TOWARDS A VACCINE AGAINST PREGNANCY-ASSOCIATED MALARIA

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Summary:

The consequences of pregnancy-associated malaria on pregnant women (anaemia), their babies (birth weight reduction), and infants (increased morbidity and mortality) are well documented. Field observations during the last decade have underlined the key role of the interactions between *P. falciparum* variable surface antigens expressed on infected erythrocytes and a novel receptor: chondroitin sulfate A (CSA) for the placental sequestration of infected erythrocytes. Identification of a distinct *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variant, VAR2CSA, as the dominant variant surface antigen and as a clinically important target for protective immune response to pregnancy-associated malaria has raised hope for developing a new preventive strategy based on inducing these immune responses by vaccination. However, despite particular structure and interclonal conservation of VAR2CSA among other PfEMP1, significant challenges still exist concerning the development of a VAR2CSA-based vaccine with profound efficacy.

KEY WORDS : *P. falciparum*, malaria, pregnancy, vaccine.

Pregnancy-associated *Plasmodium falciparum* malaria (PAM) is considered by the WHO as "one of the most important preventable causes of low birthweight (LBW) deliveries worldwide" and "a major cause of severe maternal anaemia contributing to maternal mortality" (Shulman & Dorman, 2003). Studies show that LBW (< 2,500 g) is the single most important determinant of mortality during the first year of life in African infants, and placental malaria is associated with a two-fold increased risk to give birth to a LBW baby, with the greatest effect in primigravidae (Brabin *et al.*, 2004).

P. falciparum malaria infection is frequent during pregnancy, and the specific tropism for the placenta makes parasite densities often much higher in this organ than in the peripheral blood. It is now clear that the central phenomenon mediating the pathogenesis of PAM is the accumulation of infected erythrocytes (iE) in the placenta (Brabin *et al.*, 2004). Studies clearly show that *P. falciparum* placental parasites express unique variant surface antigens (VSAPAM) that allow the parasite to sequester in the placenta by binding to chondroitin sulfate A (CSA) receptors on syncytiotrophoblast (Beeson *et al.*, 1999; Fried & Duffy, 1996; Ricke *et al.*, 2000; Tuikue Ndam *et al.*, 2004). This placental sequestration is thought to affect the overall organ architecture and function leading to placental insufficiency and impaired intra-uterine growth.

Currently the prevention of malaria in pregnancy as recommended by the WHO relies upon administering intermittent preventive treatment (IPTp) with sulfadoxine-pyrimethamine (SP) to pregnant women, given as two curative doses after the 20th week of pregnancy. This is the current Government recommendation in most African countries and seems to be effective, even though more and more parasites now in the field carry resistant genotypes (Schleiermacher *et al.*, 2002). The efficiency of this strategy is then threatened by the spread of drug resistant parasites in Africa and SP efficacy in the treatment of children is faltering. Currently, IPTp is not given during the first five months of pregnancy, and thus is not able to protect women during the early phase of pregnancy (Peters *et al.*, 2007), which may constitute an important drawback, as the relationships between time of infection and development of pathology are currently unknown.

P. falciparum variable surface antigens (VSA) expressed on infected erythrocytes play a key role in the iE binding to placenta. Specific immune responses against these antigens reduce the effect of PAM during latter pregnancies, making it possible to develop a new preventive strategy based on the enhancement of these naturally-acquired immune responses. This review will tackle the accumulating evidences and challenges associated to this approach.

NATURALLY-ACQUIRED IMMUNITY TO PAM

In areas with stable transmission of *P. falciparum* parasites, people gradually acquire protective immunity to malaria in response to repeated malaria infections episodes they experience over a number of years (Bull *et al.*, 1998; Marsh *et al.*, 1989). Naturally-acquired immunity to malaria observed at adulthood

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in those areas is largely due to the acquisition of IgG with specificity for a repertoire of parasite-encoded VSA expressed on the surface of iEs (Nielsen *et al.*, 2002). PAM occurs when a woman who is supposed to be protected against malaria becomes susceptible to *P. falciparum* infection, as she becomes pregnant. This is particularly observed during the first pregnancy, as anti-malarial immunity is largely restored during the second and subsequent pregnancies, demonstrating the rapid acquisition of immune protection, and suggesting a mechanism specific to pregnancy. Demonstration that PAM is caused by *P. falciparum* parasites expressing an immunologically and functionally unique subset of VSA (VSAPAM), and that protective immunity to PAM is mediated by IgG with specificity for those VSAPAM, resolved the enigmatic loss of immunity at first pregnancy. It is now clear that the rapid restoration of immunity to malaria in multigravid women observed as from the second pregnancy is largely due to the acquisition of antibodies that block adhesion of iEs to the placenta (Fried *et al.*, 1998).

MALARIA VACCINE APPROACH

The promise afforded by attenuated sporozoite vaccines 30 years ago led many researchers to believe that an efficacious malaria vaccine was an attainable medium-term goal. Over 30 years later, no licensed vaccine is currently available for public health intervention. In the 1960s, Nussenzweig (Nussenzweig *et al.*, 1967) showed that malaria sporozoites could be attenuated by X-irradiation and used to vaccinate animals against an infectious sporozoite challenge in several different parasite–host pairs. Most importantly, humans could be successfully immunized with attenuated *P. falciparum* sporozoites. The feasibility of developing an attenuated sporozoite vaccine has been for much of the past 30 years considered impractical, but more recently, the subject has received increased scientific and commercial attention.

The first peptide-based malaria vaccine was developed 20 years ago by Patarroyo's group on the basis of proteins isolated from infected human erythrocytes, which exhibited protection in *Aotus* monkeys. Three of the most effective peptides were chemically synthesized as a continuous peptide linked by PNANP spacer-sequences derived from the circumsporozoite protein (CSP) region repeat (Patarroyo *et al.*, 1987). The resulting peptide (designated SPf66) consisted of three peptide sequences derived from blood stages proteins. SPf66 was the first chemically synthesized anti-malarial vaccine to be extensively tested in human phase III trials (Graves & Gelband, 2000). A series of extensive vaccine studies showed great promise in early studies (Moreno & Patarroyo, 1995), but the results were not reproduced in different settings or when conducted by different investigators (Bojang *et al.*, 1997; Nosten *et al.*, 1996). While being safe and immunogenic, SPf66 showed broad variation in the resulting efficacy of all trials carried out with SPf66/alum (Graves *et al.*, 1998).

Since the 80s', malaria vaccine research has long been centred around a strategy of culturing parasites *in vitro* in human erythrocytes and extract plasmodial antigens. This was followed by a strategy based on selecting clones from an expression library using immune sera and then to produce recombinant antigens and prepare these for a vaccine study.

Merozoite proteins represented the blood-stage antigens most selected for vaccine studies. The efficacy data of the first recombinant antigen blood-stage vaccine in a study involving 120 Papua New Guinean children were only published in 2002 (Genton *et al.*, 2002). This trial showed a 62 % reduction in the parasite densities in the group of children vaccinated with a combination of three recombinant antigens, the merozoite surface protein 1 and 2 (MSP1, MSP2), and the ring-infected erythrocyte surface antigen (RESA). A number of other studies using recombinant antigens have either failed to proceed or are moving forward slowly (Chauhan & Bhardwaj, 2003; Good, 2001; Moorthy & Hill, 2002; Reeder, 2001; Richie & Saul, 2002; Wipasa *et al.*, 2002). More recently, special emphasis has been dedicated to a malaria vaccine candidate linking a recombinant protein containing part of the CSP sequence, to the hepatitis B surface antigen (RTS,S), formulated with a proprietary adjuvant system (AS02A). This vaccine targets the pre-erythrocytic stage of *P. falciparum*, and has been shown to confer protection against experimental infection (Stoute *et al.*, 1997). Short-term protection against infection was shown in immunized adult men in The Gambia in 1998 (Bojang *et al.*, 2001). Then, it was shown that, in African children aged one to four years, RTS,S reduced the risk of *P. falciparum* infection, uncomplicated malaria, and severe disease, and that this protection lasted for at least 18 months (Alonso *et al.*, 2005). The protective effect of RTS,S was confirmed in infants from Mozambique (Aponte *et al.*, 2007). Recent advances in immunity to malaria has meanwhile come to a general agreement that antibodies specific to the parasite variant antigens expressed on the surface of iEs are largely responsible for anti-parasite immunity that develops over several years of parasite exposure. Initially, Marsh and Howard showed in 1986 (Marsh *et al.*, 1986) that sera taken from children in the convalescent stage of a *P. falciparum* infection were able to agglutinate *in vitro* erythrocytes infected with the same parasite strain than the child was exposed to, but not with strains to which other children were exposed. Sera taken from immune adults, by contrast, were able to agglutinate most strains circulating in the community.

It was subsequently shown that each agglutinate formed with sera from immune adults contained parasites of the same strain, suggesting that the immune sera does not recognize a common epitope found on all iEs, but epitopes that are strain-specific (Newbold *et al.*, 1992). These strain-specific antigens later appeared, principally, to be the 'variant' antigens that are termed *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and encoded by the *var* genes (Baruch *et al.*, 1996; Smith *et al.*, 1995; Su *et al.*, 1995), of which there are about 60 variant copies present in the genome of any one parasite. However, only a single PfEMP1 antigen is expressed at any one time at the surface of iEs, and the ability of the parasite to switch expression from one variant to another is believed to be the main parasite strategy to immune evasion (Roberts *et al.*, 1992). The repertoire of unique PfEMP1 types in the global population of parasites is not precisely known, but appears to express unprecedented levels of polymorphism (Barry *et al.*, 2007).

The fact that acquired protection from this syndrome is mediated by an immune response directed against a target that is pregnancy-specific and highly immunogenic is suggested by the strong negative association between gravidity and susceptibility to malaria in pregnancy. Variant surface antigens found in PAM meet these requirements, and initial data implicated this type of antigen in protection from malaria in pregnant women (Fried *et al.*, 1998; O'Neil-Dunne *et al.*, 2001; Ofori *et al.*, 2003; Ricke *et al.*, 2000; Staalsoe *et al.*, 2001; Tuikue Ndam *et al.*, 2004). This evidence has since been strengthened, since levels of antibodies inhibiting adhesion to CSA correlated inversely with susceptibility to preterm delivery and low birthweight (Duffy & Fried, 2003). Furthermore, levels of VSAPAM-specific IgG correlated with maternal haemoglobin levels and infant birthweight, whereas levels of IgG specific for VSA found in non pregnancy-associated malaria expressed by isogenic parasites did not (Staalsoe *et al.*, 2004).

IDENTIFICATION OF THE MAIN VSA_{PAM}, VAR2CSA-PfEMP1

CSA has been consistently identified as the dominant placental adhesion receptor (Beeson *et al.*, 1999; Fried & Duffy, 1996). Although some studies point to the existence of additional receptors (Beeson *et al.*, 2000), current evidence suggests that they are less important than CSA (Fried *et al.*, 2006). iEs from the placenta of pregnant women, and iEs selected for adhesion to CSA *in vitro* fundamentally differ from other iEs in that they express antigens that are specifically recognised by plasma IgG from women (and not from men) that have been previously pregnant, moreover specific antibody levels found in women increase with parity. VSAPAM antibodies found in multigravid women or in infected pregnant women are anti-CSA adhesive antibodies that are associated with protection from PAM (Fried *et al.*, 1998; O'Neil-Dunne *et al.*, 2001; Staalsoe *et al.*, 2004). These observations combined to the fact that CSA-adhesion inhibitory activity of plasma from multigravid women was independent of the geographical origin of both parasites and plasma samples suggested that the parasite ligand that mediates adhesion to CSA is a conserved antigen (Fried & Duffy, 1998). Efforts to identify VSAPAM have largely focused on PfEMP1, a variant antigen implicated in several adhesive interactions (reviewed in Miller *et al.*, 2002). VAR2CSA, the PfEMP1 protein encoded by the *var2csa* gene, has then been identified as the VSA involved in PAM (Salanti *et al.*, 2003; Tuikue Ndam *et al.*, 2005; Duffy *et al.*, 2005; Duffy *et al.*, 2006). This protein is constituted of six Duffy binding like (DBL) domains, at least four of them possessing the ability to bind CSA. VAR2CSA show substantial sequence and structure conservation between isolates (Trimnell *et al.*, 2006), which could explain the geographical independence of antibody responses to the VSA expressed by iEs from pregnant women. The difference in susceptibility to PAM between primigravid and multigravid women is attributed to the lack in primigravidae of antibodies against VSAPAM such as VAR2CSA, and this has raised hope for the development of a vaccine to prevent PAM.

CHALLENGES TO DEVELOPING A MALARIA VACCINE

The factors that regulate anti-malarial immune protection are not completely understood. Innate immunity to malaria has been recently reviewed in (Stevenson & Riley, 2004), and vaccine strategies to induce clinical immunity by neutralizing parasite toxins are being developed (Schofield, 2007). In terms of acquired antiparasite immunity, a major component of the antimalarial response, antibodies to the surface of iEs have been extensively studied in the case of PAM.

A further challenge with VAR2CSA (the dominant PAM antigen identified) like with other blood-stage antigens including MSP1 and AMA1 (Stowers *et al.*, 2002) relates to sequence polymorphism and the question of how many variants would need to be included in a vaccine for optimal efficacy. Although *var2csa* is structurally conserved between *P. falciparum* isolates and is consistently over-expresssed by placental isolates (Duffy *et al.*, 2006; Tuikue Ndam *et al.*, 2005), multiple *var2csa* alleles were found in field isolates (Dahlback *et al.*, 2006).

The *var* gene expression was previously suggested to be hierarchically structured in field isolates, as the expression of certain *var* genes was found to be associated with severe malaria in young children (Jensen *et al.*, 2004). The situation is clearer in pregnancy malaria where a single but not a subset of *var* genes was shown to be specifically expressed by PAM parasites. Nevertheless, a similar, but more restricted, process was observed in the expression of VAR2CSA molecules as some sequence motifs on DBL3X were shown to be more likely to occur in parasites isolated from primi- *vs* multigravidae (Dahlback *et al.*, 2006). This observation suggests that some VAR2CSA variants might be more important in the acquisition of protective antibodies especially in the primigravidae where consequences of PAM are more prevalent and more severe. More research towards detailed mapping of the immune responses to VAR2CSA combined with prospective clinical studies are now a prerequisite in helping to identify those cross-reactive epitopes driving protective immunity within multiple sub-variants of the VAR2CSA antigen.

Although multiple evidence demonstrate that VAR2CSA is a target of PAM-specific immunity and IgG-mediated protective immunity (Salanti *et al.*, 2004; Tuikue Ndam *et al.*, 2006), additional targets and protective immune mechanisms could well exist. A recent study identified a ~ 22 KDa protein, but no protein within the PfEMP1 molecular weight range, as a ligand for CSA (Gowda *et al.*, 2007). Although these findings do not exclude the role of VAR2CSA in the binding of iE to CSA, they suggest that parasite binding to CSA might involve multiple binding ligands or a multiprotein complex comprising VAR2CSA PfEMP1 and other proteins, the identification of which remains an important goal. Microarray approach in transcriptome analysis of field isolates has found specific transcription of novel genes encoding potential surface antigens in fresh placental isolates (Francis *et al.*, 2007; Tuikue Ndam *et al.*, 2008). However, molecular, structural and functional data are still needed to the understanding the biological relevance of those PAM-specific antigens and thus aid in defining critical constructs as vaccine candidates.

Conserved target epitopes/antigens are usually sought for vaccines. The distinct structure of VAR2CSA compared to other PfEMP1 supports a functional and antigenic uniqueness of this VSA member, but analysis of *var2csa* from field isolates has revealed alternating areas of substantial interclonal polymorphism (Dahlback *et al.*, 2006; Trimnell *et al.*, 2006). Moreover, the fact that several VAR2CSA domains have affinity for CSA (Avril *et al.*, 2006; Gamain, 2005), and the findings that human antibodies to VAR2CSA preferentially target polymorphic rather than conserved areas (Barfod *et al.*, 2007) highlight a crucial and fundamental unresolved challenge in identifying protecting epitopes within the VAR2CSA protein. Few studies have reported data on the ability of VAR2CSA-specific antibodies to inhibit parasite binding to CSA. Mouse antibodies raised against VAR2CSA DBL domains can only partially inhibit adhesion of placental isolates to CSA (Avril *et al.*, 2006).

CONCLUSION

Taken together, it is clear that the challenges to developing a malaria vaccine are very significant, given that there is no vaccine 25 years after blood-stage antigens were first cloned. The importance of VSA-specific antibodies in the clinical protection against *P. falciparum* malaria is obvious and that of IgG with specificity for VAR2CSA has been demonstrated in protection against PAM. Range of evidence around the VAR2CSA as the main antigen target characterizing parasites causing PAM suggests for the most optimistic predictions that a VSA-based vaccine will come soon, but still at least 10 years away.

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