Clinical and molecular aspects of severe malaria

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ABSTRACT

The erythrocytic cycle of Plasmodium falciparum presents a particularity in relation to other Plasmodium species that infect man. Mature trophozoites and schizonts are sequestered from the peripheral circulation due to adhesion of infected erythrocytes to host endothelial cells. Modifications in the surface of infected erythrocytes, termed knobs, seem to facilitate adhesion to endothelium and other erythrocytes. Adhesion provides better maturation in the microaerophilic venous atmosphere and allows the parasite to escape clearance by the spleen which recognizes the erythrocytes loss of deformability. Adhesion to the endothelium, or cytoadherence, has an important role in the pathogenicity of the disease, causing occlusion of small vessels and contributing to failure of many organs. Cytoadherence can also describe adhesion of infected erythrocytes to uninfected erythrocytes, a phenomenon widely known as rosetting. Clinical aspects of severe malaria, as well as the host receptors and parasite ligands involved in cytoadherence and rosetting, are reviewed here. The erythrocyte membrane protein 1 of P. falciparum (PfEMP1) appears to be the principal adhesive ligand of infected erythrocytes and will be discussed in more detail. Understanding the role of host receptors and parasite ligands in the development of different clinical syndromes is urgently needed to identify vaccination targets in order to decrease the mortality rates of this disease.

Key words: severe malaria, Plasmodium falciparum, PfEMP1, pathogenesis, cytoadherence, rosetting, antigenic variation.

INTRODUCTION

Malaria is the most important tropical disease and causes death of more people than any other transmissible disease, except tuberculosis. Approximately 36% of the world population lives in risk areas. Worldwide estimates of patient numbers is around 515 million annually, and 1.5 to 2.7 million people die due to complications, including 1 million children of less than five years of age (one child every 40 seconds). Other groups at high risk are women during their first pregnancies and non-immune travellers. Plasmodium falciparum is responsible for most of the infections and almost all deaths, occurring in many countries but mainly in the African continent (rev. in Breman 2001 and Snow et al. 2005).

The World Health Organization (WHO) listed 101 countries or territories as endemic for malaria: 45 African, 21 American, 4 European, 14 in the east
Mediterranean, 8 in southeast Asia and 9 in the west Pacific area. However, more than 70% of all malaria cases are in Sub-Saharan Africa. Of the non-African cases, two-thirds of malaria infected people are concentrated in only 6 countries. In increasing order of incidence these countries are: the Salomon Islands, Colombia, Vietnam, Sri Lanka, Brazil and India (WHO 1996).

In Brazil, 99.7% of malaria cases are concentrated in the Amazon Region, mainly in Amazonas, Pará, and Rondônia States, which together are responsible for 85% of the cases (Figure 1). In three consecutive years (1996, 1997 and 1998), the number of cases registered in Brazil was inferior to 500,000. However, in 1999, the official number increased 34% (637,000), including an increment of 15% in the P. falciparum infections. In 2000, there was stabilization (615,000) and in 2001 there was a decrease (389,000) in the number of cases notified. In 2002 (349,000) and again in 2003 (405,000) this number remained stable. P. falciparum was responsible for 21.5% of the infections in 2003. The registration of malaria mortality varies in the Brazilian States and is limited, irregular and imprecise, but around 150 deaths due to malaria are registered annually in Brazil (http://dtr2001.saude.gov.br/svs/epi/situacao_doencas/transmissiveis00.htm).

### CLINICAL ASPECTS OF SEVERE MALARIA

Severe malaria defines infection with manifestations and complications that are potentially fatal in man causing 15 to 20% mortality in spite of effective drugs and correct medical aid. Annually, 5 to 10 million infected individuals develop complications during infection, manifested as coma (cerebral malaria), metabolic acidosis, hypoglycemia, severe anemia, renal failure and lung edema, with frequency varying according to the level of malaria transmission in the area (rev. Miller et al. 1994, 2002) (Table I).

In many parts of the world, cerebral dysfunction is the more common severe manifestation of falciparum malaria and the main cause of death in adults with severe disease. The obstruction of cerebral venules and capillaries with erythrocytes containing mature trophozoites and schizonts causes generalized convulsion and coma and a mortality rate from 4 to 50%. In Thailand and Vietnam, half of the severe falciparum cases are cerebral malaria (Tran et al. 1996), while in other places, like Papua New Guinea, this number decreases to 17% (Lalloo et al. 1996). In Brazil, the number of cerebral malaria cases is not available but the few studies published on severe falciparum malaria in Brazilian patients point to renal failure and respiratory distress as the main complications (Duarte et al. 1985, Corbett et al. 1989, Boulos 1992).

Acute pulmonary edema is also a common fatal complication, presenting interstitial edema with swollen endothelial cells and monocytes narrowing the capillary lumen. The edematous interstitium also contains macrophage with endocytes and malarial pigment (Duarte et al. 1985).

Acute renal failure is another important complication in severe malaria and is defined as an increase in the serum creatinine to above 3 mg/dL or an increase in blood urea above 40 mg%. Patients with acute renal failure without involvement of multiple organs have a good prognosis if peritoneal dialysis is accomplished. In Vietnam, half of the patients with severe malaria presented biochemical evidence of renal involvement (serum creatinine >2 mg/dL), however only 30% filled the WHO criteria for acute renal failure and half of them needed dialysis. Half of the patients with renal failure present lung edema and 45% of these die (WHO 2000).

Laboratory data are important for the diagnosis of severe malaria. Anemia (Hb < 7g/dL, Ht < 20%) is an inevitable consequence of severe malaria and jaundice (total serum bilirubin >3 mg/dL) is common in patients with acute renal failure and parasitemia above 100,000/mm³ (WHO 2000). Another important aspect of severe malaria is the degree of neutrophiles (but not monocytes) containing malarial pigment that, in hypoendemic areas, has been used to predict the gravity of infection, with sensitivity and specificity greater than 73% (Nguyen et al. 1995). In hyperendemic areas, there is no corre-
Main manifestations of severe malaria as related to age-group in areas
of different malaria endemicity.

<table>
<thead>
<tr>
<th>Transmission</th>
<th>Main manifestations of severe malaria</th>
<th>Age-group</th>
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<tr>
<td>High</td>
<td>Anaemia</td>
<td>Young children</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Cerebral malaria and metabolic acidosis</td>
<td>Children</td>
</tr>
<tr>
<td>Low</td>
<td>Renal failure and pulmonary oedema, Anaemia and hypoglycaemia</td>
<td>Adults, Children</td>
</tr>
<tr>
<td></td>
<td>Cerebral malaria and metabolic acidosis</td>
<td></td>
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It is important to note that malaria is a systemic disease where different systems are affected due to infection of the erythrocytes (Boulos 1992, rev. in Miller et al. 2002). The signs and symptoms of severe malaria indicate a complex syndrome, established by host and parasite factors. The main virulence phenotypes are related to cytoadherence,
rosetting and antigenic variation.

MOLECULAR ASPECTS OF SEVERE MALARIA

Sequestration and Cytoadherence

The erythrocytic cycle of *P. falciparum* presents a particularity in relation to other *Plasmodium* species that infect man. Mature trophozoites and schizonts are sequestered in the peripheral circulation (Bignami and Bastianelli 1889), due to adhesion of infected erythrocytes to endothelial cells (Miller 1969). Modifications on the surface of the infected erythrocytes, denominated knobs, provide adhesion sites for endothelium and other erythrocytes (Luse and Miller 1971). It is amply accepted that adhesion to other surfaces leads to better maturation in the microaerophilic venous atmosphere and allows the parasite to escape clearance by the spleen. Sequestration was also observed in primate and rodent malaria. Adhesion to the endothelium, or cytoadherence, has an important role in the pathogenicity of the disease causing occlusion of small vessels and contributing to the failure of many organs (Figure 2) (Miller et al. 1994).

Host Receptors Implicated in Cytoadherence

Several molecules have already been identified as endothelial receptors based on their ability to support the adhesion of infected erythrocytes during *in vitro* assays (Table II).

Thrombospondin (TSP) was the first molecule described as a cytoadherence receptor (Roberts et al. 1985) and demonstrated to bind to an erythrocyte membrane protein, termed PfEMP1 (Baruch et al. 1996). However, the PfEMP1 domain responsible for the adhesion to TSP has not been precisely mapped. As PfEMP1 is implicated in several aspects of severe malaria and pathogenesis, it will be described and discussed separately below.

CD36, the second molecule to be implicated in cytoadherence (Barnwell et al. 1989), is found on monocytes, endothelial cells, platelets and erythroblasts. In spite of high sequence polymorphism, PfEMP1 binds to CD36 via the CIDRα domain (Baruch et al. 1997), and all CIDR1α domains of the 3D7 clone bind to CD36 (Robinson et al. 2003). Indeed, it was demonstrated that a recombinant CIDR sub fragment of 179 residues pertaining to the CD36 binding area, inhibits and reverts adhesion of the infected erythrocytes to the receptor in four different lines of parasites (Cooke et al. 1998).

Intercellular adhesion molecule 1 (ICAM-1) is an endothelial molecule that also acts as receptor for infected erythrocytes (Berendt et al. 1989). The binding site on ICAM-1 has been mapped to the junction of the first and second immunoglobulin-like domains (Berendt et al. 1992). The DBL2β domain of PfEMP1, together with the C2 domain, binds to ICAM-1 (Smith et al. 2000a). ICAM-1 has been shown to have an important role in cerebral
### TABLE II


<table>
<thead>
<tr>
<th>Host receptors (synonymous)</th>
<th>Parasite ligands (domain)</th>
<th>Relevant remarks of each interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSP</td>
<td>PfEMP1</td>
<td>Low affinity of binding in flow conditions</td>
<td>(Roberts et al. 1985, Baruch et al. 1996)</td>
</tr>
<tr>
<td>VCAM-1 (CD106)</td>
<td>?</td>
<td>Receptor member of the immunoglobulin superfamily/Uncommon target of binding</td>
<td>(Ockenhouse et al. 1992, Newbold et al. 1997a, b)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>PfEMP1</td>
<td>Binding is Ca\textsuperscript{2+}-dependent; ligand is questioned</td>
<td>(Ho et al. 1998)</td>
</tr>
<tr>
<td>CSA</td>
<td>PfEMP1 (DBL3γ) (CIDR1)</td>
<td>Binding important in malaria during pregnancy</td>
<td>(Rogerson et al. 1995, Fried and Duffy 1996, Buffet et al. 1999, Reeder et al. 1999)</td>
</tr>
<tr>
<td>HA</td>
<td>PfEMP1</td>
<td>Receptor that mediates adhesion to placenta</td>
<td>(Beeson et al. 2000)</td>
</tr>
<tr>
<td>HS</td>
<td>PfEMP1 (DBL1α)</td>
<td>Receptor presents in endothelial cells and aorta</td>
<td>(Barragan et al. 2000b, Vogt et al. 2003)</td>
</tr>
<tr>
<td>CD36</td>
<td>Sequestrin</td>
<td>Binding is questioned</td>
<td>(Ockenhouse et al. 1991)</td>
</tr>
<tr>
<td>CD36/TSP</td>
<td>Pfalhesin</td>
<td>Ligands are fragments of a modified Band 3</td>
<td>(Crandall et al. 1993, 1994, Eda et al. 1999)</td>
</tr>
<tr>
<td>CD36</td>
<td>CLAG</td>
<td>Knockout of clag9 gene inhibits this adhesion</td>
<td>(Holt et al. 1999, Trenholme et al. 2000)</td>
</tr>
<tr>
<td>?</td>
<td>Pf60</td>
<td>C-terminal exon 7 of pf60 genes present high homology with exon II of var genes</td>
<td>(Carey et al. 1994, Bonnefoy et al. 1997, Bischoff et al. 2000)</td>
</tr>
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</table>

Endothelial leukocyte adhesion molecule 1 (ELAM-1 or E-selectin) and vascular cell adhesion molecule 1 (VCAM-1) have been identified as potential receptors for sequestration of infected erythrocytes (Ockenhouse et al. 1992). These molecules are not expressed constitutively on endothelial cells but can be induced by IL-1 and TNF-α. The parasite ligand responsible for adhesion remains unclear. Association of clinical syndromes and adhesion to these receptors was not found in clinical isolates (Udomsangpetch et al. 1996, Newbold et al. 1997b).

Interaction of infected erythrocytes with P-selectin occurs via the lectin domain and is Ca^{2+} dependent. The binding residue in infected erythrocyte is a trypsin sensitive, sialic acid, suggesting that the ligand could be part of PfEMP1 that interacts with CD36 and ICAM-1, and not CSA that is trypsin resistant (Ho et al. 1998). No study of adhesion and clinical disease has been published with this receptor.

Two other receptors are important for the binding of infected erythrocytes to the placenta. Chondroitin-4-sulfate (CSA), a glycosaminoglycan (Rogerson et al. 1995), is the main molecule involved (Fried and Duffy 1996) and uses PfEMP1 domains as ligands (DBL3, Buffet et al. 1999, CIDR, Reeder et al. 1999). The adhesion is strongly dependent on 4-O sulfation of the saccharide chains (Beeson et al. 1998). Hyaluronic acid (HA) is another receptor important for sequestration in the placenta (Beeson et al. 2000). Infected erythrocytes with dual specificity for association with these two receptors are commonly found.

Heparan sulfate (HS) has been confirmed as a host receptor, mediating cytoadherence to endothelium cells and the aorta via PfEMP1 (Barragan et al. 2000b). Recently, it has been shown that this binding is mediated by the DBL1α domain (Vogt et al. 2003).

Platelet-endothelial cell adhesion molecule 1 (PECAM-1 or CD31) is involved in cytoadherence of field isolates and uses PfEMP1 as a ligand (Treutiger et al. 1997). The analysis of PECAM-1 polymorphism in malaria patients revealed that the frequency of one genotype was a risk factor for cerebral malaria (Kikuchi et al. 2001).

Another endothelial receptor with a little investigated function during infection is alpha (v) beta3. It is an integrin and is involved in many pathological and physiological processes of adherence. The ability of infected erythrocytes to adhere to alpha (v) beta3 in endothelial cells was shown to be 7-270 times larger than for uninfected erythrocytes and the binding was inhibited by anti-alpha (v) antibodies (Siano et al. 1998).

The data reviewed above clearly indicates that wild isolates have a plethora of different host receptors to cytoadhere. However, field studies have demonstrated that there are pronounced differences in the host receptor specificity and extent of cytoadherence. Thus, in spite of the binding of CD36 and TSP to almost all parasites from infected patients (Hasler et al. 1990), CD36 is quantitatively the most important receptor and ICAM-1 the second, adhering to 80% of the isolates. In contrast, minimal or no adhesion to E-selectin, VCAM-1 or CSA, has been found in most isolates (rev. in Newbold et al. 1999). Similar results were obtained in Brazilian isolates using in vitro cytoadhesion assays (Nogueira et al. 2002). Moreover, despite the fact that CSA had been associated with malaria complications during pregnancy and ICAM-1 is thought to have important role in cerebral malaria, some authors failed in the attempt to correlate binding to a certain receptor with specific syndromes caused by malaria (Marsh et al. 1988, Ho et al. 1991). These differences in the host receptor specificity and extent of cytoadherence provide a scenario where different receptors can act synergically to determine the final pattern of adhesion (McCormick et al. 1997).

Parasite Ligands Implicated in Cytoadherence

In addition to PfEMP1 (see below), other parasite proteins located on the surface of infected erythро-
cytes also participate in cytoadherence (Table II).

The clag (cytoadherence-linked asexual gene) genes are a multigene family containing 9 genes located on several chromosomes (Holt et al. 1999). Clag9 is approximately 7 kb, and predicted to be composed of 9 exons. It is located on chromosome 9, is transcribed in mature parasites and is translated into a 220 kDa protein. The precise cellular localization of the protein remains to be determined, however, using structural prediction, four transmembrane domains were found, suggesting the protein is exposed on the membrane of infected erythrocytes. Other evidence such as immunofluorescence, the fact that knockout of the clag9 gene inhibits adhesion of infected erythrocytes to CD36 (Trenholme et al. 2000) and transfection with antisense technology indicate that CLAG proteins are indeed ligands of CD36 (Gardiner et al. 2000).

Pf60 was first identified as a multigene family (Carcy et al. 1994) containing approximately 140 genes; primary structure from one gene of this family was reported afterwards and demonstrated that it was constitutively expressed in all mature parasites and encoded a protein located in the nucleus (Bischoff et al. 2000). The N-terminal domain does not present homology with any protein previously described. In contrast, the C-terminal exon 7 presents high homology with exon II of var genes suggesting a role in cytoadherence (Bonnefoy et al. 1997).

Pf332 is a megadalton protein which is specifically expressed in mature, asexual, blood stage parasites, is translocated from the parasite to the surface of infected red blood cells and is present in all strains with marked polymorphism (Mattei and Scherf 1992). Monoclonal antibodies against Pf332 inhibit cytoadhesion in vitro in a strain independent way (Iqbal et al. 1993), while polyclonal antibodies against Pf332 inhibit growth of the parasite but not cytoadherence (Ahlborg et al. 1995). Unfortunately, its direct role in cytoadherence remains obscure.

Sequestrin is a 270 kDa protein identified by the use of antibodies that mimic CD36 adhesion (Ockenhouse et al. 1991); direct prove of this interaction however, is presently lacking.

Pfalhesin, a form of the Band 3 protein modified by the parasite, was also considered as a ligand in cytoadherence to CD36 and TSP. The binding of infected erythrocytes to TSP occurs via the T3 domain and is mediated by the peptide sequence HPLQKTY of the Band 3 protein (Eda et al. 1999).

The stevor genes (subtelomeric variant open reading frame), previously reported as 7h8 (Limpai- boon et al. 1990), seem unique to P. falciparum. They belong to a multigene family with 30-40 members located in the subtelomeric regions of all chromosomes (rev. in Blythe et al. 2004). stevor genes have 2 exons. Exon 1 is short and codifies an initiation codon and a transmembrane domain. The second exon (~1 kb) codifies 30 kDa of the protein and includes two transmembrane segments (Cheng et al. 1998). The transcription of some stevor genes is restricted to 22-32 hours post invasion (Kaviratne et al. 2002). STEVOR proteins are transported to the Maurer’s clefts and located in the sub membrane of the erythrocyte. Furthermore, these proteins are also expressed in sequestered gametocytes where no PfEMP1 is detected; thus, it is speculated that STEVOR proteins could be mediating cytoadherence (rev. in Blythe et al. 2004).

MOLECULAR ASPECTS OF SEVERE MALARIA

Rosetting

Rosetting signifies the formation of rosettes due to adhesion of erythrocytes infected with mature forms of the parasite to uninfected erythrocytes (David et al. 1988, Udomsangpetch et al. 1989). Rosettes usually appear with some uninfected erythrocytes linked to one or two infected cells although this number can be much higher (Figure 2). Although rosetting has been described in other Plasmodium species that undergo sequestration, such as P. chabaudi, P. fragile and P. coatneyi (Udomsangpetch et al. 1991), it has also been found in other species, like P. vivax, P. ovale and P. malariae, whose mature forms develop in the outlying circulation and do not usually

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Possibly, rosetting allows the parasite to invade uninfected erythrocytes more quickly (Wahlgren et al. 1992), but this has not been confirmed (Clough et al. 1998b). Moreover, rosetting can hide the infected cell thereby protecting it from phagocytosis, one of the main mechanisms of anti-parasitic immunity (Bouharoun-Tayoun et al. 1995).

Rosetting is widely distributed, existing in parasites from all the main malaria areas in the world, with reports in Latin America, Asia and Africa (Wahlgren et al. 1990). The stability of rosetting during \textit{in vitro} cultivation varies, but the rosetting rate frequently decreases after continuous culture (Wahlgren et al. 1994).

**Host Receptors Implicated in Rosetting**

Several binding combinations exist between different host receptors and parasite ligands that can induce rosette formation (Table III).

Oligosaccharides of the ABO blood group were the first host receptors identified in the rosetting process, mainly the blood group A antigens (Carlson and Wahlgren 1992, Barragan et al. 2000a). Binding probably occurs via PfEMP1, however antigens seem to influence only the size of the rosettes rather than the rosetting frequency.

Another receptor that may be involved in rosetting via PfEMP1 is CD36 (Handunnetti et al. 1992); yet, CD36 is present in low levels in mature erythrocytes and thus only rarely participates in rosetting (Wahlgren et al. 1992).

Immunoglobulins in normal serum, mainly IgM, also have a function in rosetting of some strains of parasites, via PfEMP1 (Scholander et al. 1996, Clough et al. 1998a), possibly stabilizing the interaction between infected and uninfected erythrocytes (Treutiger et al. 1999).

Complement receptor 1 (CR1, CD35, C3b/C4b receptor) is a molecule expressed on the surface of erythrocytes and presents an immune regulatory role. CR1 binds to the activated complement components C3b and C4b, and therefore participates in several functions such as clearance of immune complexes from the circulation, an increase in phagocytosis and regulation of complement activation (rev. in Ahearn and Fearon 1989). Rosettes can be formed by the binding of CR1 of uninfected erythrocytes to PfEMP1 of some laboratory-adapted parasite strains (Rowe et al. 1997). Presently, the only PfEMP1 domain implicated in rosetting is DBL1, mediating adhesion to uninfected erythrocytes by CR1 (Rowe et al. 1997, 2000) or glycosaminoglycans (GAG), like HS of the erythrocytes (Chen et al. 1998a).

Moreover, it was demonstrated that a PfEMP1 soluble area (DBL1α) requires a minimum fragment of heparin 12-mers (approximately 4 kDa) for adhesion and this fragment is able to separate naturally formed rosettes (Barragan et al. 2000b).

**Parasite Ligands Implicated in Rosetting**

Besides PfEMP1 (see below), one other molecule seem to be involved in rosetting: RIFINS or rosettins. RIFINS or rosettins are highly polymorphic proteins from 20 to 40 kDa encoded by a multigene family composed of 200 members, denominated \textit{rif}, repetitive interspersed family (Weber 1988, Helmby et al. 1993). The \textit{rif} genes are composed of one short 5’ exon, that encodes a signal peptide, a short intron and another exon of \~1.3 kb. \textit{rif} genes are clustered with \textit{var} genes in the subtelomeric regions of chromosomes (Kyes et al. 1999), are transcribed in the asexual stages and the products are exported to the surface of the infected erythrocyte, where they can be detected from 14 to 16 hours after invasion. Many RIFINS can be expressed on the surface of erythrocytes infected with only one parasite, conferring...
TABLE III


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</tr>
</thead>
<tbody>
<tr>
<td>ABO Antigens</td>
<td>PfEMP1 (DBL1α)</td>
<td>Blood group A</td>
<td>(Carlson and Wahlgren 1992, Barragan et al. 2000a)</td>
</tr>
<tr>
<td>CD36</td>
<td>PfEMP1</td>
<td>Low levels of CD36 in mature erythrocytes</td>
<td>(Handunnetti et al. 1992, Wahlgren et al. 1992)</td>
</tr>
<tr>
<td>IgM</td>
<td>PfEMP1 (CIDR1α) (DBL2β)</td>
<td></td>
<td>(Scholander et al. 1996, Clough et al. 1998a, Treutiger et al. 1999)</td>
</tr>
<tr>
<td>GAG</td>
<td>PfEMP1 (DBL1α)</td>
<td>HS in erythrocytes</td>
<td>(Chen et al. 1998a)</td>
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great antigenic variability. The function of RIFINs has not been established but they are thought to be CD31 and rosetting ligands (Fernandez et al. 1999, Kyes et al. 1999). However, their main function seems to be related to antigenic variation.

**ANTIGENIC VARIATION**

Most of the *Plasmodium* life cycle in the vertebrate host occurs in erythrocytes and is necessary for the infection of mosquitoes and parasite survival. As mature erythrocytes do not differ phenotypically, do not contain internal mechanisms of synthesis or traffic of proteins and do not express class I or II MHC molecules on their surface, they represent an ideal atmosphere for the parasite to hide from the immune system of the host. However, *Plasmodium* synthesizes proteins that cross the parasite plasma membrane, the membrane of the parasitophorous vacuole and are inserted into the erythrocyte surface. After 18 hours of invasion by *P. falciparum*, these surface antigens mediate adhesion to several receptors of the host endothelium, preventing the infected erythrocytes from passing through the spleen, where they would be destroyed. In doing so however, the erythrocyte surface proteins make the parasite “visible” to the host immune system and thus the parasite needs to vary the proteins to avoid destruction (rev. in Newbold 1999). Of notice, *in vitro* studies with *P. falciparum* clones verified that the rate of antigenic switching of a certain variant is around 2% per generation (Biggs et al. 1991, Roberts et al. 1992) (Figure 3). However, mathematical modeling predicts rates substantially slower (0.03% per generation) (Paget-McNicol et al. 2002) or faster (18%) (Gatton et al. 2003).

Antigenic variation was first described in *P. knowlesi* with the observation of the immune response in chronic infection of primates (Brown and Brown 1965). Later, it was described in *P. falciparum* (Langreth and Reese 1979), *P. fragile* (Handunnetti et al. 1987), *P. chabaudi* (McClean et al. 1982), and has been suggested in *P. vivax* (Mendis et al. 1988, del Portillo et al. 2001). The variant antigen of *P. knowlesi* responsible for antigenic variation on the surface of infected erythrocytes was identified in 1983 (Howard et al. 1983). One year later, the same group identified the homologous antigen for *P. falciparum*, which is strain-specific, has ~280 kDa, and was named *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) (Leech et al. 1984).
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Fig. 3 – Schematic representation of PfEMP1 protein switching. Merozoites released from a burst infected red blood cell expressing a particular PfEMP1 phenotype (squares), invade new erythrocytes which after completion of the asexual blood cycle give rise to schizonts expressing the original PfEMP1 phenotype in 98% of the erythrocytic population; remaining erythrocytes express different phenotypes at a rate of 2% (Biggs et al. 1991, Roberts et al. 1992).

Erythrocyte Membrane Protein 1 (PfEMP1) and var Genes

PfEMP1 is encoded by var genes (Baruch et al. 1995, Smith et al. 1995, Su et al. 1995). var genes are present in multiple copies comprising a gene family with approximately 60 copies per haploid genome. They are located in the subtelomeric regions of all 14 chromosomes (at least 1 gene per telomere) in any orientation and in internal clusters on chromosomes 4, 7, 8 and 12 (Rubio et al. 1996, Thompson et al. 1997, Fischer et al. 1997, Gardner et al. 2002). The telomeric location is not a prerequisite for var gene expression, but has been postulated to be important for the generation of diversity, together with mutation, insertion and deletion events (Hernandez-Rivas et al. 1997, Ward et al. 1999, Taylor et al. 2000). Indeed, frequent ectopic recombination facilitating gene conversion has been demonstrated in var genes and occurs in the subtelomeric regions of heterologous chromosomes (Freitas-Junior et al. 2000).

Control of var gene expression is not yet well understood and there are different views with regard to their expression. Thus, some groups believe that several var genes are transcribed during the ring stages and as the parasite matures only one var gene is expressed in mature trophozoites (~16 hours) where only one full-length mRNA message is detected (Rowe et al. 1997, Scherf et al. 1998, Chen et al. 1998b). Yet, other group raises the possibility that there is complete transcription of many var genes in trophozoite stages and that there is selective and rapid 3' to 5' degradation of the products not destined for expression (Taylor et al. 2000). Analysis of synchronized mature parasites selected for a certain receptor (a phenotypically homogeneous population) demonstrated that multiple full-length var genes transcripts could be detected (Noviyanti et al. 2001). Moreover, a monoclonal antibody against the ATS region was also able to detect several bands in extracts of these parasites, although a dominant PfEMP1 was always observed, probably determining the adhesion phenotype. Last, analysis of individual cells confirmed the transcription of multiple var genes by a parasite in the trophozoite-stage (Duffy et al. 2002). Regardless of whether only one full-length as opposed to several full-length var mRNA messages is/are present in mature asexual blood stages, it is a consensus that there is clonal expression of PfEMP1 proteins displaying different adhesive phenotypes by individually infected erythrocytes.

var genes are organized in two exons, with a total size ranging from 6 to 13 kb, excluding the 1 kb intron (Su et al. 1995, rev. in Smith et al. 2001) (Figure 4). Exon 2 (1.6 kb) codifies an acidic terminal
segment (ATS), an intracellular domain, involved in the anchoring of PfEMP1 to host proteins (spectrin and actin from the erythrocyte skeleton) or parasite proteins (KAHRP, knob-associated histidine-rich protein), located in the knob (Oh et al., 2000). Exon 1 (4-10 kb) encodes a transmembrane region and the region that is exposed on the surface of the infected erythrocyte. The extracellular region presents from 2 to 7 highly polymorphic domains, denominated DBLs (Duffy binding like domains), and one or two low polymorphic regions rich in cysteines (CIDR, cysteine rich interdomain region), exclusive to *P. falciparum*. DBLs are homologous to *P. falciparum* erythrocyte binding antigens (EBAs) and to *P. vivax* and *P. knowlesi* Duffy antigen binding proteins involved in the invasion of the erythrocyte and binding to their proteins, such as Duffy and glycophorin A (Adams et al., 1992, Sim et al., 1994). DBLs in PfEMP1 and in EBA present 10 conserved cysteines that are distributed in 10 blocks. DBLs and CIDRs are identified by the position of the domain in the gene (DBL1-7; CIDR1 or 2) and the Greek letter indicates the homology groups (DBLs: α, β, γ, δ and ε; CIDRs: α, β and γ). Among all DBL domains in PfEMP1, DBL1α is the more conserved and consequently has been a target for studies of var gene repertoires in many parts of the world (Kyes et al., 1997, Ward et al., 1999, Kirchgatter et al., 2000). These studies revealed that similarity of DBL1α var sequences is not a predictor of geographic origin.

The structure and organization of the domains from 20 PfEMP1 were determined (Smith et al., 2000b). The authors were able to verify associations of domains such as DBLα, DBLβ, DBLδ, with CIDRα, C2, CIDRβ, respectively. Moreover, two new domain-like conserved regions were also identified and were located in the extracellular part of the molecule. The first is an N-terminal segment (NTS), theoretically globular, that, starting from the methionine initiator residue, can have from 75 to 107 amino acids. NTS does not present homology with any sequence in the databases, but probably has a central α-helix. The second, from 140 to 217 amino acids, was designated C2 and is not present in all of the analyzed sequences but, like the NTS region, is supposedly globular, has an α-helix structure and a unique sequence.

The data on expression of var genes, PfEMP1 proteins and structural organization of PfEMP1 clearly indicates that the increase in expression of a non-dominant gene and its corresponding PfEMP1 variant can facilitate a switch in the adhesion phenotype leading to variants that can be associated to dif-
different clinical syndromes including severe malaria.

**Severe Malaria and PfEMP1 Genotype**

Few studies have reported associations of severe malaria and particular PfEMP1 protein sequences. Thus, a study on a hypoendemic area of French Guyana involving 19 severe malaria patients demonstrated that there was a particular DBLδ var sequence expressed exclusively by these patients as opposed to 32 patients with mild malaria (Ariey et al. 2001). Similar results were obtained in Brazil where parasites from patients with severe malaria transcribed predominantly DBL1α var sequences lacking 1-2 cysteine residues, while parasites from patients with mild malaria transcribed preferentially DBL1α var sequences without these deletions (Kirchgatter and del Portillo 2002). Recently, it was shown that *P. falciparum* strains associated with severe malaria preferentially express PfEMP1 encoded by group A var genes, which are transcribed towards the telomere (Jensen et al. 2004). These data indicate that there are indeed associations between severe malaria and particular PfEMP1 sequences. However, these studies are complicated by the extensive variation and simultaneous transcription of var genes and technical difficulties such as primer bias. Moreover, as cited here, in addition to the var multigene family, other multigene families such as rif, stevor, clag, and Pf60, likely to be involved in virulence, were described in *P. falciparum* (Duffy et al. 2002). High throughput methodologies can now be envisaged to discover and compare the repertoire of genomic and expressed virulence genes circulating in endemic regions with particular clinical syndromes of severe malaria to pursue the development of PfEMP1-based malaria vaccines.

**PfEMP1 Vaccines**

It is amply accepted that acquired immunity in malaria is developed after continuous exposure of patients to different isolates expressing highly variant surface antigens, mainly PfEMP1, and that antibodies against them play a major role in this acquisition. Indeed, the presence of anti-PfEMP1 antibodies has been associated with the development of clinical immunity (David et al. 1983, Reeder and Brown 1996, Bull et al. 1998, Barragan et al. 1998, Giha et al. 1999, Bull et al. 1999, Giha et al. 2000). Immune responses against PfEMP1 however, are variant-specific (Newbold et al. 1992). Thus, individuals with low exposure to *P. falciparum* show limited recognition of the surface of the infected erythrocyte (Marsh and Howard 1986, Bull et al. 1998), whereas sera from adults resident in endemic areas can agglutinate infected erythrocytes from different strains and isolates (Aguiar et al. 1992, Reeder et al. 1994). This data indicates that a vaccine against variant-specific PfEMP1 epitopes might be unrealistic; yet, the use of relatively conserved PfEMP1 domains or new vaccine strategies to generate cross-reacting antibodies may circumvent this problem.

The CIDR1 domain of PfEMP1 is a relatively conserved domain (Smith et al. 2000b). Of importance, in spite of CIDR1 domain being unable to induce high antibody titers during infection (Baruch et al. 1996, 1997), monoclonal antibodies produced against different regions of this domain reacted with several strains, expressing different PfEMP1 variants (Gamain et al. 2001). Moreover, a monoclonal antibody was capable of recognizing 90% of the strains tested, only failing to react with those that do not bind to CD36. Furthermore, some immunization experiments accomplished in monkeys using a 179-amino acid region of the CIDR1 domain, induced protection against a lethal strain (Baruch et al. 2002). More recently, induction of crossreactive antibodies was also obtained after immunization of mice with three different CIDR1 that have critical function of binding to CD36 (Gratepanche et al. 2003). Thus, CIDR1α-based general malaria vaccines have served the proof-of-principle of the possibilities of developing vaccines against variant antigens and other PfEMP1 domains are also being exploited for this purpose (Lekana-Douki et al. 2002, Chen et al. 2004).
PERSPECTIVES

The pathogenesis of *falciparum* malaria involves complex interactions of host and parasite factors further complicated by the fact that antigenic and adhesive properties of circulating parasites may be quite different to those sequestered. The complete sequences of the human and *Plasmodium falciparum* genomes, high-throughput methodologies and reverse genetics, along with clinical data from different epidemiological regions, will hopefully lead to a better understanding of the role of these complex interactions in different clinical syndromes of human malaria and to new opportunities for interventions to treat or prevent severe malaria.

RESUMO

O ciclo eritrocítico do *Plasmodium falciparum* apresenta uma particularidade em relação às outras espécies de *Plasmodium* que infectam o homem. Trofozoítas maduros e esquistões são seqüestrados da circulação periférica devido à adesão de eritrócitos infectados às células endoteliais. Modificações na superfície dos eritrócitos infectados, denominadas “knobs”, permitem adesão ao endotélio e a outros eritrócitos. A adesão fornece um melhor maturação na atmosfera venosa microaerofílica e permite que o parasita escape do clareamento pelo baço, que reconhece a perda de deformabilidade do eritrócito infectado. A adesão ao endotélio ou citoaderência, tem importante função na patogenicidade da doença, causando obstrução de pequenos vasos e contribuindo para danos em muitos órgãos. Citoaderência designa também a adesão de eritrócitos infectados a eritrócitos não infectados, fenômeno amplamente conhecido como “rosetting”. Aspectos clínicos da malária grave bem como receptores do hospedeiro e ligantes do parasita envolvidos em citoaderência e “rosetting”, são revisados aqui. A proteína de membrana do eritrócito 1 de *P. falciparum* (PfEMP1) parece ser o principal ligante adesivo dos eritrócitos infectados e será discutida em maiores detalhes. Uma melhor compreensão da função dos receptores do hospedeiro e dos ligantes do parasita no desenvolvimento de diferentes síndromes clínicas é urgentemente necessária para identificar alvos para vacinação visando diminuir as taxas de mortalidade desta doença.


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