Variant Antigens Characterize Pathologic Immune Complexes in Severe *Plasmodium falciparum* Malaria

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ABSTRACT

Introduction: *Plasmodium falciparum infection is characterized by deadly complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM). The exact mechanisms underlying pathogenesis of these severe forms of Plasmodium falciparum malaria are not fully understood yet they are associated with a lot of morbidity and mortality. Studies have shown a link between severe P. falciparum malaria and levels of circulating immune complexes (CIC) but the exact role of these CICs and the specific malarial antigens involved in the pathogenesis of severe P. falciparum malaria is still unclear.*

Objectives: *This study aimed to investigate the qualitative differences in P. falciparum antigens in serum immune complexes (ICs) between children with the severe forms of Plasmodium falciparum malaria and those with uncomplicated malaria. It was aimed at identifying and characterizing the predominant P. falciparum antigens that contribute to IC formation in these clinical groups.*

Methods*: ICs were purified using polyethylene glycol (PEG) precipitation and dissociated using an acidic buffer (Glycine-HCL pH 2.0). These were then electrophoresed on one-dimensional and two-dimensional polyacrylamide gel blotted by Western transfer and revealed using human hyperimmune sera.*

Results: *Six distinct P. falciparum antigens were found to be associated with severe malarial anaemia while another three antigens were associated with cerebral malaria when compared to their respective controls. An antigen with approximately 91 kDa was highly associated with SA (P < 0.01) while a slightly lighter antigen of about 87 kDa was significantly associated with CM (P < 0.01).*

Conclusion: *These findings may point to differences in qualitative characteristics of ICs in children with SMA and CM and give insight into potential mechanisms of the disease. The findings further suggest differing target of humoral immunity in the severe forms of malaria.*

Keywords- *Circulating immune complexes, Malaria, Pathogenesis, Anemia, Cerebral malaria*

INTRODUCTION

Malaria has plagued mankind with a myriad of mechanisms for avoiding host immune responses. Strategies against both the parasite and the disease are crucial. Malaria infection leads to significant elevation of blood concentrations of immunoglobulins (Igs) and this interacts with the multiple malaria antigens leading to formation of immune complexes (ICs). The elimination of malaria parasites in vivo relies on cellular and antibody-mediated mechanisms directed against malarial antigens 1, 2, and antibody response is the prominent part in immune response against malaria 1, 3. The most important biological functions of antibodies are related to their effector functions aimed at inactivation or removal of infectious agents and their products. Antibody based protective mechanisms form the basis of exposure-based acquired immunity and passive transfers of IgG have provided protection against P. falciparum blood stage in humans 4.

Once formed, ICs may persist in circulation and be deposited in susceptible tissues and hence become pathological. A large body of evidence suggests that immune complexes form during malaria infection 5, 6, 7 8, 9, 10, and have suggested a possible role for CIC in the pathogenesis of severe anemia (SA) and cerebral malaria (CM) 7,10. However, the contribution of these complexes to the pathogenesis of severe malaria is not well understood.

The pathologies associated with ICs differ according to class/subclass of the antibody and size of the ICs and it is becoming apparent that the functional specificity of antibodies to malaria antigens may play an important role in the protective immune responses 11,12, 13. The nature of the antibody is related to differences in biological properties where the Fab region of the antibody molecule combine with the antigen resulting in IC which triggers the effector mechanisms mediated via the Fc part of the immunoglobulin molecule.

The relationship between IgE & CM especially on sequestration of PRBC has been suggested 14, 27, and it is thought IgE-IC might be inducing local overproduction of TNF-α, which plays a major role in CM pathogenesis. IgA-Immune complexes have been shown to bind CR1, just like IgG-IC in patients with glomerulonephritis 15. It is felt that the same could be happening in severe malarial anemia where there is interaction of IC and CR1 and lysis of RBC.

Polyethylene glycol (3.5% w/v) has been frequently used to isolate immune complexes from serum or plasma 16, 17, 18, 19. PEG solution at 3.5% w/v precipitates mainly immune complexes while very few free antibodies are precipitated and very little of other proteins comes down. PEG at 3.5% is thus effective in purifying immune complexes. PEG at 2.0% w/v was used to precipitate ICs for the current study. Once the ICs have been precipitated out, they can be dissociated by an acidic buffer. Ab-Ag complexes are held by weak covalent bonds, which dissociates in an acidic environment. Glycine-HCL at pH less than 3 is normally used 20, 21. Glcine-HCL pH 2.0 was used to dissociate ICs for the current study.

Gel electrophoresis is one method which has been extensively used to separate proteins and sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been described and shown to be efficient in separating proteins and thus is useful in separation and identification of antigens 21, 22, 23, 24, 25. The principle is based on the fact that proteins will migrate at different speeds depending on the molecular weight and the net charge. Two-dimensional gel electrophoresis, (2-DE) coupled with protein identification through proteomics analysis is currently the tool in protein identification technology 26. 2-DE enables the separation of complex protein mixtures and delivers a map of intact proteins 26. 2-DE couples isoelectric focussing, (IEF), in the first dimension with SDS-PAGE in the second dimension, and enables separation of complex mixtures of proteins according to their isoelectric points, mass, solubility and relative abundance. Depending on the gel size and pH used, 2-DE can resolve more than 5000 proteins simultaneously and can detect less than 1ng of protein per spot 26. SDS-PAGE was utilised for the initial screening of IC samples for *P. falciparum* antigens followed by 2-DE for identification of the specific target *P.falciparum* proteins for proteomics analysis.

Although a pattern of association has been established between ICs and *P. falciparum* infection, the exact composition of these complexes in terms of the antigen and the antibody class involved, is not fully understood; so far no clear pattern of association between antigenic composition and protection or severity of malaria has been demonstrated. The current study aimed to further delineate the composition of the immune complexes in terms of the parasite antigens involved in their formation. The parasite antigen composition of the CIC was compared between children with complicated and uncomplicated *P. falciparum* malaria and this was also analysed to reveal relationship with disease severity in an effort to establish any relationship and whether there are qualitative differences between these two groups that could explain their distinct clinical presentations.

MATERIALS AND METHODS
Study Design and Patient Population

This study was reviewed and approved by the Kenya National Ethical Review Committee and by the Human Subjects Research Review Board of the Office of the Surgeon General, US Army. The recruitment of human subjects and study procedures were in accordance with all applicable regulations and informed consent was obtained from all parents or guardians.

We executed a case-control study for qualitative analysis of ICs and results on antibody class and subclass analysis were recently reported 27. SA cases were defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained thick or thin blood smear and hemoglobin $\leq$ 6 g/dL and were recruited from the Pediatric Ward of the Nyanza Provincial General Hospital (NPGH), Kisumu. The NPGH catchment area is the malaria holoendemic region of the Lake Victoria basin, western Kenya. CM cases, defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained blood smear and a Blantyre coma score of $\leq$ 2 28, were

recruited from the pediatric ward of NPGH and the Kisii District Hospital (KDH). KDH is located in the highlands of western Kenya and has seasonal malaria transmission. Consequently, more CM cases were seen at KDH than at NPGH. Children were excluded if they had a history of blood transfusion within three months preceding enrollment. In addition, cases were excluded if there was clinical evidence of other concomitant infections or malignancy. Two types of controls were recruited and matched by age ± 2 months and gender to each case. Symptomatic controls were children with uncomplicated *P. falciparum* malaria that were recruited from the outpatient clinic of the hospital where the respective case was enrolled. Inclusion criteria for these controls were a Giemsa-stained blood smear positive for asexual *P. falciparum* and an axillary temperature ≥ 37.5 ^{o}C or, in the absence of the latter, two of the following signs or symptoms: nausea/vomiting, irritability, poor feeding, myalgias, or headache. Exclusion criteria for controls were the same as for cases with the addition of any evidence of malaria complication manifested by respiratory distress, palmar or conjuctival pallor, hypotension, seizures, hemoglobin ≤ 6 g/dL, or coma.

Collection and Processing of Blood Samples

Approximately 2.5 ml of whole blood was collected at enrollment and at convalescence. Thick and thin blood smears were prepared and stained with Giemsa. The diagnosis of *P. falciparum* parasitemia was confirmed microscopically after scanning a minimum of 200 high power fields. The number of parasites per 500 WBCs was determined. A complete blood count (CBC) was determined using a hematology analyzer (Coulter Corp., Hialeah, FL). The number of parasites/μL was calculated using the white cell density. The serum/plasma was collected, aliquoted into cryovials, and stored at –70 °C until use.

Immune Complex Dissociation and Electrophoresis

ICs were precipitated from plasma by adding 100 μL of 4% PEG (w/v) solution (PEG 6000, Fluka, St. Louis, MO), to 100 μL of plasma diluted 1:5 in borate buffer pH 8.5 (Pierce, Rockford, IL). This was mixed well and incubated overnight at 4 °C then centrifuged at 5,000 RPM for 10 minutes at 4 °C. The IC precipitate was washed twice with 200 μL 2% PEG solution. IC pellet was then re-suspended in 100 μL borate buffer pH 8.5 and stored frozen until use. The purified immune complexes were dissociated using an acidic buffer. 100μl of the purified IC sample was mixed with an equal volume of 1.5M glycine-HCL pH 2.0 and incubated for 1h at 37°C. The samples were then neutralized by adding 100μl 1.5M Tris-HCL buffer pH 8.5. The dissociated immune complexes were then separated by electrophoresis on Novex 4-12% Tris-glycine precast gels (Invitrogen). 10μl of the diluted samples and standards were loaded to each well in the gel and electrophoresis done at 200 volts constant for 50 minutes.

Western blotting was then done according to the procedure provided (Novex, Invitrogen). The membrane was probed with human malaria hyperimmune serum. Goat anti-human IgG HRP-

conjugated was used as a secondary antibody. Bands were then revealed by addition of chemiluminescense substrate (Pierce), followed by signal acquisition by exposure of photographic film, Clear Blue X-Ray film (Pierce). Preliminary parasite specific band identification and analysis was done and those samples showing specific parasite proteins were then subjected to 2-dimensional gel electrophoresis (2-DE) and the specific parasite spots punched out for preliminary proteomics analysis to confirm *P. falciparum* origin.

Statistical Analysis

Fishers' Exact test was used to test for association of severity to specific *P. falciparum* malarial antigens. All tests were two-tailed with α ≤ 0.05.

RESULTS

Demographics

The demographic characteristics of the study participants were recently reported 27 and are summarized in Table 1. The mean age (range) for CM cases was 31.2 months (3 months to 7 years) and for SMA cases was 16.8 months (5 months to 7 years). Despite the overlap in ages, the mean difference in age for the two clinical groups was significant ($P < 0.001$ by an independent samples t test). There were no significant differences in the mean parasite densities although CM cases had higher parasitaemia.

Table 1. Demographics of the study groups. SD - Standard Deviation; N, Sample Size

Variable	Severe Anaemia		Cerebral Malaria	
	Cases (N=75)	Controls (N=74)	Cases (N=32)	Controls N=52
Mean Age (SD) in Months	16.9 (13.7)	16.8 (13.3)	33.1 (19.2)	33.6 (16.4)
No. Female (%)	33 (44)	32 (43)	16 (50)	29 (56)
Mean Haemoglobin (SD) in g/dl	4.7 (0.9)	8.8 (1.7)	9.0 (1.7)	9.3 (1.6)
Mean Parasite density (SD)	4468 (6742)	4427 (4687)	6493 (6274)	5180 (5887)

P. falciparum Parasite Specific Antigens Which Participate in IC Formation

Immune complexes were purified from patient's sera using 2% polyethylene glycol (PEG), and 1–dimensional polyacrilamide gel electrophoresis

(PAGE) and Western blotting done. There were several different *P. falciparum* antigens detected from the blots probed with human hyperimmune serum. *P. falciparum* antigen analysis was done with Fisher's exact test and six specific *P. falciparum* antigens (ranging in approx. weight from 60-120 Kda) were found to be significantly associated with severe malarial anaemia (Table 2) while one Ag#5 (approx. 113 Kda) was found to be

associated with both SMA and CM (though weakly associated to CM). Another three different antigens (Table 3) were found to be significantly associated with cerebral malaria (ranging from approx. 40 to 110Kda).

When the two severe groups, SA and CM were compared together, it was found that Ag #15 was highly associated with SA (P = 0.002), while Ag #17 was significantly associated with CM (P = 0.003) (Table 4). Figures 1-4 are representative blots showing the significant parasite bands.

Figure 1. *P. falciparum* antigen profiles of representative test samples showing some of the significant *P. falciparum* parasite bands. ICs were dissociated, electrophoresed on 1D gel blotted by Western transfer and probed with human hyperimmune serum. Note lanes: MW- Molecular weight marker, Pf – Crude extract positive control, IgG – human IgG negative control, S1-S5 – test IC samples.
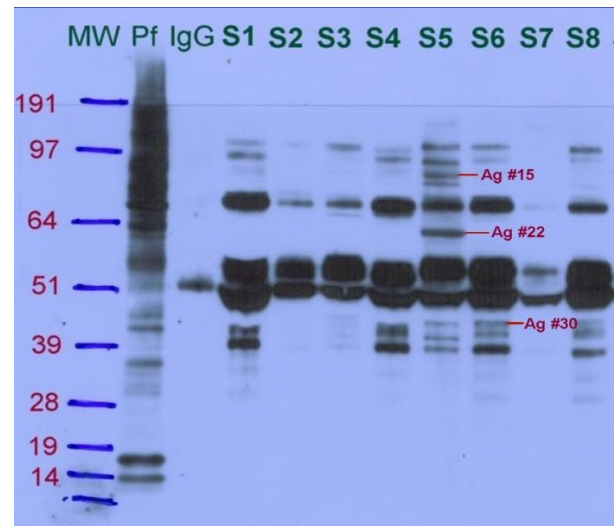
Figure 2. *P. falciparum* antigen profiles of representative test samples showing some of the significant *P. falciparum* parasite bands. ICs were dissociated, electrophoresed on 1D gel blotted by Western transfer and probed with human hyperimmune serum. Note lanes: MW- Molecular weight marker, Pf – Crude extract positive control, IgG – human IgG negative control, S1-S8 – test IC samples.

Severe malarial anaemia when compared with symptomatic controls, was significantly associated with six distinct *P. falciparum* antigens; Ag #5, (P = 0.004), Ag #13 (P = 0.03),

Table 2: *Plasmodium falciparum* specific antigens significantly associated with severe malarial anaemia. Analysis was done by 2X2 contingency tables Fisher's Exact Test. SA – Severe Malarial Anaemia, Pf – Plasmodium falciparum, Ag – Antigen, MW – Approximate Molecular weight (kDa)

| Pf Antigen | MW | SA Cases | | Controls | | |
		Positive	Negative	Positive	Negative	*p* value
Ag 5	113	21	50	6	57	0.004
Ag 13	109	20	51	8	55	0.03
Ag 15	91	33	38	12	51	0.001
Ag 18	85	35	36	17	46	0.01
Ag 22	73	12	59	1	62	0.002
Ag 25	65	15	56	2	61	0.002
Ag 26	62	40	31	24	39	0.03

Table 3: *Plasmodium falciparum* specific antigens significantly associated with cerebral malarial. Analysis was done by 2X2 contingency tables Fisher's Exact Test. CM – Cerebral malaria, Pf – Plasmodium falciparum, Ag – Antigen, MW – Approximate Molecular weight (kDa)
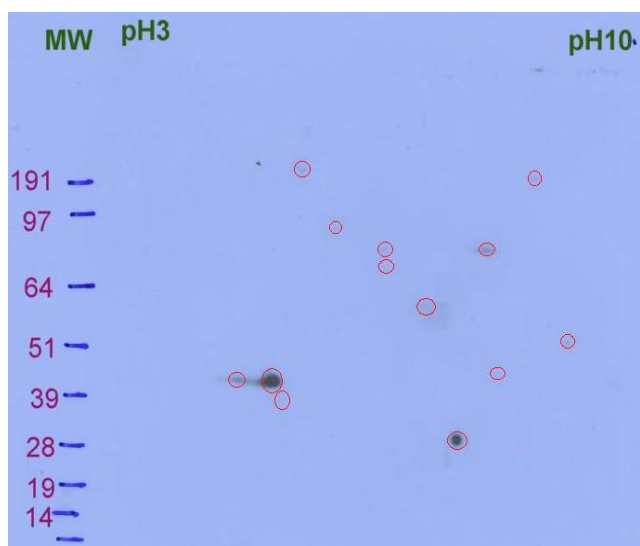
| Pf Antigen | MW | CM Cases | | Controls | | |
		Positive	Negative	Positive	Negative	*p* value
Ag 5	113	5	23	0	26	0.05
Ag 6	109	9	19	1	25	0.01
Ag 17	87	16	12	5	21	0.005
Ag 30	40	14	14	5	21	0.02

Table 4: *Plasmodium falciparum* specific antigens significantly associated with cerebral malarial. Analysis was done by 2X2 contingency tables Fisher's Exact Test. CM – Cerebral malaria, Pf – Plasmodium falciparum, Ag – Antigen, MW – Approximate Molecular weight (kDa)
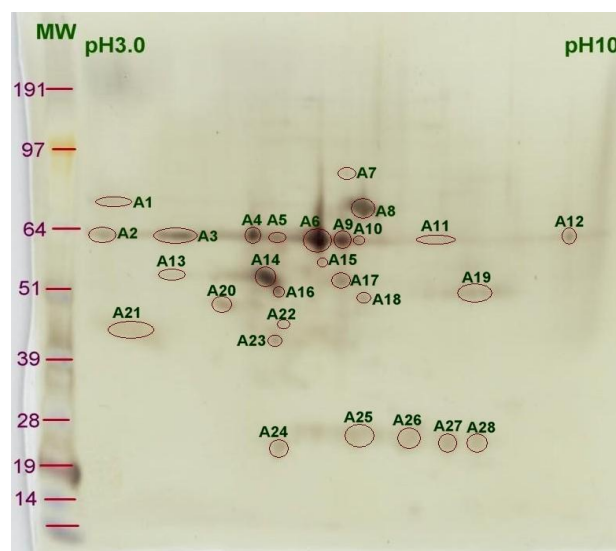
| Pf Antigen | MW | SA Cases | | CM Cases | | |
		Positive	Negative	Positive	Negative	*p* value
Ag 15	91	33	38	4	24	0.002
Ag 17	87	17	54	16	12	0.003

Ag #15 (P < 0.001), Ag #18 (P = 0.01), Ag #22 (P = 0.002), Ag #25 (P < 0.001) and Ag #26 (P = 0.03) (Table 3). While another three distinct antigens were significantly associated with cerebral malaria; Ag #6 (P = 0.01), Ag #17 (P = 0.005) and Ag #30 (P = 0.02) (Table 4).

Representative samples with the significant *P. falciparum* antigens were further processed on 2-D gels identified and punched out and eluted. Initial proteomics analysis of the 2-D separated proteins revealed the antigens detected were indeed of *P. falciparum* origin though they were not specifically identified. Representative blots are shown in Figures 2 and 3.

Figure 4. *P. falciparum* antigen profiles of one SA sample showing the diverse separated protein spots. ICs were dissociated in glycine-HCl pH 2.0, solubilized in NaOH, IgG/human albumin depleted then electro-focussed and 2-DE done. The gel was then silver stained to reveal the protein spots which were then punched out for preliminary identification. Note: MW- Molecular weight marker (kDa), A1-A28 – Protein spots for preliminary proteomics identification.

Figure 3. *P. falciparum* antigen profiles of representative SA sample showing some of the significant *P. falciparum* parasite spots. ICs were dissociated, electro-focussed and 2-DE done, blotted by Western transfer and probed with human hyperimmune serum. Note: MW- Molecular weight marker (kDa).

DISCUSSION

Plasmodium falciparum malaria infection leads to generation of ICs. The role of antibodies in malaria protection is not yet fully clear and the effect of each isotype is not fully understood and remains a controversial subject. ICs can lead to cell and end organ damage by their deposition on cell surfaces and by initiating complement activation resulting in the deposition of complement activation products on erythrocytes and other organs such as kidneys 29. By cross-linking Fc receptors on effector cells like macrophages and monocytes, ICs can also stimulate the production of pro-inflammatory cytokines 30,31

that have been proposed to have a role in the pathogenesis of severe malaria 32. Despite the accumulating evidence linking immune complexes to malaria, no clear pattern of association between antibody isotype and nature of antigen and protection against or susceptibility to malaria has emerged. Furthermore most of the study findings have been based on free antibodies and not complexed antibodies and not the antigen part of the immune complexes. The main objective of this study was to profile the parasite antigens involved in IC formation and to assess the association to severity of *P. falciparum* malaria.

A recent study using the same sample group found that IC levels of children with CM were higher than those with SA and the difference was significant for IgG total, IgG1, IgG2, IgG4, IgA and IgE antibody classes/subclasses 27. The study found out IC levels diminished in all groups in response to malaria treatment, as earlier shown from the same study area 2727, suggesting that malaria played an important role in IC formation. These studies found that there was no difference in mean parasite densities between CM and SA cases that could account for the differences in IC levels.

Soluble malarial antigens as well as antibodies to various plasmodial constituents have been demonstrated in the sera of patients infected with either *P. falciparum* or *P. malariae* 6, 33, 34, 35, 36. These antigens may be available for binding by reactive antibodies and this sets the stage for the formation of CICs 7 37 and i*n vivo* complement activation leading to hypocomplementamia which are more common in patients with malaria

complications such as severe anaemia, cerebral malaria and thrombocytopenia of *P. falciparum* 6. Furthermore, repeated exposure to parasite antigens contribute to development of acquired immunity to malaria 38. Immunity to malaria only occurs after many years of recurring infections. This is believed to be due to antigenic variation and the time taken by individuals to develop immunity to invariant parts of otherwise very polymorphic antigens. Immunity to malaria manifests as lessened disease symptoms and lower parasitaemia. A large body of evidence shows that antibody responses against *P. falciparum* variable antigens on the surface of RBC contribute a lot to acquired immunity against malaria 39, 40, 41 42. Furthermore, adhesion of infected RBC to vascular endothelium via these variable antigens is thought to contribute to the pathogenesis of malaria 11, 42, 43.

Several protective malaria antigens were isolated from immune complexes and the significant *P. falciparum* antigens were different in CM and SMA cases (Table 5 – Table 7). This finding may point to different immunological responses in the two categories. Although several antigens detected were shared across the clinical categories, there were those which were found to be characteristic to a particular group.

When the two severe malaria clinical groups, SA and CM were compared together, a *P. falciparum* antigen with a molecular weight of approximately 91Kda, was found to be highly associated with SA (P = 0.002), while a slightly lighter antigen of about 87Kda was significantly associated with CM (P = 0.003). As to whether the two antigens are related or

different remains to be known although initial analysis of the profiled purified proteins revealed they are *P. falciparum* derived. Identification of these various antigens is of paramount importance as it will enable purification of such antigens which elicit higher levels of protective or appropriate immune responses thus being considered as vaccine candidates.

Malaria antigen analysis is vital in helping to understand and profile the various antigens in relation to their contribution to protection and/or pathogenesis of the disease. Malaria infection is complex and involves many inflammatory responses which may enhance cell to cell interactions (cytoadhearance) and cell stimulation involving both malaria-derived antigens or toxins and host derived factors such as antibodies and cytokines 44, 45, 46. Malaria antigens and toxins are mostly stage-specific. It has been observed that during malaria infection, the peaks of parasitaemia fluctuate over time 47. This is due to presentation of different antigenic determinants and this is a survival strategy of the parasite. Merozoites and late stage trophozoites or schizonts undergo antigenic variation thus rendering the host immune responses ineffective. This variation originates from genetic recombination in the mosquitoes and also through existence of several variable antigens gene families in the genome of the parasite. The large reservoir of variant antigens gives the parasite the ability to avoid specific and non-specific immune clearance by the host 48.

Several blood stage antigens may be the target of protective antibodies and some of which have been correlated with clinical immunity. Such malaria antigens have been included in malaria vaccine preparations in humans 49. RESA, MSP-1 and MSP-2 were shown to be targets of protective immunity in experimental models 50, 51 and also in humans 52. These antigens are also recognised by naturally acquired antibody 53, 54.

Malaria toxins are parasite derived molecules which induce the human host to over produce serum-bound factors and include parasite-derived molecules secreted or released from parasite at late stages (Trophozoites and schizont stages) and are contained among the glycosylphosphatidylinositol (GPI)-anchored proteins. Parasite products either directly damage host tissues or more importantly stimulate the overproduction of host cytokines 46. Studies have shown that *P. falciparum* cultures contain antigens which stimulate the secretion of TNF-α and IL-1 and other cytokines from various host cells 55. Moderate amounts of cytokines such as TNF-α, IFN-γ and IL-1 are necessary for the human host in fighting invading microorganisms but over induction of host cytokines can vary the disease pathogenesis. High circulating levels of TNF-α and high fever occur at the rapture of schizonts and this suggests antigens from schizonts might have potential toxic effects 56. Also *P. falciparum* metabolite hemozoin released after schizont rapture is toxic and has been suggested to induce IL-2 production 57.

Previous studies on human antibody responses to malaria have focused on the antigenic specificity of the response but it is increasingly becoming apparent that it is important to consider the quality

of the immune response. This involves both the affirnity of the antibody for the antigen and the nature of Fc which determines the effector functions of the antibody including antibody mediated cellular cytotoxicity which has been described as a potentially important mechanism of anti-malarial immunity. It has also been proposed that a predominance of IgG1 and IgG3 is required for an effective anti-malarial immunity 11. Thus an ideal vaccine candidate should be one which elicits the appropriate antibody class/subclass and also an effective cell mediated immunity.

COCLUSION

These results together with complete antigen analysis and identification will guide future studies and enable picking those specific antigens which stimulate the appropriate immune response in the human host and will guide on malaria vaccine development as has been shown by the ability of different antigens to stimulate different arms of the immune system with varying levels of protection.

ACKNOWLEDGEMENTS

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