Sequestration of Plasmodium falciparum–infected erythrocytes to chondroitin sulfate A, a receptor for maternal malaria: monoclonal antibodies against the native parasite ligand reveal pan-reactive epitopes in placental isolates

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Plasmodium falciparum parasites express variant adhesion molecules on the surface of infected erythrocytes (IEs), which act as targets for natural protection. Recently it was shown that IE sequestration in the placenta is mediated by binding to chondroitin sulfate A via the duffy binding-like (DBL)–γ3 domain of P. falciparum erythrocyte membrane protein 1 (PfEMP1CSA). Conventional immunization procedures rarely result in the successful production of monoclonal antibodies (mAbs) against such conformational vaccine candidates. Here, we show that this difficulty can be overcome by rendering Balb/c mice B cells tolerant to the surface of human erythrocytes or Chinese hamster ovary (CHO) cells before injecting P. falciparum IEs or transfected CHO cells expressing the chondroitin sulfate A (CSA)–binding domain (DBL–γ3) of the FCR3 varCSA gene. We fused spleen cells with P3U1 cells and obtained between 20% and 60% mAbs that specifically label the surface of mature infected erythrocytes of the CSA phenotype (mIECSA) but not of other adhesive phenotypes. Surprisingly, 70.8% of the 43 mAbs analyzed in this work were IgM. All mAbs immunoprecipitated PfEMP1CSA from extracts of 125I–labeled IE CSA. Several mAbs bound efficiently to the surface of CSA-binding parasites from different geographic areas and to placental isolates from West Africa. The cross-reactive mAbs are directed against the DBL–γ3CSA, demonstrating that this domain, which mediates CSA binding, is able to induce a pan-reactive immune response. This work is an important step toward the development of a DBL–γ3–based vaccine that could protect pregnant women from pathogenesis.

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In this study we have developed a new technique for overcoming the difficulties encountered in producing mAbs against minor and conformational parasite surface antigens. We tested the hypothesis that distinct isolates involved in human placental infection share common surface epitopes. Balb/c mice were rendered B-cell tolerant to human erythrocytes or Chinese hamster ovary (CHO) cells. We then injected these mice with intact IEs of the CSA-binding phenotype or CHO cells expressing a single domain (DBL-γ3CSA) of the FCR3 varCSA gene. A large number of specific mAbs that specifically recognized the P. falciparum CSA ligand at the surface of the IE were obtained for each fusion. Several of these mAbs bound strongly to the surface of CSA-binding parasites from different geographic regions and to placental isolates from central African women via the DBL-γ3CSA domain. This study gives strong support for the development of an antimalaria vaccine based on the FCR3 DBL-γ3CSA domain to protect pregnant women against disease.

Materials and methods

Parasites

We cultured and maintained P. falciparum strains B358, BXII, FCBR, Suk, H, IBR, and FCR3 under standard culture conditions as previously described, replacing 10% human serum with 5% Alumax. Tissue cryosections of 6 P. falciparum–infected placentas from Cameroonian women (no. 24, 42, 42DJ, 193, 939, and 940) have been described elsewhere.11 Placenta isolates from the same 6 Cameroonian women from whom the parasite populations listed above were obtained by flushing with CSA.8 They were snap-frozen immediately after delivery and stored in liquid nitrogen until use. For liquid-phase immunofluorescence assay (L-IFA), we used 7-μm unfixed placenta cryosections mounted on standard microscope slides.

Selection of CSA, CD36, and intercellular adhesion molecule 1 adhesive phenotype

Highly synchronized (4 ± 2 hours) parasites in mature blood-stage-infected erythrocytes of the CSA adhesive phenotype (mIECSA) were obtained by regular panning on Sc17 Saimiri brain microvascular endothelial cells as described elsewhere,11 and successive sorbitol treatments.12 We investigated the adhesive specificity of such mIECSA of the FCR3 strain by using concentrated synchronized parasites obtained by gelatin flotation using Plasmagel (Fresnius France Pharma, Couvier, France).13 These parasites were incubated with a CSA chain bearing recombinant human thrombomodulin-coated magnetic beads (Dynabeads M450; Dynal, Oslo, Norway), as described elsewhere.14,15 Bound mIEs were expanded in culture and cytotoxicity inhibition assays were regularly performed16 to assess the specificity of binding to CSA. Typically, the adhesion of mIEs selected in this way was inhibited, by more than 95%, by 100 μg/mL soluble CSA (Fluka, Isle Abeer, Chesnes, France) or prior 1 U/mL chondrotinase ABC treatment of the endothelial cells used for the assay. We obtained mIECD36 and mIEICAM-1 by panning FCR3 IE preparations enriched by gelatin flotation on Sc2 and Sc3A4 Saimiri brain microvascular endothelial cells, which express either CD36 or intercellular adhesion molecule 1 (ICAM-1), as described elsewhere.11 Placenta parasite populations that bound CSA on endothelial cells and placenta syncytiotrophoblasts were obtained by flushing 6 full-term placentas from Cameroonian women with malaria with a soluble 50-kd CSA.8

Induction of B cell–mediated tolerance to CHO cells and normal human erythrocytes in mice

We rendered the B cells of 24- to 48-hour-old Balb/c mice (Ifa Credo, L’Arbresle, France) tolerant to normal human O erythrocytes (nEs) or normal CHO-745 cells (nCHOs) by antigenic overload, as described in Figure 1.

The subcutaneous injection into the dorsal region of 2 × 10^9 nEs or CHO-745 cells suspended in 0.2 mL 0.9% NaCl was sufficient to induce B cell–mediated tolerance to these cells. We gave a booster injection of 5 × 10^9 nEs or 5 × 10^6 CHO-745 cells suspended in 0.4 mL 0.9% NaCl 21 days after the initial injection. Three weeks later, we tested mice for antibodies directed against surface antigens of nEs or nCHO cells, by L-IFA with a 1:10 dilution of serum.

Immunization of tolerant mice with P. falciparum IEs and CHO cells expressing DBL-γ3CSA

Mice with B-mediated cell tolerance, for which no signal or only faint immunofluorescence (IF) was observed with nEs or nCHO cells, were selected for the specific immunization protocol. Approximately 5 × 10^9

Tolerization

1st. injection (i.c. or i.p.) of 2 × 10^8 IE CSAs or 2 × 10^6 CHO-745 cells

2nd. injection (i.p.) of 5 × 10^9 nEs or 5 × 10^6 nCHO-745 cells

Immunization

1st. injection (i.p.) of 5 × 10^9 IE CSA or 5 × 10^6 CHO-DBL-γ3CSA transfectant

2nd. injection (i.p.) of 5 × 10^9 IE CSA or 5 × 10^6 CHO-DBL-γ3CSA transfectant

3rd. injection (i.p.) of 5 × 10^9 IE CSA or 5 × 10^6 CHO-DBL-γ3CSA transfectant

IFA screening of live IECSA

Figure 1. Procedure for the development of mAbs specific for P. falciparum IE surface antigens. B cells of 24- to 48-hour-old Balb/c mice were rendered tolerant to nEs and CHO-745 by subcutaneous injection of 2 × 10^9 cells. Mice were given an boost 21 days later intraperitoneal injection of 5 × 10^9 nEs or 5 × 10^6 nCHO-745 cells. Tolerant animals were identified by L-IFA with a 1:10 dilution of serum, 21 days after the boost (day 42). Animals given no IF or only weak IF were then immunized and boosted with 5 × 10^9 mIECSA or 5 × 10^6 CHO-DBL-γ3CSA transfectant. Spleen cells from animals that responded (day 74) to mIECSA or CHO-DBL-γ3CSA were then used to raise mAbs by fusing these cells with P3U1 cells. The specific antiparasite surface immune responses were assessed by L-IFA.

Figure 1. Procedure for the development of mAbs specific for P. falciparum IE surface antigens. B cells of 24- to 48-hour-old Balb/c mice were rendered tolerant to nEs and CHO-745 by subcutaneous injection of 2 × 10^9 cells. Mice were given an boost 21 days later intraperitoneal injection of 5 × 10^9 nEs or 5 × 10^6 nCHO-745 cells. Tolerant animals were identified by L-IFA with a 1:10 dilution of serum, 21 days after the boost (day 42). Animals given no IF or only weak IF were then immunized and boosted with 5 × 10^9 mIECSA or 5 × 10^6 CHO-DBL-γ3CSA transfectant. Spleen cells from animals that responded (day 74) to mIECSA or CHO-DBL-γ3CSA were then used to raise mAbs by fusing these cells with P3U1 cells. The specific antiparasite surface immune responses were assessed by L-IFA.
highly synchronized mIECSA or $5 \times 10^6$ of transfected CHO cells expressing the DBL-γ3 domain of varCSA were injected into each mouse as described in Figure 1.

Development of mAbs

Mice giving positive IFA results with mIECSA or CHO–DBL-γ3 were used for the development of mAbs. We produced mAbs by fusing mouse spleen cells with P3U1 cells as described elsewhere.17,18 IFA+ cells were cloned by limiting dilution and reassessed by L-IFA; positive clones of interest were reisolated by limiting dilution. The mAbs that reacted strongly with the cell surface were expanded and isotyped by enzyme-linked immunosorbent assay (ELISA), using the Immunopure Monoclonal Antibody Isotyping Kit (Pierce, Rockford, IL).

Indirect L-IFA and air-dried IFA

We used 2 different types of indirect IFA for assessing the polyclonal antibody responses of mice and for the initial screening of mAbs: with thin air-dried indirect fluorescence smears (AD-IFA) and L-IFA performed at 4°C to prevent endocytosis with nEs or nCHO cells and asynchronous and synchronized mIECSA, mIECSA, mIECSA and CHO–DBL-γ3/3varCSA transfectants. Air-dried indirect fluorescence smears and fresh placenta cryosections were washed twice with phosphate-buffered saline (PBS; pH 7.4). Smears were incubated for 30 minutes at room temperature with 1 μg/mL 4,6-diamidino-2-phenyl-indole dihydrochloride (DAPI; Molecular Probes, Eugene, OR) for nuclear staining and with mAbs containing culture supernatants or 10 μg/mL purified mAbs. The smears were washed and incubated with a goat (Fab’2); Alexa Fluor 488-labeled antihuman immunoglobulin (Ig) G or IgM (Molecular Probes) at a dilution of 1:200 for an additional 30 minutes at room temperature. The slides were then washed and mounted in 30% (vol/vol) glycerol in PBS. For L-IFA, we washed 10 μL nEs or asynchronous or synchronized mIECSA, mIECSA, mIECSA twice with culture medium without Albumax and incubated these cells in 5 μg/mL DAPI at 37°C for 45 minutes. The nEs and IEs were washed and incubated with culture supernatant or 10 μg/mL purified mAb at 4°C for 30 minutes, washed twice, and incubated at 4°C for an additional 30 minutes with a goat (Fab’2); Alexa Fluor 488-labeled antihuman IgG or IgM (Molecular Probes) at a dilution of 1:200. In some cases, mIECSA were incubated with 100 μg/mL trypsin or chymotrypsin before the addition of mAbs, as previously described.19 For the staining of sequestrated mIEs in placenta cryosections from women with malaria, we used the AD-IFA procedure with Evans blue counterstaining (1:10 000 dilution) and simultaneous incubation with goat (Fab’2); Alexa Fluor 488-labeled antihuman IgG or IgM (Molecular Probes) at a dilution of 1:200. Immunofluorescence staining was analyzed with a Nikon E800 microscope and images were acquired with a DDx Nikon camera (Tokyo, Japan).

ELISA

The ELISA was performed with a slightly modified version of a published protocol.20 Briefly, 96-well polystyrene microtiter plates (Nunc-Polylabo, Strasbourg, France) were coated with 10 μg/mL recombinant DBL-γ3/3varCSA of the FCR3 strain (rDBL-γ3/3varCSA) produced in an insect cell expression system (Fusai et al, manuscript in preparation). The plates were incubated overnight at 4°C, and unbound antigen was removed with washing 0.05% Tween-20 in PBS (PBST). Possible residual free sites were saturated by treatment with 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C, and the plates were washed 4 times with PBST. We then added 100 μL mAb supernatant or 10 μg/mL purified mAb to duplicate wells, and incubated the plates for 2 hours at 37°C. Wells were washed with PBST and the plates were incubated at 37°C for 1 hour with a peroxidase-labeled goat antiguinea IgG (Sigma, 1’Isle Aube Chesnes, France) diluted 1:4000 in PBST. Bound immunocomplexes were detected with o-phenylene-diamine (Sigma). Absorbance was read at 405 nm on a Multiskan Ascent ELISA reader (Labsystem, Helsinki, Finland). A positive result was considered to have been obtained for a mAb (+) (Table 1) if the OD value was above the cutoff point set at 3 SDs above the mean background absorbance of P3U1 supernatant or unrelated mouse IgG isotypes or IgM.

Immunoprecipitation of 125I surface-labeled mIECSA

We used mAbs to immuno precipitate the corresponding proteins from surface 125I-labeled synchronized IECSA trophozoite stage parasite extracts, as previously described.10 IgM mAb immune complexes were recovered by incubation with an antiguinea μ chain-specific goat IgG (Sigma) followed by precipitation with protein G-Sepharose. A pool of sera from multiparous Cameroon women3 was used as a positive control and unrelated mouse IgM and IgG isotypes were used as negative controls.

Results

Induction of B cell–mediated tolerance to human erythrocytes and CHO cells

The number of Balb/c mice found to be tolerant after 2 injections of human erythrocytes or CHO cells (details are in Figure 1) was variable. About 10% to 40% of the mice injected (depending on the series) with nEs did not develop antibodies. Another 20% to 40% gave faint IF, and the other mice presented positive IF signals of various intensities. The proportion of mice displaying B cell-mediated tolerance to nCHO cells was much lower, at 2% to 5%. The best results for the production of specific antibodies against new surface antigens were obtained with “IF-negative” animals,

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Table 1. Characterization of anti-mIECSA and anti–DBL-γ3 mAbs by IFA and ELISA

<table>
<thead>
<tr>
<th>mAbs</th>
<th>Isotype</th>
<th>CSA</th>
<th>CD36</th>
<th>ICAM-1</th>
<th>CSA</th>
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<td>+</td>
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Eleven mAbs have been analyzed in detail.

L-IFA indicates liquid immunofluorescence assay using intact IEs; AD-IFA, air-dried immunofluorescence assay of IEs; (+) positive; (–) negative.
but satisfactory results were also achieved with animals that gave faint IF signals.

**mAbs against *P. falciparum* IE surface antigens**

The scores for specific mAbs directed against surface-exposed antigens on IEs in general were high and similar for mice immunized against trophozoite-IECSA or CHO cells expressing DBL-γ3. Typically, 20% to 60% of the 460 wells screened per fusion reacted with the surface of IEs but not with nEs. The initial selection of positive wells was based on the screening by L-IFA of mature parasite stage IEs of the CSA adhesive phenotype. The 43 mAbs chosen for this study, obtained from mice immunized against DBL-γ3 and against IECSA of the trophozoite stage, gave surface positive IF signals only with mIECSA (Figure 2A), but not with other parasites that express the CD36 or ICAM-1 adhesive phenotypes.

This IF was completely abolished by treating mIECSA with trypsin and chymotrypsin (100 μg/mL for 30 minutes at 37°C). All 43 mAbs reacted with the parasitophorous vacuole and vesicle-like structures (Maurer clefts) of mIECSA (Figure 2B). Unlike L-IFA, cross-reactivity with other adhesive phenotypes was observed for some mAbs with air-dried parasites (Table 1).

We found that, by AD-IFA, about 33% of the anti-mIECSA mAbs cross-reacted with similar cell structures in mIECD36 and mIECAM1. This suggests the existence of cross-reactive epitopes on intra-IE-PfEMP1, which are not accessible to antibodies once the protein is exposed on the IE surface. Anti-nE mAbs were observed only at very low frequency (0.5%), demonstrating the efficacy of this novel immunization protocol.

We isolated the mAbs used in this study and found that the anti-mIECSA and anti-DBL-γ3 mAbs were predominantly of the IgM isotype: 75% of anti-CHO-DBL-γ3 mAbs were IgM and 25% were IgG2a. For anti-mIECSA mAbs, 66.7% were IgM, 25% were IgG2a, and 8.3% were IgG1 (Table 1). All mAbs carried a light chain.

We investigated the reactivity of mAbs with parasite surface molecules, using extracts of synchronized 125I surface-labeled mIECSA. Both types of mAbs, anti-mIECSA and anti-DBL-γ3, immunoprecipitated a molecule of approximately 400 kD, previously shown to correspond to PfEMP1CSA (Figure 3).10

No other proteins, such as rifins, were detected, indicating that the immune response to the native IE is largely directed against the PfEMP1 molecule. There is no cross-reactivity with 125I surface-labeled mIE of the CD36 phenotype that matches the L-IFA data.

**DBL-γ3CSA is the target of most anti-mIECSA mAbs**

The specificity of anti-mIECSA mAbs for PIEMP1CSA was further analyzed by testing their reactivity to the domain that binds to CSA. To this end, a rDBL-γ3CSA was produced by an insect cell expression system. This recombinant consisted of the same DBL-γ3 region of FCR3 expressed by the CHO transfectant that specifically binds CSA.10 The recombinant DBL-γ3CSA protein inhibits the cytoadhesion of mIECSA to endothelial cells and syncytiotrophoblasts by more than 60% (100 μg/mL) and mAbs raised against it inhibit also IE adhesion to CSA by more than 90%. We assume that rDBL-γ3CSA protein carries conformational epitopes, because the same domain expressed as bacterial GST-fusion protein did not inhibit parasite adhesion to CSA and the antibody response in mice did not react with the native parasite molecule (Fusai et al, manuscript in preparation).

The rDBL-γ3CSA protein reacted specifically with 15 of 23 anti-mIECSA mAbs in ELISA. As expected, almost all anti-CHO-DBL-γ3 mAbs recognized rDBL-γ3CSA (85%). The intensity of surface IF and the absorbance values obtained in ELISA were not correlated (Table 1). We conclude that the DBL-γ3 domain not only mediates adhesion to CSA but also acts as an immunodominant region of PIEMP1CSA at least when using this immunization procedure described here.

**Pan-reactivity of anti-CHO-DBL-γ3CSA and anti-mIECSA mAbs**

Two mAbs, 2HS/D3 and 1B11/A5, respectively anti-mIECSA and anti-CHO-DBL-γ3, were arbitrarily chosen because of their typical reactivity of the other mAbs with multiple variants of a number of CSA-binding parasites from different geographic regions (Brazil, Thailand, and West Africa). Surface staining by L-IFA showed that all 7 laboratory strains analyzed (Table 2) reacted with both mAbs, 2HS/D3 and 1B11/A5, at varying degrees (2%-98%) in laboratory strains not previously selected for CSA binding (Table 2).

Pan-reaction of each of these parasite strains on Sc17 cells, which carry CSA as the only adhesion receptor, resulted in a considerable enrichment in mIE, which reacted with both mAbs (> 94%) in most laboratory strains. Cytoadhesion inhibition assays on Sc17 cells with these 6 panned parasite subpopulations resulted in the inhibition of mIE adhesion, by 90% to 96%, by 100 μg/mL CSA or 1 U/mL chondroitinase ABC treatment of the endothelial cells (Table 2). Analysis of such CSA panned parasites by reverse transcription–polymerase chain reaction showed the expression of one type of varCSA gene20 (data not shown).

The reactivity of 2HS/D3 and 1B11/A5 with placental isolates from 6 different women infected with malaria was investigated.

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**Figure 2. Surface staining by L-IFA and AD-IFA.** Surface staining of live FCR3 mIECSA by L-IFA with 10 μg/mL anti-mIECSA mAb 2HS/D3 and 10 μg/mL anti-DBL-γ3 mAb 1B11/A5 (A). Staining by AD-IFA of the parasitophorous vacuole and vesiclelike structures in mIECSA with 10 μg/mL anti-mIECSA mAb 2HS/D3 and anti-DBL-γ3 mAb 1B11/A5 (B).

**Figure 3. Immunoprecipitation of PIEMP1CSA from surface 125I-labeled FCR3-mIECSA extract.** Lanes 1, 2, and 3: anti-mIECSA mAbs 2H5/D3, 2A11/B7, and 2A2/G6. Lanes 4, 5, and 6: anti-DBL-γ3 mAbs 1B11/A5, 4F10/G9, and 1B4/G10. Lane 7 positive control: pool of sera from multigravidae from Cameroon. Lane 8: unrelated IgM.
using placental tissue cryosections. All sections showed large numbers of adhering parasites and gave strong signals with the 2 mAbs. A typical example of the antibody staining is shown in Figure 4.

However, only a fraction of the pigmented erythrocytes in the placenta were stained with 2H5/D3 and 1B11/A5 (approximately between 40% and 60%), suggesting the presence of parasites that might bind to a distinct placental receptor such as the Fc/IgG receptor or hyaluronic acid.21,22 We conclude that the 2 mAbs, 2H5/D3 and 1B11/A5, directed against FCR3 DBL-3CSA and DBL-3CSA, define cross-reacting epitopes that are conserved in geographically and genetically distinct CSA-binding parasite populations, including clinical isolates, and are involved in human placental infection.

**Discussion**

The immunization protocol developed in this work was highly efficient at generating large sets of mAbs specifically directed against the native form of present on the surface of IEs. The analysis of these mAbs led to a number of novel and important observations. First, it was noticed that mice immunized with intact parasitized erythrocytes developed mainly variant specific mAbs. No cross-reactivity was observed with the surface of IE expressing a PfEMP1 able to bind to CD36 or ICAM-1. This shows that the immune response against a native PfEMP1 molecule on the surface of IE is primarily variant specific. Although we obtained a large number of mAbs directed against the surface of mIE with this novel immunization protocol, all of those analyzed in more detail immunoprecipitate the same large molecule of approximately 400 kd, indicating that the major immunodominant surface molecule is PfEMP1 and that other molecules, such as rifins,23,24 are probably only minor targets of the antibody response against the IE surface.

Second, the mAbs against mIE CSA and CHO–DBL-3CSA were predominantly of the IgM subclass. This result contrasts with the exclusive development of IgG mAbs against a purified recombinant DBL-3/CHO–DBL-3/CSA produced in insect cells (Fusai et al, manuscript in preparation), using “Titer Max” as adjuvant (Pierce) in a conventional immunization procedure. These data strongly suggest that the type of immune response to variant surface molecules depends on the context in which the antigen is presented to the immune system. It would be of interest to investigate whether the immune response in malaria patients to *P falciparum* IEs includes a major IgM component.
The quality of the antibody response in mice to the native P\(\text{IE17MP1}\) or DBL-\(\gamma/3\) variant domains expressed on the surface of CHO cells contrasts with the type of immune response obtained with a DBL-\(\gamma/3\) variant domain expressed in Escherichia coli (GST fusion protein). The antibodies produced in response to the recombinant GST fusion protein (in mice, guinea pigs, or rabbits; data not shown) were not able to react with the surface of mIECSA. This result demonstrates the importance of conformational epitopes in the natural immune response to the surface of IEs. These are important considerations for epidemiologic studies attempting to correlate antibody reactivity to the var gene domains expressed in E coli with clinical immunity status in patients with malaria.

Previous work revealed that the CSA-binding region of the P\(\text{IE17MP1}\) protein might be a vaccine candidate that could protect pregnant women from malaria. However, it was pointed out that the genetic diversity of the DBL-\(\gamma/3\) variant domain in clinical isolates could be a major obstacle in the development of this vaccine. Our results add a new dimension to the validation of this vaccine candidate. Here we demonstrate that mAbs that react specifically with the DBL-\(\gamma/3\) variant domain have a strong pan-reactive component. Genetically different laboratory parasite strains selected on the basis of CSA binding (from Brazil, Thailand, and West Africa) and 6 placental isolates from central Africa specifically reacted with mAbs directed against the surface of DBL-\(\gamma/3\) variant of the FCR3 parasite. These data clearly demonstrate the presence of conserved conformational epitopes in CSA-binding parasites and confirm experimentally a prediction made by previous epidemiologic studies on placental malaria. This model, established by the work of Fried and Duffy, is based on the observation that multiparous women develop antibodies that inhibit IE-CSA adhesion in isolates from different endemic areas.

In conclusion, these newly generated mAbs are unique tools for screening for new Plasmodium surface antigens, mapping adhesive domains, purifying antigens, and studying the prevalence of defined adhesive phenotypes in peripheral blood and necropsies. Furthermore, the immunization method described here may be extended to any cell surface modification induced by a pathogenic process. In particular, it could be used for other Plasmodium species and human and animal pathogens that infect erythrocytes such as Bartonella and Babesia and could also be extended to tumor markers on cancer cells.

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References

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