J Vector Borne Dis 49, September 2012, pp. 151–156

Evaluation of host humoral antibody production against *Plasmodium falciparum* recombinant circumsporozoite antigen in Nigerian children

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ABSTRACT

Background & objectives: The challenge of malaria and efforts targeted at developing malaria vaccines triggered this study on the reactivity of IgG and its subclasses in the test serum specific to CSP. This work was directed at assessing the influence of age and gender on host humoral antibody against *Plasmodium falciparum* recombinant circumsporozoite antigen in Nigerian children.

Methods: In all, 67 serum samples (>10,000 parasites/µl of blood) collected from malaria-infected children at the University College Hospital, Ibadan during the transmission season were analyzed by ELISA.

Results: The mean absorbance values of IgG subclasses reactive against *P. falciparum* CSP appeared to be age-dependent and ranged from 0.01 for IgG4 in younger children to 0.95 for IgG3 in older children. The sixty-seven subjects investigated in this study had significantly higher mean IgG1 and IgG3 than the uninfected controls (p <0.01). This follows the order IgG3 >IgG1>IgG2>IgG4 which confirmed the prevalence of the cytophilic antibodies (IgG1 and IgG3) in 65% of the malaria infected children over the non-cytophilic subclasses (IgG2 and IgG4). Similarly, there was low production of IgG4 and IgG2 levels in 35% of the subjects compared with control. IgG was detected in the serum of North American Subjects (NAS) which served as negative control for CSP-specific IgG subclasses. Although the NAS titre was lower than that of the malaria subjects in Nigeria, its IgG2 was, however, higher (0.16) than that of other subclasses. The mean absorbance values of total serum IgG subclass were higher than those of IgG subclasses specific to *P. falciparum* circumsporozoite antigen. The mean absorbance values of the total serum IgG subclass follows the order IgG2>IgG1>IgG4>IgG3.

Interpretation & conclusion: Age and gender-dependent correlations of results suggest that acquired immunity could play a significant role in protection from malaria. Antibody levels are higher in male than female children of the same age group. Antibody levels also increase with age in both the male and female children.

Key words Cytophilic antibodies (IgG1 and IgG3); ELISA (enzyme-linked immunosorbent assay); malaria vaccine; non-cytophilic subclasses (IgG2 and IgG4); *Plasmodium falciparum* circumsporozoite protein (PfCSP)

INTRODUCTION

Immunoglobulin-G (IgG) antibodies are produced against a number of malaria vaccine candidate antigens including the pre-erythrocytic antigen CSP[1]. It has been associated with some degree of protection from clinical malaria in areas of stable transmission[1]. The pre-erythrocytic *Plasmodium falciparum* antigen CSP is a component of a multistage malaria vaccine[2,3]. However, the mechanisms by which this antigen induces protection against malaria in humans have been the subject of multiple investigations.

Information obtained to date was based primarily on observations of naturally infected individuals living in areas where there is stable malaria transmission. Residents of such areas could develop partial protection against severe malaria morbidity coupled with high density asexual parasitemia with increasing age[3]. Although the protective mechanisms have not been completely fully understood, they are postulated to involve both cellular and humoral immune responses elicited by both pre-erythrocytic and blood stage antigens as a consequence of repeated sporozoite and blood stage infections[4,5]. It has also been suggested that IgG and its subclass antibodies to CSP could mediate or represent surrogate markers of resistance to infection and malaria morbidity in areas of Africa where malaria is holoendemic[6].

In the same vein, studies of pre-erythrocytic immunity in animals and humans have focused largely on T-cell-mediated immunity[4,7], a role for antibodies to pre-erythrocytic antigen in protection from infection and disease in humans cannot be excluded. For example, antibodies to CSP have been reported to impair the liver-based differentiation of sporozoites into merozoites in rodent malaria models [8,9].

Most of those infected with malaria recover and go on to acquire immunity to it, but there may, however, be hidden damage even among those who survive[10] especially children living in countries where the diseases is endemic (*e.g.* Tropical Africa) where the majority of children who

developed malaria pass through several crisis involving convulsions, perhaps even coma, which may lead to blockage of blood vessels in the brain and such damage might impair intellectual performance later in life but the intensity and level of such damage is difficult to evaluate[10]. In spite of the fact that circulation by invading sporozoites rapidly leave the circulation by invading host liver cells or through generalized phagocytosis, ability to induce protective immunity has been demonstrated in avians, rodents, primates and humans[11] . The protection is both species and stage-specific and has both humoral and cellular components[12]. The target antigen of the protective immunity is the CSP, a major polypeptide covering, which consists of conserved central region of repeating tetramers (B-cell determinants) that is the primary target of humoral response[8] and the non-repetitive flanking regions that are the main targets of cellular response[11]. Antibodies to the CSPs occur frequently in individuals living in areas of hyperendemic malaria[13]. The antibody levels and prevalence are also proposed to be indicators of malaria transmission[12]. The levels of antibodies, therefore, demonstrate seasonal variation in areas of high malaria transmission. A number of different recombinant and synthetic (NANP)n peptides have been produced and used to develop immunoassays for detection of antisporozoite antibodies in human serum. These peptide-based methods, mainly enzyme-linked immunosorbent assays (ELISAs) have been used in molecular epidemiological research and the results obtained are similar to those obtained with immunofluorescent assay (IFA)[11,12].

In this paper, the responses of IgG and its subclass antibodies against CSP antigen contained in recombinant R32tet32 protein which might serve as correlate of protective immunity in human vaccine trials were evaluated. Investigation was also carried out to determine whether low or high level titres of individual IgG subclasses and total against R32tet32 could be associated with protection from *P. falciparum* infection in children.

MATERIAL & METHODS

Ethical considerations

All the aspects of the study were approved by the University of Ibadan/University College Hospital, Ibadan Joint Ethical Review Board.

Collection of blood samples

Screening and collection of blood samples were done at the General Outpatients Clinic (GOP) in the Department of Pharmacology, University College Hospital, Ibadan, Oyo state, Nigeria.

Informed and free consent was obtained from the parents or legal guardians of all children included in this research work. The proper detection of human antibodies specific to the malaria antigen (R32tet32) by ELISA was done at Cellular Parasitology Laboratory in the Department of Zoology, University of Ibadan, Nigeria. Serum samples were obtained from 67 children of <10 yr already confirmed positive to *P. falciparum* parasitemia by thick blood smear. About 1–2 ml of blood was taken from the children aseptically by venipuncture. Serum was obtained by centrifugation and stored at –4ºC at the Institute for Advanced Medical Research and Training Laboratory, College of Medicine, University of Ibadan, Nigeria.

Control serum

Two control sera were used, namely the negative control serum (NAS) and pooled negative control serum (PNCS). The NAS was obtained from an individual (North American subject) who has never been exposed to malaria with negativity to the antigen compared and defined[14]. The PNCS was also obtained from individuals living in Igboora area of Oyo state, Nigeria. Previous studies showed that the pooled negative control did not produce antibodies against the antigen R32tet32[14].

Detection of IgG subclasses specific to P. falciparum CSP by ELISA

The detection of IgG subclasses specific to *P. falciparum* CSP by ELISA was carried out using a modification of the method of Ozurumba *et al*[14]. Recombinant circumsporozoite antigen (R32tet32) solution (1 mg/ml) was diluted to a working concentration of 1: 500 (6 µl antigen in 3000 µl solution A) with solution A (40 ml boiled casein/50 ml phosphate buffer saline). 50 µl antigen was placed in the wells and solution A was placed in the two blanks. Incubation was done at 4ºC overnight. On the following morning, contents were aspirated and dried. Fresh blocking buffer (0.5% boiled casein, 0.2% tween 20) was prepared immediately. 200 ml of blocking buffer was dispensed to block each of the wells on the plate. The system was left to stand for 1 h. The test sera were diluted with blocking buffer (1:10); 45 ml of serum was added to 450 ml of blocking buffer and the content mixed with the multichannel pipette. 50 µl of the solution was added to the wells leaving the two last rows at the bottom of the plate for the addition of the control serum. The control sera were treated along with the test sera and added to the wells. The test sera were all assayed in duplicates. The plate was covered, incubated for 1 h at 37ºC, the wells were aspirated and washed twice with wash solution (Dulbeccos phosphate buffered saline) and flick-dried.

Monoclonal antibodies were raised against each of the IgG subclasses at dilution 1: 500 (Anti IgG1; Anti IgG2; Anti IgG3 and Anti IgG4). 10 ml of artificial monoclonal antibody was diluted to 5000 µl with blocking buffer and mixed gently. 50 µl of the mixture was added to the wells, covered and incubated at 37ºC for 1 h. After 1 h incubation, contents were aspirated, washed thrice and flick-dried. Dilute goat antimouse IgG-alkaline phosphatase was prepared at dilution 1:2000. 2.5 ml of goat antimouse IgG-alkaline phosphatase was added to 5000 µl blocking buffer mixed gently. 50 µl of the mixture was then put in the wells. The system was kept at room temperature for 2 h. 50 µl of substrate and substrate buffer solution of cyclohexylammonium of paranitrophenol phosphate (PNPP) at equal volume (1 mg/ml) to Diethanolamine-/-p-Nitrophenylphosphate (DEA/PNPP) were then added to each well and incubated for 45 min to 1 h at 37ºC for the colour to develop. 50 µl sodium hydroxide was then added to terminate the reaction and to increase the intensity of the colour formed. Absorbance value was measured at 405 nm with the ELISA reader.

Measurement of total IgG subclasses specific to CSP by ELISA

Recombinant circumsporozoite antigen solution (1 mg/ml) was diluted to a working concentration of 1:500 (6 µl antigen in 3000 µl solution A) with solution A (40 ml boiled casein/50 ml phosphate buffer saline). 50 ml Antigen was placed in the wells. Incubation was done at 4ºC overnight. On the following morning, contents were aspirated and dried. Fresh blocking buffer (0.5% boiled casein, 0.1% tween 20) was prepared immediately. 200 ml blocking buffer was added to block each of the wells on the plate. The system was left to stand for 1 h. 3 ml serum was added to 600 ml blocking buffer (1:200) and the content mixed with the multichannel pipette dipped into the labeled tubes. 50 µl of the solution was added to the wells. The serum from the positive children occupied the first three rows, while the adult control serum occupied the last two rows on the microtitre plate.

The plate was incubated for 1 h at 37ºC after which the wells were aspirated and washed twice with wash solution, flicked and banged dried. 1.25 ml mouse antihuman IgG-alkaline phosphatase was added to 500 ml of blocking buffer and mixed gently (1: 400). 50 µl of mixture was then put in the wells. The plate was covered and incubated at room temperature for 2 h. 50 µl of substrate and substrate buffer solution of dicyclohexylammonium salt (DEA) of paranitrophenol phosphate (PNPP) at equal volume (1 mg/ml) to DEA/PNPP were then added to each well and incubated for 45 min to 1 h at 37ºC. 50 µl sodium hydroxide was then added to terminate the reaction. Absorbance value was measured at 405 nm with the ELISA reader.

Measurement of total IgG in the test serum (Mab assay)

Plate was coated with monoclonal antibody (Mab) raised against each of the IgG subclasses: Anti IgG1, Anti IgG2, Anti IgG3 and Anti IgG4 at dilution 1:500 in tris saline each at 50 µl per well, incubated at 4ºC overnight. Content was aspirated the following morning and dried. 200 µl blocking buffer was added to each well, incubated again, flicked and banged dried. Two dilutions of test sera were made (1:160) of 4 ml serum added to 600 µl blocking buffer for IgG1 and IgG3 and dilution (1:20) of 12.5 µl serum dispensed into 250 ml blocking buffer for IgG2 and IgG4 respectively. 50 µl of the mixture were put in each well. Incubation was done at 37ºC for 1 h. After incubation time has elapsed, content was aspirated, washed twice and flick-dried. 2.5 µl of mouse antihuman IgG-alkaline phosphatase was added into 10 µl of blocking buffer (1: 400). 50 µl of the mixture was put in each well and incubated at room temperature for 2 h. Wells were aspirated, washed thrice and flick-dried.

Substrate dilution of DEA/PNPP was prepared in ratio 1:1. 50 µl of the mixture was added to the wells and incubated for 1 h. The reaction was then terminated by the addition of 50 µl of sodium hydroxide. Absorbance was measured at 405 nm with ELISA reader.

Determination of cut-off values

Cut-off is evaluated by the addition of the mean of negative control and three times standard deviation of negative control. Subjects were considered positive when the value for the subject is higher than the cut-off for each IgG subclass.

Statistical analysis

Values were expressed as mean ± standard deviation. Values were considered statistically different at $p < 0.01$.

RESULTS

Calculation of cut-off values

Cut -off values were calculated as follows:

$$IgG_x = X_{NAS} + (3 \times SD)$$

Where, X = Mean; and SD = Standard deviation.

If the mean of each of the IgG subclasses in the group is higher than the cut-off values obtained by using the above formula then such subject in the group is, therefore, con-

sidered (+ve) for such subclass.

The mean absorbance value of IgG subclasses reactive against *P. falciparum* CSP are shown in Table 1. The mean absorbance values of IgG subclasses reactive against *P. falciparum* CSP appeared to be age-dependent and ranged from 0.01 for IgG4 in younger children to 0.95 for IgG3 in elder children. All the malaria-infected children under study had significantly higher mean IgG1 and IgG3 than the uninfected control (p <0.01). They were also positive to the cytophilic IgG1 and IgG3 and negative to the non-cytophilic IgG2 and IgG4.

The order of prevalence of IgG subclasses is as follows: IgG3>IgG1>IgG2>IgG4 which confirmed the prevalence of the cytophilic antibodies (IgG1 and IgG3) over the non-cytophilic subclasses (IgG2 and 4). Generally, there was low production of IgG4 and IgG2 level in 35% of the subjects.

The results also showed that all the subjects produced IgG, but male subjects produced higher levels of IgG subclasses specific to the circumsporozoite antigen than their female counterparts. The mean titre of IgG subclasses specific to circumsporozoite proteins also increases as age increases. IgG was also detected in the NAS which served as negative control for CSP-specific IgG subclasses, although NAS titre was lower than each of the malaria subjects. Its IgG2 was, however, higher (0.16) than other subclasses (Table 1). The mean total IgG specific to CSP concentration was higher than the subclasses except for IgG3 of the malaria subjects (Table 1).

The mean total serum IgG subclasses were higher than each of the CSP specific IgG subclasses (Tables 1 and 2). IgG2 values were highest of all the subclasses in the malaria subjects (Table 2). The mean total serum IgG subclasses follows the order IgG2>IgG1>IgG4>IgG3 (Table 2). Generally, there was an increase in the IgG levels as age increased. In other words, there was a direct relationship between the levels of IgG subclasses and age. IgG levels may, therefore, be age-dependent. Furthermore, the level of IgG subclasses was shown to be higher in male subjects of a given age group than in the female subjects.

Table 1. Mean absorbance values of IgG subclasses specific to CSP by ELISA (ë = 405 nm)

Age groups (yr)	IgG1		IgG2		IgG3		IgG4		Total IgG	
	M	F	M	F	M	F	M	F	M	F
0–2	0.15 ± 0.02	0.09 ± 0.08	0.12 ± 0	0.10 ± 0.01	0.47 ± 0.01	0.19 ± 0.02	0.11 ± 0	0.01 ± 0.03	0.30 ± 0.04	0.14 ± 0.02
3–5	0.18 ± 0.03	0.22 ± 0.13	0.14 ± 0.01	0.10 ± 0.09	0.84 ± 0	0.77 ± 0.18	0.15 ± 0.04	0.08 ± 0.05	0.34 ± 0.05	0.29 ± 0.01
6–8	0.27 ± 0.02	0.24 ± 0.03	0.17 ± 0.02	0.14 ± 0.02	0.92 ± 0.04	0.86 ± 0.05	0.17 ± 0.01	0.10 ± 0.08	0.39 ± 0.05	0.35 ± 0.09
9–11	0.33 ± 0.01	0.27 ± 0.04	0.21 ± 0.01	0.16 ± 0.02	0.95 ± 0.04	0.95 ± 0.02	0.19 ± 0.04	0.12 ± 0	0.40 ± 0.02	0.40 ± 0.08
NAS	0.04 ± 0.01		0.16 ± 0.06		0.01 ± 0		0.02 ± 0.01		0.40 ± 0.15	
PNCS	0.12 ± 0.02		0.08 ± 0.01		0.05 ± 0.04		0.01 ± 0		0.20 ± 0.07	
Cut-off	0.07		0.33		0.04		0.23			

DISCUSSION

In recent years, a limited number of studies have focused on the mechanisms mediating premunition in humans. However, there is only limited knowledge of the very long delay required to reach this state of premunition[15]. Previous *in vitro* studies held to the idea that naturally occurring antibodies may by themselves be unable to directly limit parasite growth but required the activities of blood monocytes through the Fc receptors after binding to

Table 2. Mean absorbance values of total serum IgG subclasses in malaria positive children (ë = 405 nm)

Age groups (yr)	IgG1		IgG2		IgG3		IgG4	
	M	F	M	F	M	F	M	F
0–2	1.19 ± 0.01	0.95± 0.05	1.35 ± 0.26	1.21 ± 0.35	0.66 ± 0.07	0.53 ± 0.06	0.10 ± 0	0.66 ± 0.04
3–5	1.20 ± 0.08	1.21 ± 0.13	1.50 ± 0.28	1.80 ± 0.11	0.80 ± 0.17	0.86 ± 0.25	1.02 ± 0.15	1.24 ± 0.17
6–8	1.33 ± 0.01	1.28 ± 0.09	1.89 ± 0.02	1.81 ± 0.06	0.95 ± 0.15	0.96 ± 0.17	1.26 ± 0.16	1.27 ± 0.03
9–11	1.33 ± 0.06	1.33 ± 0.01	1.92 ± 0.04	1.85 ± 0.22	1.04 ± 0.01	1.26 ± 0.32	1.40 ± 0.08	1.44 ± 0.54
NAS (Control)	1.69 ± 0.01		1.72 ± 0.01		0.89 ± 0.07		1.37 ± 0.09	
PNCS	1.62 ± 0.18		1.94 ± 0.05		1.31 ± 0.10		1.67 ± 0.18	

their parasite target[16]. Cytophilic antibodies have been associated with protection, whereas non-cytophilic antibodies against the same epitopes may block the protective activity of the protective ones[17].

In vivo and *in vitro* comparative analysis also confirmed that IgG from protected African adults was able to transfer passively clinical protection to non-immune infected children[16]. The acquisition of a state of resistance appears to correlate with the ability to develop antibodies of the proper subclasses mostly IgG3 and IgG1 and to reduce the proportion of non-cytophilic subclasses (IgG2 and IgG4) of the same specificity that might want to block the effector mechanisms. The isotype and subtype of an antibody confer specific functional activity. Binding of the Fc portions of cytophilic antibodies, immunoglobulin G1 (IgG1) and IgG3, to Fc receptors on phagocytic cells triggers a range of effector functions including phagocytosis, production of cytokines and chemokines, cytotoxicity, and generation of reactive oxygen and nitrogen species[18]. There is also a higher possibility that IgG2 and IgG4 may compete with IgG1 and IgG3[19], hence, among IgG1 or IgG3 containing samples, IgG2 and IgG4 were absent in almost two-third of the samples. Also in IgG2 containing samples where IgG1 and IgG3 antibodies were present, IgG4 antibodies were absent in one half of the samples. A number of factors including age of the patient, duration of infection prior to the collection of blood samples, transmission pattern and season, previous experience of the parasite and the intensity of the infection known to influence the presence and occurrence of antibody responses to *P. falciparum* could have been responsible for these. Among these factors, previous malaria experience is probably the most important[20].

In this study, IgG3 and IgG1 cytophilic subclass antibodies were the most frequent and were coexpressed in the malaria infected children, a relationship previously described for *P. falciparum* antibody and *P. falciparum* CSP[20] showing that natural antigenic stimulations first trigger B cells to produce IgG4 and lastly IgG2, suggesting that subclass expression reflects sequential activation of specific B-cells by repeated antigenic stimulations. The immune competence of the host may determine various escape mechanisms which can develop. In young children living in endemic areas who do not seem to produce high level of IgG2, the parasite may produce preferentially the synthesis of blocking IgM antibodies. Similarly, the low levels of all antibody classes produced generally suggest the existence of other escape mechanisms such as the induction of T-suppressor cells or the production of anti-idiotypes. Since the patients have malaria, there is little merozoite phagocytosis[14] .The mechanisms leading to the early production of IgG2, IgM (effector blockers) or both are far being known. It could be proposed that malarial proteins with repetitive epitopes and defective T-helper epitopes behave immunologically as polysaccharides and would, therefore, induce IgG2 antibodies preferentially in adult patient[15]. Whether the induction of inappropriate (i.e. blocking) subclass per say is a feature of the antigens being targeted by the antibody dependent cellular inhibition (ADCI) mechanism or whether it is induced by some other molecule(s) presented at the same time to the immune system by the parasite can be debated[21].

Conclusively, the role of antibodies in protection against malaria is still unclear, and the exact effect of each subclass is controversial[20]. Generally, the relative level of IgG3 gradually increased and overtook that of IgG1 probably at a time when subjects tended to experience less malaria attacks. The levels of all IgG specific subclass except IgG4 increased noticeably between the seasons of lowest and highest transmission which might be 6 months apart[20]. In contrast, the pattern of specific subclasses was remarkably similar in the two periods of relatively low

transmission during the year; hence, cytophylic subclasses (IgG 1 and IgG 3) could have a potential role in the development of protection and valuable targets of the CSP vaccine development.

Conflict of interest: We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Mark Nwagwu and the Cellular Parasitology Group, Department of Zoology, University of Ibadan, Nigeria for their technical support. Huge thanks to the malaria positive children, the medical and non-medical personnel of the General Outpatient Clinics, University College Hospital, Ibadan.

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Received: 17 October 2011 *Accepted in revised form:* 1 June 2012