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Cytoadhesion of *Plasmodium falciparum*-infected erythrocytes and the infected placenta: a two-way pathway

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Abstract

Malaria is undoubtedly the world's most devastating parasitic disease, affecting 300 to 500 million people every year. Some cases of *Plasmodium falciparum* infection progress to the deadly forms of the disease responsible for 1 to 3 million deaths annually. *P. falciparum*-infected erythrocytes adhere to host receptors in the deep microvasculature of several organs. The cytoadhesion of infected erythrocytes to placental syncytiotrophoblast receptors leads to pregnancy-associated malaria (PAM). This specific maternal-fetal syndrome causes maternal anemia, low birth weight and the death of 62,000 to 363,000 infants per year in sub-Saharan Africa, and thus has a poor outcome for both mother and fetus. However, PAM and non-PAM parasites have been shown to differ antigenically and genetically. After multiple pregnancies, women from different geographical areas develop adhesion-blocking antibodies that protect against placental parasitemia and clinical symptoms of PAM. The recent description of a new parasite ligand encoded by the *var*2^{CSA} gene as the only gene up-regulated in PAM parasites renders the development of an anti-PAM vaccine more feasible. The search for a vaccine to prevent *P. falciparum* sequestration in the placenta by eliciting adhesion-blocking antibodies and a cellular immune response, and the development of new methods for evaluating such antibodies should be key priorities in mother-child health programs in areas of endemic malaria. This review summarizes the main molecular, immunological and physiopathological aspects of PAM, including findings related to new targets in the *P. falciparum var* gene family. Finally, we focus on a new methodology for mimicking cytoadhesion under blood flow conditions in human placental tissue.

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Introduction

Globally, malaria is the most widespread human parasitic disease, affecting 300 to 500 million people per year. Four species of *Plasmodium* can infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. No complications are observed in most malaria cases, but some *P. falciparum* infections develop into severe forms of the disease, such as cerebral malaria and pregnancy-associated malaria (PAM), which cause more than two million deaths annually. It is estimated that 2.4 billion people, almost half the world's population, are at risk of contracting malaria. In subtropical regions, and sub-Saharan African countries in particular, this disease limits economic development. The control of this disease has been hampered by the alarming spread of drug-resistant parasites, insecticide-resistant mosquitoes, and the lack of an effective vaccine.

The situation has been aggravated by the deterioration of socioeconomic conditions in rural areas and disordered human migration in countries in which malaria is endemic. These factors have contributed to the re-emergence of malaria. As a result, much of the current research into malaria continues to focus on attempts to develop a vaccine capable of controlling parasite transmission. Some promising results have been obtained, but it seems unlikely that a vaccine conferring significant levels of immune protection, particularly against severe infection, will be developed in the near future.

Severe malaria and *Plasmodium falciparum* cytoadhesion

Severe malaria is a multifactorial phenomenon involving the sequestration of *P. falciparum*-infected erythrocytes (IE) in deep vascular beds and the production of inflammatory cytokines, such as TNF-α and IFN-γ (1). IE adhere directly to various host endothelial receptors, including CD36, intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), E-selectin, P-selectin, hyaluronic acid (HA), and chondroitin sulfate-A (CSA), or to other IE. They may also form rosettes by adhering to non-infected erythrocytes (Figure 1B) (2). It has been suggested that adhesion to host receptors expressed on the surface of endothelial cells enables IE to avoid spleen-mediated filtration and host immune attack, potentially implicating cytoadhesion in parasite survival (3). In addition to direct parasite adhesion to host receptors, platelets can act as a bridge between IE and endothelial cells, providing additional CD36 receptors for cytoadhesion (Figure 1A).

Following infection, *P. falciparum* proteins are exported to the erythrocyte surface, altering host cell conformation and generating electron-dense structures. These structures are known as knobs (Figure 1), and correspond to IE-binding sites adhering to the host endothelium. Knobs are composed of several polypeptides, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1; Figure 2A). PfEMP-1 is a variable protein 200 to 350 kDa in size, encoded by the members of the *var* multigene family, which are present as about 60 distinct copies per haploid parasite genome. These proteins mediate both antigenic variation and cytoadhesion (2,4), and therefore play an important role in parasite virulence. It has been suggested that *var* gene diversity is largely based on the clustering of these genes at the ends of several chromosomes, creating a "hot-spot" recombination zone facilitating the alignment of homologous sequences located on heterologous chromosomes. Despite this variability, only one antigenic variant is expressed on the surface of the IE at a given time (5). PfEMP-1 contains a transmembrane and an extracellular region, which has been implicated in binding to the cytoadhesion-binding site and as a target for the host immune response (Figure 2A). This extracellular region has a succession of bind-

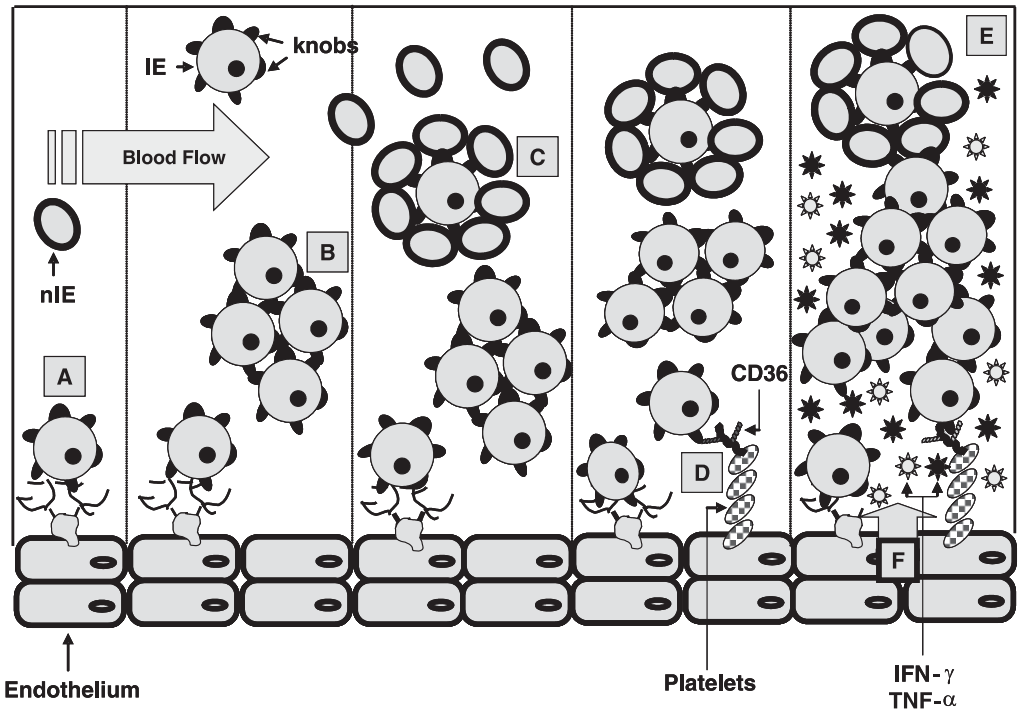

Figure 1. Sequestration mechanisms involved in *Plasmodium falciparum* infections. *P. falciparum*-infected erythrocytes (IE) adhere directly to different receptors on the host endothelium via knobs (A); to other IE by auto-agglutination (B); to non-infected erythrocytes (nIE), forming rosettes (C); to platelets, which act as a bridge in IE cytoadhesion via the CD36 receptor (D). All these phenomena are thought to contribute to blood flow occlusion (E) and production of the inflammatory cytokines, TNF-α and IFN-γ (F); thus leading to the poor clinical outcomes observed in severe malaria.

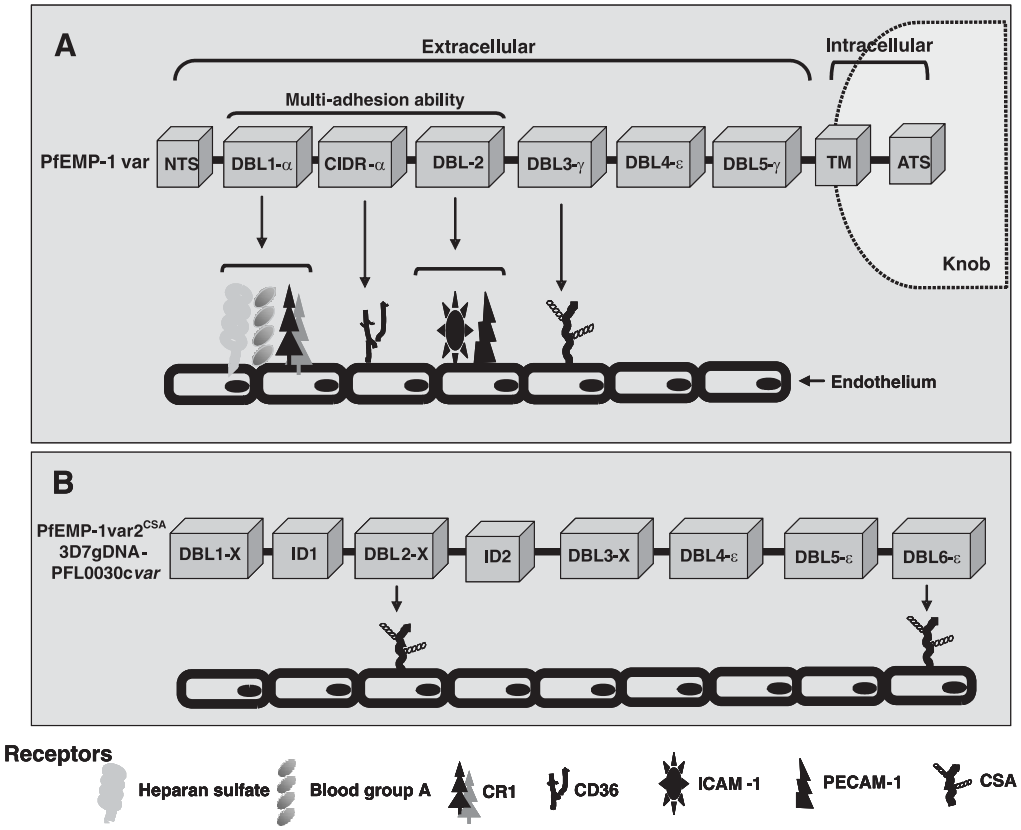

Figure 2. Schematic structure of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEPM-1). The intracellular and the immunogenic extracellular regions of a PfEMP-1 are represented. *A*, One PfEMP-1 encoded by a *var* gene contains several Duffy binding-like (DBL) domains intercalated by cysteine-rich interdomain region domains (CIDR), responsible for mediating parasite cytoadhesion to different receptors directly on the host endothelium, multiadhesion, or adhesion to non-infected erythrocytes, forming rosettes. NTS = N-terminal segment; TM = transmembrane domain; ATS = acidic teminal segment; CR1 = complement receptor 1; ICAM-1 = intracellular adhesion molecule 1; PECAM-1 = platelet endothelial cell adhesion molecule; CSA = chondroitin sulfate A. *B*, The PfEMP-1 encoded by the *var*2^{CSA} gene 3D7gDNA-PFL0030c*var* contains DBL domains capable of CSA-binding and inter-domain regions (ID).

ing sites arranged in tandem at the N-terminal end of the molecule (Figure 2A). These motifs are known as Duffy binding-like (DBL) domains, as they were first identified in *P. vivax* Duffy binding protein, intercalated by cysteine-rich interdomain region domains (CIDR). Both CIDR and DBL regions can be identified on the basis of their amino-acid sequences (2,4).

Different PfEMP-1 molecules have binding sites for adhesion to different host receptors (Figure 2A), such as CD36, ICAM-1, VCAM-1, E-selectin, P-selectin, CSA, and others dependent on multiple functional binding domains within PfEMP-1. For example, adhesion to CD36, ICAM-1 and CSA is mediated by different PfEMP-1 variants, as *var* genes are expressed in a mutually exclusive manner, with only one PfEMP-1 expressed on the surface of an IE at a given time (5). Thus, placental parasites can bind CSA, but not the CD36 receptor (6). This dichotomous behavior may result from differences in gene location and transcription orientations between CSA-binding and non-binding parasites (7-8).

General aspects of PAM

After years of exposure to the parasite, individuals living in areas of endemic malaria acquire high levels of immunity, limiting parasitemia and attenuating the clinical outcome of malaria. However, pregnant women remain susceptible, especially in their first pregnancy, in which case the risk of contracting malaria is two to ten times higher than that in non-pregnant women living in the same area. Until recently, it was thought that this particular susceptibility of women to malaria during pregnancy was due to pregnancy-related immune suppression and hormonal alterations. However, it has been recently shown that the placenta provides an ideal environment for the development of a subpopulation of malaria parasites that adhere to receptors in the placental syncytiotrophoblast. In most cases, the parasites remain on the maternal side of the placenta, but this maternal-fetal syndrome, known as PAM, has adverse effects on both mother and unborn child, causing maternal anemia and low-birth weight (LBW) babies (9). PAM is thought to be responsible for 62,000 to 363,000 infant deaths in sub-Saharan Africa annually (10). Unfortunately, these figures are probably underestimates since peripheral parasitemia is not always observed and the symptoms are not well characterized in some cases.

In PAM, parasite adhesion to CSA, HA and other placental receptors may trigger an inflammatory process involving cytokine-secreting mononuclear cells. The inflammatory component, which may appear after parasite accumulation in the placenta, is associated with the immune-pathological consequences of PAM, such as cytotrophoblast proliferation and cytotrophoblast membrane thickening (10). This inflammatory process, characterized by massive cell deposition, is thought to alter local blood flow, disrupting metabolic pathways and hindering IgG transfer across the placenta and the exchange of nutrients between mother and fetus, resulting in placental lesions and LBW (10,11). However, the clinical outcomes of PAM, such as fetal growth restriction and preterm delivery, the strict association with LBW at term and placental parasitemia, have been observed in primigravidae (9). It is also known that infants born to infected Cameroonian mothers are significantly more susceptible to plasmodial infections, especially in the first two years of life (11). A recent epidemiological analysis in Tanzania, including twice as many infants as the Cameroon study (11), also showed that children born to women with placental malaria presented parasitemia earlier in life than those born to non-infected peers (12). Surprisingly, this study also showed that parity played a role, as the offspring of primigravidae had a significantly lower risk of

parasitemia than infants born to multigravidae (12). The precise reasons for this remain unclear, but the marked change in cytokine profile and the timing of cytokine production in primigravidae may be involved (12).

Most studies of maternal malaria have been carried out in *P. falciparum*-infected women. However, pregnant women are susceptible to all four human malaria parasites, including *P. vivax*, the most prevalent parasite in Brazil and elsewhere outside sub-Saharan Africa. A study in Thailand revealed that primigravidae had a significantly higher risk of *P. vivax* infection than multigravidae. Moreover, *P. vivax* infection has also been shown to be significantly associated with maternal anemia and risk of LBW, although these outcomes were more marked in multigravidae (13). The deposition of malaria pigment in the placenta has been observed in *P. vivax*-infected women (14), and variant antigens encoded by a specific *P. vivax* multigene family have been identified (15). However, hard data on *P. vivax* cytoadhesion to the placental syncytiotrophoblast or endothelial cells remain scarce.

In Brazil, where malaria transmission is unstable, *P. falciparum* and *P. vivax* infections account for 15.1 and 84.4% of cases among non-pregnant women (16). However, the corresponding proportions for pregnant women are 29.7% for *falciparum* and 67.7% for *vivax* malaria (16). This corresponds to a significant, 2.5 times increase in the frequency of *P. falciparum* infection for the 195 cases of malaria in pregnant women analyzed (16). The precise reason for this shift in prevalence is unclear and further studies with a larger number of patients are required.

Ligands and receptors involved in PAM

The DBL-γ3 domain of PfEMP-1, encoded by the *var*1^{CSA} gene, was initially thought to be the ligand responsible for parasite cytoadhesion in PAM (17). However, several recent studies have suggested that the protein encoded by the *var*2^{CSA} gene may be the principal ligand involved in placental cytoadhesion. Unlike *var*1^{CSA}, *var*2^{CSA} is up-regulated in placental parasites after selection for adhesion to the CSA receptor *in vitro* (18-20); indeed it is the only gene shown to be transcriptionally up-regulated following such selection. *var*2^{CSA} knockout parasites are unable to recover their initial CSA binding, even after repeated exposure to CSA (21,22). The PfEMP-1 encoded by the *var*2^{CSA} gene has been shown to have a structure different from that of other *var* genes, in that it lacks the CIDR, DBL-γ and N-terminal DBL-α domains (Figure 2B). The PfEMP-1 encoded by the 3D7 *var*2^{CSA} gene (3D7gDNA-PFL0030c*var*) has six DBL motifs: DBL4-ε, DBL5-ε and DBL6-ε, and a further three motifs that do not fit into the current classification: DBL1-X, DBL2-X and DBL3-X. The DBL2-X and DBL6-ε domains are able to bind CSA (Figure 2B) (18).

A recent mass spectrometry-based proteomics study identified novel parasite antigens, which might be expressed on the IE surface, exclusively in CSA-binding or placental parasites, but did not evaluate the binding of these antigens to host receptors (23).

There is evidence that CSA is not the only placental receptor and that a subpopulation of parasites collected from infected placentas may also bind to HA and to non-immune IgG via their F(ab') moieties. However, recent cytoadhesion assays using placental parasites collected from 60 pregnant Tanzanian women with malaria showed that almost all placental parasites capable of binding to at least one host receptor were also able to adhere to immobilized CSA, and that only three of 46 of these parasites adhered to immobilized HA. In binding inhibition assays using soluble CSA as a competitor,

adhesion to placental sections was significantly abolished in all placental parasites tested. In contrast, soluble HA, non-immune IgG and protein A failed to inhibit parasite binding to placental cryosections. These findings strongly suggest that CSA is the major placental receptor, and support the development of vaccines targeting parasite ligands to CSA (24).

The CSA or chondroitin 4-sulfate (C4S) receptor is a glycosaminoglycan present in the extracellular matrix, and was first identified as a receptor for parasite binding to *Saimiri* monkey endothelial brain cells and to Chinese hamster ovary cells (25,26). Gysin et al. (27) showed that thrombomodulin with a CSA chain was the dominant proteoglycan involved in the sequestration of CSA-binding parasites in the placenta and that a CSA chain at least 9 kDa in size was required to sustain the adhesion of CSA-binding parasites (28). Achur et al. (29) subsequently purified and identified several types of chondroitin sulfate proteoglycan (CSPG) from the human placenta, showing that these natural CSPGs present in the intervillous space contained unusually low levels of sulfate and served as receptors for PAM parasite adhesion. The same group went on to define the structures required for parasite cytoadhesion as C4S dodecasaccharides, and showed that these placental CSPGs have a unique distribution of sulfate groups throughout the second and third semesters of pregnancy (30-32). It was recently shown that the ability of antiadhesive molecules to inhibit C4S-specific binding also depends on the sulfation partner of these CSPGs (33).

Does the antibody-mediated immune response play a role in PAM?

Despite pregnancy-related immunosuppression, pregnant women with malaria develop antibodies that inhibit the binding of IE to CSA, and these antibodies are associated with protection against placental infection. Primigravidae have a much higher susceptibility to maternal malaria than multigravidae, because the antibodies acquired after multiple pregnancies are associated with a reduction in the number of IE in the placenta (34). In addition, higher levels of these CSA adhesion-blocking antibodies are correlated with less pronounced maternal anemia and with higher birth weight for babies born at term (35,36).

For *var*2^{CSA} parasites, a recent study showed that rabbit antibodies raised against two VAR2CSA recombinant proteins, corresponding to the DBL1-X and DBL5-ε domains, recognize only the surface proteins of PAM parasites (20). Plasma samples from Ghanaian individuals recognized these recombinant proteins in a sex- and parity-dependent manner in ELISA tests; this was particularly true for the recombinant protein based on the DBL5-ε domain (20). High plasma levels of anti-VAR2CSA antibodies in women were also found to be correlated with a lower risk of LBW (20). Finally, monoclonal antibodies (mAbs) that recognize VAR2CSA DBL domains inhibit, to various extents, the adhesion of a placental isolate to placental cryosections under flow conditions. Moreover, sera from mice immunized with native VAR2CSA domain complexes with specific mAbs strongly inhibit PAM parasite cytoadhesion to CSA on the surface of endothelial cells (37).

These observations suggest that one probable mechanism controlling placental parasitemia and attenuating clinical outcome may be based on adhesion-blocking antibodies against CSA-binding domains. However, as primigravidae and multigravidae present significant levels of adhesion-blocking antibodies at term, the timing of acquisition of these antibodies may be important in immune protection. O'Neil-Dunne et al. (38) studied 198 pregnant Cameroonian women and showed that multigravidae began mounting an antibody-based immune response af-

ter just 12 weeks of gestation, whereas primigravidae took eight weeks longer to develop such adhesion-blocking antibodies. However, as cytophilic antibodies have been collected from infected pregnant women (39), it should be borne in mind that other antibody-dependent mechanisms, such as phagocytosis and complement activation, may also help to protect against PAM, in addition to blocking adhesion. Consistent with the existence of additional antibody mechanisms, Megnekou et al. (40) showed that IgG1 and IgG3 were the most prevalent subclasses of PAM antibodies in Cameroonian women, and that larger amounts of these antibodies were found in pregnant multiparous women.

Is the cell-mediated immune response involved in PAM pathogenesis or protection?

The precise mechanism by which PAM parasites evade the immune system and the possible involvement of a cell-mediated immune response in protection remains unresolved. However, it has been shown that massive sequestration of the parasite in the placenta leads to a switch in the cell-mediated immune response, typically from T_H2 to T_H1, resulting in the clinical manifestation of PAM, characterized by an increase in the level of pro-inflammatory cytokine production (10). Several studies have shown that high levels of IFN-γ and TNF-α, mainly secreted by placental macrophages, are associated with poor clinical outcome in patients with PAM and with the concentration of hemozoin in the placenta (41). Placental infection increases the levels of α- and ß-chemokines, which, in turn, increase immune cell recruitment to the placenta (10,42).

In contrast, IFN-γ levels have been reported to be higher following *in vitro* stimulation of blood mononuclear cells from multigravidae than following the stimulation of such cells from women in their first or second pregnancy. The cells collected from women in their second pregnancy secreted high levels of IL-4 and IL-10 (43). Multigravidae have been shown to present higher levels of lymphocyte proliferation and natural killer cell cytotoxic activity in response to CSA-binding parasites than primigravidae women (10). These observations suggest that IFN-γ is involved in immunity to PAM. Further evidence for the protective role of IFN-γ is provided by the higher susceptibility to PAM of women with both malaria and HIV infection (10). Indeed, it has been recently shown that neonates born to mothers with active placental infection have lower levels of PAM-parasite antigen-specific IFN-γ T cells and higher levels of IL-10 CD4 T cells than do pregnant infected women treated for malaria (44). However, TGF-ß, an anti-inflammatory cytokine, is produced in larger amounts in multigravidae than in primigravidae, suggesting a possible role in controlling the manifestation of PAM clinical symptoms.

A recent study in monkeys showed that infection with *P. coatneyi* did not result in higher levels of CD4 and CD8 T lymphocytes than observed in infected non-pregnant monkeys. Indeed, the pregnant infected monkeys had lower levels of monocytes and macrophages in peripheral blood than did the non-pregnant infected monkeys (45). Conversely, high levels of mononuclear cell accumulation have been associated with poor PAM outcomes (46). It should be noted that, in these studies, cells were counted in peripheral blood, so we cannot exclude the possibility that this modulation may alter the levels of these cells in placental compartments. In PAM, T cells collected from peripheral blood proliferate more efficiently than those collected from the intervillous blood, whereas intervillous and peripheral monocytes are equally able to present antigens (47).

These observations suggest a dual effect on cytokine production in PAM and that a fine balance in the timing and levels of pro-

and anti-inflammatory cytokines dictates whether an individual will manage to control PAM or whether the clinical symptoms associated with the disease will develop. This dual effect may depend on regulation of the macrophage migration inhibitory factor, a specific cytokine that counter-regulates the immunosuppressive effects of pregnancy. Placental infection was recently shown to increase macrophage migration inhibitory factor production in the presence of cytotrophoblast-adherent IE (48).

Is an anti-PAM vaccine feasible?

The first evidence that it might be possible to develop an anti-PAM vaccine was provided by the study of Fried and Duffy (9,35) and Staalsoe et al. (34,36) showing that multiparous women were less susceptible to PAM than women in their first pregnancy, that infected women developed high levels of adhesion-blocking antibodies against PAM parasites after several pregnancies (9,34) and that these antibodies were associated with attenuation of the clinical outcome of PAM. Antibodies against *var*2^{CSA} parasites have also been shown to cross-react with genetically different *P. falciparum* strains (49). Cross-reactivity between the DBL-γ3^{CSA} domain and *var*2^{CSA}-encoded antigens has been observed (50). Furthermore, mAbs raised against *var*2^{CSA} parasite surface antigens have also proved to be pan-reactive with CSA-binding parasites from different geographical origins (37). Moreover, molecular analysis of the *var*1^{CSA} DBL-γ3 minimal binding domain revealed 37% sequence identity to the *var*2^{CSA} DBL3-X domain (51). These somewhat surprising pan-reactivity results are probably related to conformational similarities.

In light of the antibody-mediated immune response in PAM, an efficient vaccine would probably elicit large amounts of adhesion-blocking antibodies, mainly against conserved binding motifs. However, as the cell-mediated immune response also seems to be involved in immune protection against PAM, immunization regimes and adjuvants should aim to induce high levels of IFN-γ-secreting T cells. Immunization regimens based on naked DNA for priming and recombinant viral vectors or proteins for boosting have been shown to elicit high levels of CD4- and CD8-producing T cells able to induce immune protection against several viral and protozoan diseases (52). Finally, anti-PAM vaccines may contain other parasite antigens since sera collected from Cameroonian women showed a significant correlation between low or null levels of antibodies against the carboxyl-terminal 19-kDa segment of the *P. falciparum* merozoite surface protein-1 and the risk of PAM (53).

Is it possible to model PAM?

Reliable *in vitro* adhesion models are required for the evaluation of potential vaccine candidates and the antibody-mediated immune response directed against them. Most of the existing *in vitro* models of IE sequestration were developed for studying IE adhesion in parasites thought to be involved in cerebral malaria. Many knob^{+} laboratory-adapted *P. falciparum* strains adhere *in vitro* to various cell types, including human umbilical vein endothelial cells, C32 amelanotic melanoma cells, human dermal microvascular endothelial cells, human brain capillary endothelial cells, human monocytes and platelets, and transfected COS cells (54). However, in 1995, Gay et al. (55) described the use of *Saimiri* microvascular brain endothelial cell clones differing in terms of the expression of several combined surface molecules such as CD36, ICAM-1, E-selectin, and CSA, permitting for the first time the selection by cytoadhesion of distinct monomorphic adhesion phenotypes.

Each model attempts to simulate the interaction between IE and the cerebral endothelial cells, but none can be considered

ideal. The *Saimiri* cell model has some advantages in that it allows *in vitro* cytoadhesion studies, the results of which can be confirmed or rejected using the homologous *Saimiri/P. falciparum* monkey model (56). This model is also considered to be more relevant than non-primate cell models due to the phylogenetic proximity to humans. The use of organ-specific endothelial cells appears to be particularly useful for structure-function studies in which the native conformation of a receptor is critical. A placental BeWo-derived cell line has been successfully used to select monomorphic CSA-binding parasites (57). Since placental CSPGs have an unusual sulfation pattern, BeWo cells provide an alternative to cells of non-placental origin in CSA-binding studies.

In all the parasite-binding assays described above, cytoadhesion was investigated under static conditions, in which suspensions of IE were allowed to settle on a confluent monolayer of cultured cells. This method is technically simple, making it possible to carry out a large number of assays simultaneously. However, static assays do not model the dynamic blood flow conditions encountered by IE *in vivo*.

In 1995, Cooke and Coppel (54) developed an *in vitro* assay for visualizing and quantifying the adhesion of IE to endothelial cells or to immobilized adhesion receptors under flow conditions. The flow assembly used consisted of a parallel-plate flow chamber or a glass microcapillary tube (microslide) on which a monolayer of endothelial cells, such as human umbilical vein endothelial cells, C32 melanoma cells, or *Saimiri* microvascular brain endothelial cell clones (58), can be cultured, or a plastic slide coated with purified proteins, such as CD36, ICAM-1 or thrombospondin, by adsorption. Adhesion under dynamic flow conditions can be quantified by counting adherent IE directly under the microscope.

Nevertheless, with the exception of this flow adhesion model using endothelial cells and immobilized proteins, very few models have been developed for studying placental malaria. One example is the *Saimiri* (squirrel monkey) microvascular endothelial cell line Sc17, which expresses a thrombomodulin bearing only a CSA chain and a CD44-csa isoform. The presence of the chondroitin sulfate of thrombomodulin, or a CD44 isoform, on endothelial cells mimics, to some extent, the presence of CSA on the surface of the syncytiotrophoblast, thereby providing a clear advantage over previous cell models or commercial CSA preparations from various non-placental sources (59). Conversely, one major disadvantage of the use of cell lines as models for CSA binding is the presence of unidentified or unknown adhesion receptors in addition to CSA on the surface of endothelial cells. Another disadvantage of these assays is that CSA preparations from various sources, used by different laboratories, can generate conflicting results. Thus, the conformational modification of CSA by adding dipalmitoyl-diphosphatidylethanolamine may bias results, especially when this system is used to select CSA-binding IE by panning. The addition of charged groups seems to be problematic for the specific selection of CSA-binding IE from laboratory strains and field isolates (28). Furthermore, cytoadhesion inhibition assays with *Saimiri* brain endothelial or Chinese hamster ovary cells cannot distinguish between subpopulations of CSA-binding parasites in field samples.

However, most of these problems can be solved by using normal and at-term human placental cryosections (60), as IE bind almost exclusively to syncytiotrophoblast and in the intervillous space containing proteoglycans with low levels of sulfation (61). The use of human placental tissue makes it much easier to count IE under flow than under static conditions, in which parasites also bind to CSA and to other receptors within the villi. A comparison of inhibition assays carried out under flow and static con-

ditions revealed significant differences in the presence of soluble CSA, or inhibitor mAbs in serum samples from primi- and multigravidae (61). Static parasite adhesion assays are subject to considerable inter-experimental variation, due primarily to differences in washing procedures.

The use of placental cryosections made it possible, for the first time, to measure the shear-stress resistance of CSA-binding IE. Distinct subpopulations within the CSA-binding phenotype were identified by increasing the flow rate gradually from 0.2 to 3.2 Pascal (Pa). For example, at 0.6 Pa, which exceeds the normal shear stress in the placenta (0.05 Pa), 70% of IE remained adherent for the laboratory strain FCR3^{CSA}, and 25% of IE resisted a shear stress >3.2 Pa (60).

These results strongly suggest that the initial FCR3^{CSA} strain was composed of a mixture of strong (≥3.2 Pa) and weak (≤0.8 Pa) CSA-binding parasites, confirming the existence of distinct adhesion subpopulations among the CSA phenotypes of various strains (58; Nogueira PA, Costa FT and Gysin J, unpublished data), and supporting the hypothesis that only some subpopulations of CSA-binding IE have the potential for sequestration in the microvasculature (58). This hypothesis is based on the notion that IE in the placenta are not normally exposed to shear stresses exceeding 0.05 Pa, whereas shear stresses in the postcapillary venules vary from 0.1 to 1 Pa (58).

Conclusions

There is now considerable evidence that PAM is a particularly severe form of malaria, and that primigravidae and their offspring have a higher risk of developing PAM than do multigravidae and their children. These observations, and others, provide an impetus for the development of an anti-PAM vaccine. However, several issues concerning the expression of antigens by PAM parasites and the precise immunological mechanisms involved in protection remain unresolved in the context of PAM. First, does the unique set of hormones and cytokines induced during gestation play a role in antigen or placental host receptor expression? Second, why is CSA the major PAM receptor, given that this glycosaminoglycan is found in various organs other than the placenta? Third, how many antigenically different PAM parasites exist, and what are their relative prevalences in infected pregnant women? Fourth, what is the evolutionary importance of this pan-reactivity and the presence of multiple CSA-binding domains? Can these domains be ordered into a hierarchy? Finally, as some of the poor placental outcomes observed in *falciparum* malaria are also observed in *vivax* malaria, is IE cytoadhesion in the placenta an exclusive feature of *P. falciparum* parasites? Or, like the inhabitants of "Plato's Cave", are we merely watching the "theater of shadows" of real PAM parasite interactions and mechanisms reflected on the cave wall?

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