An epidemiological approach to malaria vaccine discovery: hypothesis and preliminary results

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Astract Despite sustained efforts, an effective vaccine against malaria is still awaited. The overall objective of the present investigation was to identify antigens and epitopes that are preferentially recognized by immune subjects as potential candidates for protective immunity against *P. falciparum*. We proposed that in the malaria endemic zone antigens specifically recognized by immune subjects are probably implicated in the development of protective immunity to malaria whilst those that are exclusively or preferentially recognized by susceptible subjects are likely to be pathogenic. We tested this hypothesis using an epidemiological approach. We selected and pooled two sets of high-titred sera respectively from 23 parasitised children and 25 immune (non-parasitised) adults. After absorption with immobilized E.coli proteins, the antisera were employed in differential immunoscreening of a 3D7 *P. falciparum* gene expression library obtained from MR4. From 20000 clones screened, 9 clones specifically recognized by sera from the immune adults were, sequenced and compared. It turned out that only two clones designated UB05 and UB9 represented full-length *P. falciparum* antigens. The other clones were fragments of these two clones. Using bioinformatics analysis (BLAST and Swissplot) it was confirmed that UB05 is a hypothetical malaria antigen found on the 10th chromosome of *P.falciparum*. UB09 on the other hand was 100 and 96% identical to a protein related to the well-known malaria vaccine candidate, the circumsporozoite protein and to export protein 1 respectively. This demonstrated that the sero-epidemiological approach could identify a vaccine candidate –the circumsporozoite protein. UB05 has been submitted to Genbank under the accession number DQ235690 and is currently being characterized for its role in protective immunity against malaria. Supported by grants from ISP/IPICS (Project CAM 01)

Introduction

Malaria remains a major public health hazard with 300-500 million cases per year worldwide and 1-3 million deaths annually most of them occurring among African children under the age of five. Malaria ranks first among the most serious causes of morbidity and death in Cameroon. Chemotherapy, the most efficient method of malaria control has recently been undermined by the emergence and spread of drug resistant strains of *P. falciparum* a phenomenon, which highlights the need for a preventive vaccine.

1.1. *Evidence for a malaria vaccine*

Despite sustained efforts, an effective vaccine against malaria is still awaited. Pessimists have it that vaccine development against a complicated parasite such as *P. falciparum* is impossible. But there are both clinical-

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epidemiological and experimental evidence in support of protective immunity and vaccination against malaria (Rechie *et al*, 2002; Ballou *et al*, 2004).

It has been observed that children residing in high transmission areas succumb to malaria attacks more often and more severely than adults in the same area. Furthermore some children are more likely, because of their genetic dispositions, to develop severe-complicated malaria when infected with high doses of the parasite. Serum transfer experiments demonstrated the involvement of IgG antibodies as effectors of this acquired immunity (Cohen et al, 1961). These observations have led to the theory that adults develop anti-disease immunity enabling them to harbor malaria parasites without developing clinical symptoms (Palsoske *et al*, 1994). Under these conditions it might be anticipated that the adults were sensitized by weak immunogens, which take long to induce protection. Alternatively an entirely different set of antigens may be recognized by immune adults in contrast to susceptible ones. By comparing the immune responses of the children with uncomplicated malaria to those with severe complicated malaria one may identify parasite factors that precipitate severe disease and either eliminate them from vaccine formulations or design methods to counteract them.

Other pairs of susceptible/immune individuals have been described in malaria endemic zones (Bigoga *et al*, 2002). Women who are pregnant for the first time (primagravida) are more prone to placental malaria than those who have been pregnant twice or more (multigravida). Recently we described distinct differences in the genetic profile (HLA-DBQ) and *P. falciparum* antigen recognition patterns of pregnant women at delivery with and without parasites in their placentas. Heterozygous pregnant women resisted *P. falciparum* infection better than their homozygous ones; at the same time they recognized preferentially *P. falciparum* antigens with molecular weights of (17, 27, 58, 102, 143, and 223) 10^3. Such antigens may be protective against placental malaria and need to be cloned and characterized. In general since pregnancy results in a depression of the immune system, pregnant women are more likely to succumb to malaria than their non pregnant counterparts (Miller et al, 2000; Bigoga *et al*, 2002).

Two additional sets of observations corroborate the feasibility of vaccination against malaria, whilst at the same time suggesting new avenues towards the perennially elusive vaccine. The now classical experiments in which irradiated sporozoites were used to induce sterilizing immunity against *P. falciparum* infection (in humans) have often been replicated in rodents and non-human primates (Kocken *et al*, 1999; Ballou *et al*, 2004). Realizing that sporozoites would be difficult to produce in large amounts for direct use as vaccines, considerable efforts have led to the identification of the circumsporozoite protein (CSP) as a primary candidate for sterilizing immunity. In other studies more than a score antigens have been identified as potential malaria vaccine candidates targeting the blood stages (Richie *et al*, 2002). These include the merozoite surface antigen (MSP1), the apical membrane antigen (AMA1) and the *P. falciparum* eryth-

rocyte membrane protein 1 (pfEMP1) of which there are 50 polymorphic forms. A prime candidate for a transmission blocking vaccine that would prevent the further development of the parasite in mosquitoes is the Pfs25. These and other antigens have been cloned and tested in stage 1/2 clinical trials, where they have only produced partial protection, (Ballou *et al,* 2004) indicating that the entire set of critical antigens for an effective vaccine cocktail has still to be constituted.

Apart from the fact that sub-unit vaccines often failed to induce immunity in a considerable fraction of subjects tested, they seem to be parasite-strain dependent and effective in one geographical region, but not in others. These observations have led researchers to think of a composite vaccine constituted either from conserved epitopes or the most commonly effective immunogens from a broad range of strains. Though the number of malaria antigens cloned is increasing by leaps and bounds, thanks to the elucidation of the *P. falciparum* genome, it is likely that not all the critical protective antigens have yet been identified. It is estimated that the *P. falciparum* genome codes for 5000-6000 antigens, 40% of which are of unknown function (Hoffman et al, 2000). Systematic testing of these new antigens for protective immunity is not feasible. But a rational approach, which employs sera and cells from protected individuals for screening, may limit the number of potential candidates. The systematic comparison of the malaria antigen recognition patterns between susceptible and immune individuals has been conducted in a few endemic areas leading to the identification of some antigens exclusively recognized by immune subjects to the exclusion of individuals infected with malaria (Lobo et al, 1994). Finally, though previous efforts aimed at vaccine development have concentrated largely on humoral immune responses, it is now evident that T-cell responses play a crucial role as well (Golding et al, 1994).

1.2 Objectives of the study

The overall objective of the present investigation was to identify antigens and epitopes that are preferentially recognized by immune subjects as potential candidates for protective immunity against *P. falciparum.*

1.3 Hypothesis

In the endemic zone malaria parasite antigen recognition pattern is not identical for immune and susceptible individuals. The antigens specifically recognized by immune subjects are probably implicated in the development of protective immunity to malaria. Antigens that are exclusively or preferentially recognized by susceptible subjects are likely to be pathogenic. We decided to test this hypothesis using an epidemiological approach.

The epidemiological approach

The epidemiological approach which we propose consists of the following three essential steps:

2.1. Identification of cohorts of protected/susceptible subjects in a malaria endemic zone. We consider a meso or hyper endemic zone of continuous (non seasonal) transmission of malaria as a suitable site for such a study.

The village of Bolifamba on the flanks of Mount Cameroon fulfills the criterion (Akenji et al, 2005). Using a well structured questionnaire information is obtained about the episodes of fever that the subjects have experienced during the past year. Blood obtained from finger pricks is used to prepare such thick and thin smears for the detection of malaria parasites after staining with Fast^{T}µ stain. The number and type of malaria parasites are quantified. A fever registry is then opened, and based on weekly visits over the next 12 months the immune/susceptible status of the subjects are further confirmed. This type of follow up leads to the identification of two sets of immune/susceptible subjects viz: infected children/healthy adults; healthy adults/infected adults. Although microscopy failed to show the presence of *P. falciparum* in the healthy adults, PCR did in a few cases. However, low levels of infection not detectible by microscopy are not considered to be clinically significant.

2.2 *Differential immunoscreening*. This method involves testing for the reactivity of separated malaria antigens simultaneously with sera from immune and infected ones. Those antigens, which are recognized by the immune subjects but not by infected subjects, are deemed to be potentially protective. Antigen separation before screening may be achieved in different ways.

2.2.1 Immunoprecipitation followed by SDS-PAGE.
Cultured malaria parasites can be radio-labelled *in situ* with S^{35} methionine. Extracted radiolabelled antigens could be immuno-precipitated using the immune/infected antisera, and analyzed by SDS-PAGE in one or two dimensions. Any antigens exclusively recognized by immune sera could be cut out of the gels and microsequenced to obtain information that can be employed to identify it or construct primers/probes for its cloning from the genomic DNA or cDNA gene libraries of the parasite. The major limitation of this approach is the small quantity of antigen that can be obtained from an SDS-PAGE gel or a 2-D gel spot.

2.2.2 A cDNA gene expression library of *P. falciparum* can be screened differentially for expressed antigens that are recognized preferentially by the immune subjects. The detection of expressed genes using antibody probes is well established (Young and Davies, 1983) and has been applied for the detection for protective parasite antigens (Lobo *et al*, 1994, Ghogomu *et al*, 2002). The major draw-back of this approach is that the expression library may be skewed to exclude or under-represent some important antigens. But the method has important merits including the availability of *P. falciparum* cDNA expression libraries from MR4 and well established gene techniques that can allow for rapidly characterizing any identified clones. The use of cDNA libraries from different zones should address the question of skewedness at least in part.

2.2.3 *Other screening strategies can be envisaged.* The use of peptide recombinatorial gene- libraries has the advantage of identifying protective epitopes and using these to identify the parent molecules in the parasite. Also malaria cDNA can be employed to construct phage-display libraries, which can be differentially screened.

2.3 Selection and validation of protective malaria antigens based on in vitro studies. There is currently no agreement on the *in vitro* correlates of protective immunity to malaria. The only acceptable proof for the protective nature of an antigen is that such an antigen should induce protection in humans, a goal that so far has been achieved only partially for the antigens tested (Richie *et al*, 2002; Ballou *et al*; 2004). Several criteria may however indicate that an antigen is involved in protection and these should be checked before too much additional effort is deployed on a candidate antigen.

2.3.1. *The protective antigen should be accessible to immune effector mechanisms.* In order for the immune responses (antibody and/or cellular) to block the antigen, they must react with it. This is possible if the vaccine candidate is deployed on the surface of the parasite or its host cell (the erythrocyte) or if the antigen is excretory/secretory. If the antigen is not surface-associated or excretory/secretory, it is difficult to imagine how it could be exposed to immune attack. Yet certain constitutive antigens have been proposed as candidate vaccines (Cancela et al, 2004). One direct method of localizing the antigen could be to carry out an indirect immunoflourescence test (IFAT) using specific antibodies directed to the target antigen. Another is to characterize the antigen by bioinformatics looking for signal peptides and transmembrane domains, which may indicate whether or not the antigen is membrane bound and/or excretory-secretory.

2.3.2 *The protective antigen should be essential for parasite survival.* Several approaches have been employed to address this question. Growth inhibition studies using antibodies have been conducted to evaluate sera from immune subjects (Bolad and Berzins, 2000). Monoclonal antibodies raised against a protective antigen was included into continuous culture of *P. falciparum* and shown to inhibit parasite growth (Daubenberger *et al*, 2003). An alternative but more demanding approach is to carry out gene silencing (gene-knockout) experiments to block the expression of the target antigen in continuous culture of *P. falciparum*. If gene silencing leads to a lethal mutation or growth inhibition, the target can be deemed essential for survival of the parasite. However, *P. falciparum* knockout systems are not yet widely available particularly in endemic countries.

2.3.3 *The protective antigen should be widely and preferentially recognized by immune subjects, and should not be recognized or only rarely recognized by infected subjects.* It is easy to verify if antibodies to the target are available in the sera of target populations (Lobo *et al*, 1994). An arbitrary cut off point of 30% positives can be envisaged to consider an antigen as being epidemiologically important.

2.3.4 *The protective antigen should be relatively well conserved* i.e. it should not be highly polymorphic. Extensive polymorphism is likely to hamper vaccine design especially if the immune responses to the various polymorphic forms vary from one locality to another. Using the polymerase chain reaction (PCR) it can be easily verified if the gene coding for the

target antigen is conserved in field isolates. We propose that an antigen, which exhibits five or less polymorphic forms like the MSP, can be considered suitable for further study.

Limitations of the Sero-epidemiological approach.

The sero-epidemiological approach for the identification of protective antigens has a number of important limitations, which need to be taken into consideration when interpreting the results.

3.1. Firstly, not all protective responses operate through antibodies. In fact cell mediated protective mechanisms play an important role in malaria and would be missed by this approach. Yet all protective malaria antigens described so far have been shown to elicit antibodies in patient populations (Golding *et al,* 1994; Richie *et al,* 2002; Ballou *et al,* 2004).

3.2 Polyclonal activation leading to the production of irrelevant antibodies to dominant repetitive epitopes are well documented in malaria and may serve as a "smoke-screen" to relevant protective responses. Differential screening is, however, sufficiently selective to allow for the identification of specific responses (see preliminary results below).

3.3 Genetic factors play a role in immune responsiveness to malaria antigens as well as susceptibility to malaria and this may complicate the identification of immune and susceptible subjects. Selection of study populations in various sites and making sure that subjects with diverse ethnic backgrounds are employed can largely eliminate genetic bias.

Experimental

The study site, study subjects, parasitological and serological assays employed in the present study were described in detail previously (Titanji *et al,* 2003; Akenji *et al,* 2005). The methods for the immunoscreening of gene expression libraries were essentially as previously described (Ghogomu et al, 2002). The sub-cloning and the expression of cloned malaria genes in *E coli* followed the methods described by the vendors of the expression vectors pBAD, pET and pQE30. All other Molecular Biology techniques were performed using standard protocols (Sambrook *et al,* 1989; WHO, 1985).

Preliminary Results

5.1. *P. falciparum* prevalence and parasite counts decrease as a function of age. In a survey that was conducted in the principal study site of Bolifamba (Titanji *et al,* 2002; Akenji *et al* 2005) and 13 selected villages of the Mbonge sub-division we confirmed that the prevalence of malaria rises to a maximum between the ages of 1-5years and then declines steadily to a minimum that is attained by the age of 30 years and maintained throughout the sixth decade of life. A corresponding profile was observed for the *P. falciparum* counts, which also rose briefly and then steadily, declined with increasing age. It has been postulated that protective immunity to malaria develops slowly, and this drop in parasite prevalence and density in adults may be a reflection of the protective immune responses.

5.2 *Immunoglobulin G. levels to P. falciparum antigens were higher in adults than in children in the study site.*(Titanji *et al,* 2002) We compared the total IgG and IgG1, 2, and 3 responses in four sets of subjects: children with and without parasitaemia, and adults (aged 18years or higher) with and without parasitaemia. Analysis of the results revealed significantly higher antibody titres to crude *P. falciparum* antigens in adults than children (1-5years). Malaria parasite bearing individuals in both age groups had developed higher antibody titres than their non-infected counterparts. Interestingly, the levels of IgG2 to crude *P. falciparum* antigen was significantly higher in adults than in children in agreement with its postulated protective role (Nguer *et al,* 2001).

5.3 *The differential immunoscreening revealed antigens preferentially recognized by immune sera* (Titanji *et al, 2003*). Based on the serological studies described in 5.2 above, we selected and pooled two sets of high-titred sera respectively from 23 parasitised children and 25 immune (non-parasitised) adults. These sera were subsequently depleted of *E. coli* antibodies using glutaraldehyde insolubilised *E. coli* proteins. The absorbed anti-sera were then employed for differential immunoscreening of a 3D7 *P. falciparum* gene expression library obtained from MR4. From 20,000 clones screened, a total of 120 positive signals were recorded as follows: 63 were recognized by adult immune sera, 37 by infected children and 23 by both groups. After four cycles of screening the adult immune sera recognized 9 clones, the infected children 2 and both groups none simultaneously.

The DNA inserts of the clones recognized by the immune sera were sequenced and compared. It turned out that only two clones designated UB05 and UB09 represented full length *P. falciparum* antigens. The other clones were fragments of these two clones. Using bioinformatics analysis (BLAST and Swissplot) it was confirmed that UB05 is a hypothetical malaria antigen found on the 10th chromosome of *P. falciparum*. UB09 on the other hand was 100 and 96% identical to a protein related to the well-known malaria vaccine candidate, the circumsporozoite protein and to export protein 1 respectively. This demonstrated that the sero epidemiological approach could identify vaccine candidates. UB05 has been submitted to Genbank under the accession number DQ235690.

5.4 Further characterization revealed properties of UB05 that are compatible with those of a protective antigen.

Secondary structure prediction using PHD of the UB05 sequence suggested that it is an all-alpha helical protein, with 63.9% alpha helices and 36.1% loops. Structural prediction of the protein using InterProScan software revealed the presence of an N-terminal signal peptide preceding two transmembrane regions suggesting that UB05 may be surface associated. Three possible C-terminal protein kinase C sites also suggest a regulatory role.

Using plague purified antibodies, the parent protein of UB05 was found in crude *P. falciparum* extracts by Western blotting to be a component of 38kd which was about thrice the protein size of 13.7kd predicted from the

cloned insert. However difference in molecular size could be attributed to post sythetice N-glycosylation for which there is a predicted site on UB05.

Primers were designed to exclude the signal peptide that was on the N-terminal end of the peptide, and employed to screen for polymorphic forms of UB05 in field isolates of *P. falciparum* from patients in Bolifamba. The PCR amplified two fragments respectively of 500bp and 250bp from about 54% (17/31) of the field isolates. The remaining isolates showed only the 250bp allele. It is concluded that UB05 is not highly polymorphic at the studied site.

Attemps so far to mass-express UB05 in *E. coli* have met with variable results. One attempt in pBAD expression vector yielded a fusion protein with thioredoxin of 26kd, which would predict an antigen of 13kd considering the size of thioredoxin.

Conclusions

The proposed sero-epidemiological screening approach is capable of identifying antigens recognized by a well-defined clinical sub-set of patients. The antigen UB05 identified in this study is a potential marker of protective immunity to malaria and should be further investigated.

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