous report by one of the consortium labs (26).

DISCUSSION

Naturally acquired immunity to pregnancy malaria in the form of cross-reactive antiadhesion antibodies is likely targeting a conserved protein(s) or conserved epitopes. A pregnancy malaria vaccine is expected to induce immune responses with qualities similar to those of naturally acquired immunity, including broad recognition and functional activity against diverse placental parasites.

Previous studies reported that immunization with recombinant VAR2CSA domains can elicit antibodies that recognize the surface of homologous (33–35) and heterologous (22, 27, 28, 36, 37) IRBCs. Naturally acquired antiadhesion antibodies are associated with protection from PM (29), and therefore, an effective PM vaccine should elicit antibodies that can inhibit parasite adhesion. Identifying the immunogen that will mimic the native protein and will induce broadly reactive antibodies is challenging. Immunization with recombinant full-length VAR2CSA protein induces antibodies that inhibit the binding of homologous parasites to a high degree (17, 18).

Individual domains were previously analyzed in individual laboratories for their ability to induce antiadhesion antibodies. The DBL4 domain of the FCR3 allelic form almost completely inhibited FCR3 parasite binding to CSA (17, 34, 35). Antisera against individual domains of the 3D7 allele inhibited adhesion of the heterologous isolate FCR3 more than 50%, and the greatest inhibition was obtained with antibodies raised against DBL1 (27). Among VAR2CSA domains of the HB3 allele, only antibodies raised against DBL3 inhibited the binding of FCR3 parasites (27). Antibodies against DBL5 and the tandem DBL5-6 domain of the HB3, 7G8, and Dd2 allelic forms inhibited binding of FCR3 parasite lines more than 60% (26).

The goal of the study was to identify recombinant DBL domains, including allelic forms and domain boundaries, as PM vaccine antigen targets, using standard animal immunization and adjuvanting procedures. Immunogens that elicit functional antibodies were to be prioritized for future assessment with adjuvants and platforms that are used for human immunization. Here, we systematically compared the level of activity of antibodies raised against different VAR2CSA domains and alleles produced by the consortium laboratories using different expression systems.

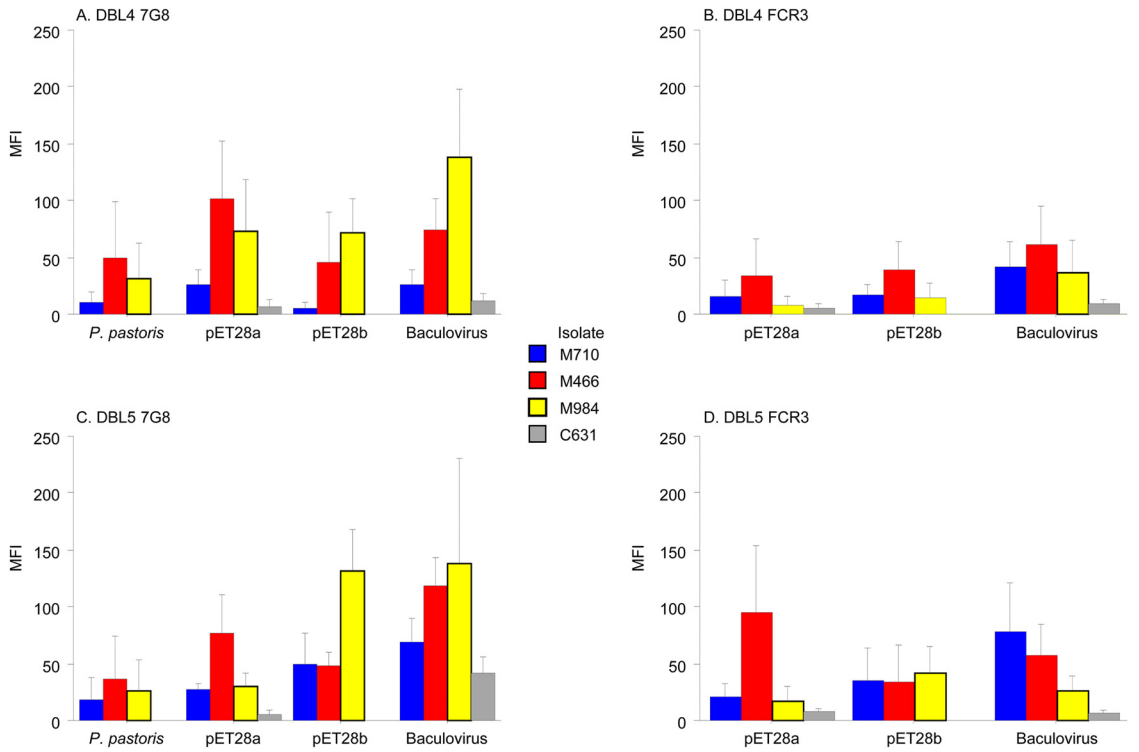

FIG 8 Surface recognition of maternal parasites by purified IgG to VAR2CSA domains DBL4 (A and B) and DBL5 (C and D). The results are presented as means and SEs obtained from three independent assays. Isolates M710, M466, M900, M984, and M1010 were collected from pregnant women, and a control child isolate (C631) was used to determine the level of nonspecific reactivity.

Initial evaluation was conducted with CS2 parasites that express the VAR2CSA allele of FCR3 parasites. We anticipated that antibodies elicited against the FCR3 allelic form would therefore inhibit CS2 parasites to a greater degree than antibodies elicited against the 7G8 form. However, the level of inhibition of CS2 parasites by antibodies to DBL5 of the 7G8 or FCR3 allele types was similar, and these results are consistent with previous results obtained with antibodies directed to DBL5 (26).

When tested on cultured CSA-binding parasites, the DBL4 and DBL5 domains from different alleles or expression systems yielded antibodies that inhibited binding of maternal isolates to a variable degree, while antibodies from multigravid women inhibited all the isolates to a level of ≥80% compared to nonimmune human sera (data not shown). The differences appeared to be strain dependent, rather than the superiority of one immunogen over the other. For example, maternal isolate M466 was inhibited to a level of more than 50% by almost all the antibodies, while maternal isolate M1010 was poorly inhibited by the same antibodies, even though the two isolates bound CSA to a similar degree (see Fig. S4 in the supplemental material).

When the same reagents were evaluated in functional assays performed on fresh maternal isolates that were not adapted to *in vitro* culture, IgG to DBL5-FCR3 demonstrated the broadest reactivity and was superior to the other reagents. Among the maternal isolates that were inhibited by antisera to DBL5-FCR3, the mean inhibition level was more than 70%. We cannot explain the differences in the pattern of inhibition by the different IgG antibodies between adapted maternal isolates and freshly collected parasites. While adapted maternal isolates maintained their polyclonality, other unknown changes associated with adaptation to *in vitro* culture might account for the various degrees of inhibition by antibodies raised against PMI immunogens. The data presented here have been gathered from a limited number of isolates but show that recombinant VAR2CSA domains expressed in scalable expression systems elicit functional antibodies that block adhesion of maternal parasites. Further studies to confirm the broad reactivity by antibodies to DBL5-FCR3 are needed to ensure its suitability as a pregnancy malaria vaccine. Recent studies reported that antibodies raised against other VAR2CSA domains also show a high degree of activity against maternal isolates. Rat IgG to DBL4-FCR3 inhibited 10 out of 13 parasite isolates collected from pregnant women at a level of 58 to 97% (38). Similarly, sera from mice immunized with the amino-terminal portion of VAR2CSA (NTS-DBL2X) inhibited the majority of maternal isolates under study to various degrees (39). Mouse sera to DBL4-FCR3 failed to inhibit the binding of maternal isolates (39), consistent with the results presented here. The various results obtained in multiple studies, including those that test identical VAR2CSA domains, emphasize the importance of systematic comparisons of potential vaccine candidates to identify the most promising targets for developing an antiadhesion-based malaria vaccine. On the basis of the process described here, candidate pregnancy malaria vaccine immunogens from multiple sources can be directly assessed in a head-to-head fashion. Our results support continued evaluation of DBL5 as well as DBL4 as target antigens and the potential for both *E. coli* and baculovirus expression systems to yield active immunogens. Future studies should further assess DBL domain boundaries and combinations of domains as approaches to improve the efficacy of candidate vaccines.

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