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Immunoglobulin G Subclass Responses to *Plasmodium falciparum* Circumsporozoite Protein among Nigerian Children

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Abstract: The response of Immunoglobulin G (IgG) subclass to *Plasmodium falciparum* Circumsporozoite Protein (CSP)-R32tet32 was evaluated among febrile Nigeria children aged 2-10 years (n = 23) by the Enzyme Linked Immunosorbent Assay (ELISA). The mean absorbance of normal IgG subclass and anti-CSP specific antibody of each subclass was negatively correlated and was significant for IgG3 (r = - 0.358, p = 0.05) and IgG4 (r = - 0.403, p = 0.03). The predominant anti-CSP antibodies were IgG1 and IgG3, co-expressed in nearly 60% of the subjects. Cytophilic IgG1 and IgG3 were associated with positive recognition of the CSP antigen (Fisher's exact probability test: p = 0.0001). Some 34.78% of the children did not produce any anti-CSP specific antibodies despite previous exposure to sporozoite inoculation. This study demonstrates that cytophilic IgG subclasses are the main antibodies produced against the CSP but some children living in a holoendemic malaria transmission area may not produce anti-CSP specific antibodies. There is a need to investigate the antibody response of this group of children with the CSP based RTS, S/AS vaccine candidate molecules.

Key words: Immunoglobulin G subclass, *Plasmodium falciparum*, antigen, malaria, circumsporozoite protein, sporozoite

INTRODUCTION

Malaria is a leading cause of childhood and maternal deaths in Africa. Recent estimates by the WHO (2010) have shown that there were 225 million clinical cases of the disease in 2009 and nearly 800,000 deaths. About 176 million of global cases were in Africa and *Plasmodium falciparum* was implicated in 90% of mortality cases. The increasing ability of *P. falciparum* to develop acquired resistance to available chemotherapeutic agents for the treatment of malaria, vector acquired resistance to insecticides and the inability to produce a potent vaccine to immunize against the disease in spite of numerous attempts have been the major obstacles that stand in the way of effective malaria control over the years. If an effective vaccine against malaria is developed, the problems of parasite and vector resistance can be bypassed. A major challenge is to identify and include the right complement of parasite antigens (Sutherland, 2007) in a malaria vaccine (Doolan and Hoffman, 2001) that would elicit protective immune responses from patients that have been exposed to malaria infection (Yazdani *et al*., 2006).

The *P. falciparum* Circumsporozoite Protein (CSP) is one of several vaccine candidate molecules that have been identified and characterized as potential antigens for inclusion in the production of a multi-stage malaria vaccine (Doolan and Hoffman, 2001). The recombinant form of this protein was produced for potential inclusion in a human malaria vaccine in the mid 1980s (Young *et al*., 1985). In early experiments to evaluate its potential in protective immunity, the CSP was shown to elicit strong immune responses which possessed biological activity that were associated with protective immunity. These antibodies protected human subjects against subsequent challenge with malaria infected mosquitoes (Ballou *et al*., 1987). But there were also some reports which suggested that antibody response to the CSP did correlate with protection (Hoffman *et al*., 1987; Nwagwu *et al*., 1998). The most successful malaria vaccines so far produced are the CSP antigen based vaccines RTS,S/AS02A which protected nearly 30% of <5 years old children at Phase II trial in Mozambique (Alonso *et al*., 2005) and RTS,S/AS01$_E$ which has further achieved over 50% protection against malaria infection in children

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(Olotu *et al*., 2011; Asante *et al*., 2011). The characterization of immune responses to the CSP antigen is still a key to understanding the mechanism of its induced protection in some individuals which appears to be very complex (Good and Doolan, 1999).

The aim of this study was to evaluated the immune response of human malaria patients against the recombinant form of the CSP antigen $R32tet_{32}$, based on the previous observations that majority of protective antibodies against some malaria antigens were cytophilic Immunoglobulin G (IgG) subclass (Bouharoun-Taoun and Druilhe, 1992; Dubois *et al*., 1993). Cytophilic IgG1 and IgG3 were found to correlate positively with protection in malaria while non cytophilic IgG2, IgG4 and IgM correlated with non protection against malaria (Dubois *et al*., 1993). The researchers tested whether children that experienced clinical malaria in a holoendemic malaria transmission region made IgG subclass antibodies that recognized the CSP antigen and measured the most prevalent subclasses of IgG antibodies that were directed against the CSP epitopes. The findings are discussed in the light of the current efforts to produce a malaria transmission blocking vaccine that incorporates a CSP repeat antigen.

MATERIALS AND METHODS

Study area: The study area was General Outpatient Department of the University College Hospital Ibadan, Nigeria. The study was conducted in accordance with the ethical standards of the hospital. Malaria is holoendemic in this area (Salako *et al*., 1990).

Subjects and blood sample collection: Subjects were male and female Nigerian children of mean age 4.7 years, range (2.5-10.0) years who reported to the hospital with symptoms of clinical malaria whose parents or guardian gave oral informed consent after the purpose of the research was explained to them. For each febrile subject that gave consent to participate, malaria infection was checked and confirmed by microscopy then, 1.5 mL of venous blood was collected into sterile EDTA tubes, coded with subjects particulars (n = 23). The collected samples were transported in cool flasks with ice packs to the Cellular Parasitology Laboratory at the Zoology Department, University of Ibadan where the samples were further processed. Sample collection and processing were done between January to March 1997.

Centrifugation of blood sample and recovery of serum: Each blood sample was centrifuged at 14000 g for 20 min in Beckman microfuge. The serum was recovered and stored at -70°C for 3 weeks before the Enzyme Linked Immunosorbent Assay (ELISA) was performed to test the reactivity of the serum IgG subclass antibodies to the recombinant CSP antigen.

Quantification of titres of serum IgG subclass: The enzyme linked immunosorbent assay was used to quantify the titres of antibodies following a modification of the original procedure described by Wirtz *et al.* (1989) and Dubois *et al.* (1993). To determine the titres of IgG subclass in each subject, 50 µL solution of mouse monoclonal antibodies: anti-(IgG1-IgG4) purchased from (Catlag Laboratories San Francisco, Calif. USA) were transferred into duplicate wells on 96 well U-bottom Immulon microtiter plates (Dynatech Laboratories, Alexandria VA), excluding the wells at the edge of the plate (blank wells). Anti-IgG1: on wells 2 and 3, anti-IgG2: on 4 and 5, anti-IgG3: on 6 and 7, anti-IgG4: on 8 and 9. The plates were incubated overnight at 4°C, aspirated the following day and washed twice with washing solution-(Phosphate Buffered Saline (PBS)-0.05% Tween 20) and air dried. About 50 µL of each test serum diluted 1:10 was added to each coated well and the plates incubated at 37°C for 1 h. The plates were aspirated, washed thrice, banged and dried. About 50 µL well^{-1} solution of mouse anti human alkaline phosphatase conjugated monoclonal antibody diluted 1:4000 was added to the plates and incubated at room temperature for 2 h. It was followed by addition of 50 µL well^{-1} solution of freshly prepared substrate (P-Nitrophenyl phosphate mixed 1:1 with substrate buffer (10% Diethanolaine/1 mM $MgCl_2$; pH 9.8)) and incubated at 37°C for 1 h. About 50 µL of stop solution (2M NaOH) was added to stop the enzyme substrate reaction. Each plate absorbance was measured at 405 nm on a V. Max kinetics micro plate reader. The absorbance value of each serum tested well was measured by subtracting the mean background absorbance value of the blank wells.

Quantification of anti-CSP specific IgG subclass: The capture antigen was $R_{32}tet_{32}$, a recombinant circumsporozoite protein with the sequence 30 (Asn-Ala-Asn-Pro) tetrapeptide repeats, 2 (Asn-Val-Asp-Pro) tetra peptide repeats fused to 32 COOH-terminal amino acid sequence derived from a gene encoding bacteria tetracycline resistance (Young *et al*., 1985). It was a kind gift of Dr. R.A. Wirtz, Walter Reed Army Institute of Research Washington DC, to the Cellular Parasitology Programme, University of Ibadan. Stock solution (1 mg mL^{-1}) of CSP capture antigen was diluted in a solution made of 0.5% boiled casein-PBS to 0.1 µg/50 µL of capture antigen and distributed on the well on a

microtiter plate using wells on column 2-9. It was followed by overnight incubation at 4°C and subsequent washing. Test sera dilutions at 1:10 from each subject were distributed on the column wells 2-9. A pooled malaria negative North American serum collected from individuals that had not been exposed to malaria was also included as control. It was followed by 1 h incubation at 37°C and washing. Then, 50 μL well^{-1} of mouse anti human monoclonal antibodies diluted 1:500 were distributed on the wells; anti-IgG1, on column wells 2 and 3, anti-IgG2, on 4 and 5, anti-IgG3, on 6 and 7 and anti-IgG4 on 8 and 9. The plates were incubated for 1 h at 37°C then, aspirated and washed 3 times.

The step was repeated with goat anti mouse monoclonal antibodies diluted 1:500 incubated for 2 h at room temperature then, after washing, substrate addition (P-Nitrophenyl phosphate was added and incubated at 37°C for 1 h. About 2 M NaOH was used to stop the enzyme substrate reaction and the plates absorbance were read at 405 nm wavelength. Positive reactivity of IgG subclass to the CSP capture antigen was defined as mean absorbance of the North American serum +3 standard deviations (Dubois *et al*., 1993). Cut off values for each subclass was: IgG1>0.14, IgG2>0.17, IgG3>0.12 and IgG4>0.07.

Data analysis: The data were analyzed with StatsDirect Statistical Software Version 2.7.8 (2010). Absorbance values of normal and anti-CSP specific IgG subclass antibodies were compared by Pearson correlation coefficient-r with t-values at n-2 degree of freedom. Association between group of IgG subclass and reactivity to CSP was determined by Fisher's exact probability test. The probability value of ≤0.05 was considered significant.

RESULTS AND DISCUSSION

The mean absorbance titre values of normal IgG subclass and anti-CSP specific IgG subclass antibodies revealed that only a small fraction of titres of IgG subclass antibodies produced were specific to the CSP antigen. Specifically, 10.23, 3.76, 8.92 and 4.13% of titres corresponding to IgG1-IgG4 were directed to the epitopes on the CSP molecule. The absorbance of the normal and anti-CSP specific antibodies of each IgG subclass antibodies (IgG1-IgG4) were negatively correlated (Table 1). The negative correlation (-r) was significant for both IgG3 and IgG4 but not IgG1 and IgG2. Based on the cut off value of IgG1 = 0.14, IgG2 = 0.17, IgG3 = 0.12 and IgG4 = 0.07, corresponding to positive reactivity to the CSP molecule of the different IgG subclass, >60% of the subjects (n = 23) produced IgG1 and IgG3 that were co-expressed in response to the CSP antigen. Similarly, IgG1, IgG3 and IgG4 were co-expressed in 13.04% of subjects. However, none of the subjects produced IgG2 that recognized the CSP (Fig. 1).

Table 1: Mean Optical Density (OD) values of normal IgG subclass and anti-CSP specific subclass titres in febrile Nigerian children (n = 23) and malaria negative pooled North American sera

IgG subclass	*Mean±SD	**Mean±SD	(r)	t	p-value
IgG1	2.15±0.38 (2.79±0.04)	0.22±0.14 (0.08±0.02)	-0.256 (ND)	-1.214	= 0.11
IgG2	1.86±0.41 (2.98±0.01)	0.07±0.03 (0.14±0.01)	-0.027 (ND)	-0.124	= 0.45
IgG3	1.57±0.43 (2.07±0.08)	0.14±0.03 (0.09±0.01)	-0.358 (ND)	-1.757	= 0.05
IgG4	1.21±0.41 (1.87±0.39)	0.05±0.01 (0.04±0.01)	-0.403 (ND)	+2.018	= 0.03

ND = Not Done, df = (n-2) = 21; *OD of normal IgG subclass titres; **OD of anti-CSP specific IgG subclass titres; r = Pearson correlation coefficient

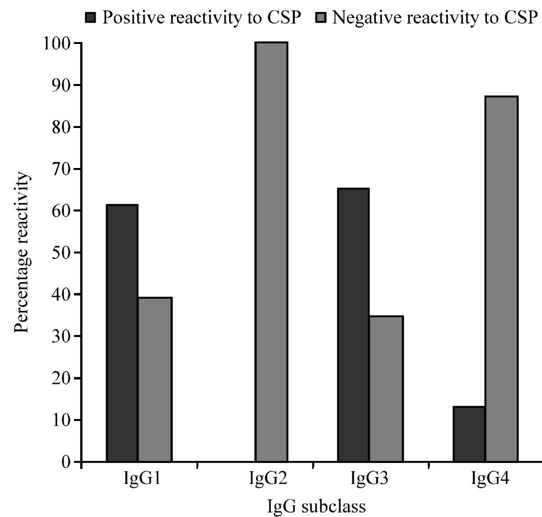

Fig. 1: Reactivity of IgG subclass to CSP antigen (n = 23)

One subject (4.35%) produced only IgG3 that reacted positively to the CSP antigen while 8 subjects (34.78%) did not produce any IgG subclass that responded positively to the CSP antigen. When the reactivity of the IgG subclass were grouped on the basis of being cytophilic (IgG1 and IgG3) and non cytophilic (IgG2 and IgG4), a highly significant association was observed between reactivity to the CSP antigenic molecule and the group of IgG subclass (Fisher's exact probability test, p = 0.0001, Table 2).

The ratio of cytophilic IgG subclass to non cytophilic IgG subclass was approximately 1:1 in normal antibody subclass titres but was 3:1 in specific response to the circumsporozoite protein.

The circumsporozoite protein is one of the several vaccine candidate molecules that have been evaluated for inclusion in a malaria vaccine. The $(NANP)_n$ repeat domain

Table 2: Association between groups of IgG subclass and combined mean reactivities to the recombinant circumsporozoite protein molecule in febrile Nigerian children (n = 23)

IgG subclass combinations	Positive reactivity to CSP molecule (%)	Negative reactivity to CSP molecule (%)
Cytophilic IgG1+IgG3	14.5	8.5
Non cytophilic IgG2+IgG4	1.5	21.5

Fisher's exact probability test, p = 0.0001

of the CSP which has a common B-cell epitope among different field isolates of *P. falciparum* CSP (Singh *et al*., 2009) is a key component of the RTS,S/AS01$_E$ malaria transmission blocking vaccine that has entered phase 3 trials (Ballou, 2009; Cohen *et al*., 2010) after encouraging results at phase 2 clinical trials (Alonso *et al*., 2005; Asante *et al*., 2011). The researchers determined whether Nigeria children, naturally exposed to malaria infection produce IgG subclass antibodies that recognize the circumsporozoite protein. The findings are that over 60% of these children produced cytophilic IgG1 and IgG3 as the predominant anti-CSP IgG subclass but some subjects did not produce any anti-Csp specific antibodies. Anti-CSP IgG4 was also produced by only 3% of subjects. In each case, there was negative correlation between non specific IgG subclass levels and anti-CSP specific levels; demonstrating that IgG subclass antibodies that recognized the CSP antigen did so with smaller amounts of specific antibody levels dedicated to the antigen epitopes, compared to the whole titres of a particular antibody subclass that was produced by individual subjects. It demonstrates clearly that only a small fraction of each IgG subclass produced was actually specific to the CSP among those subclasses that reacted positively to the CSP antigen. Moreover, this observation suggests that the recognition of the CSP molecule may depend more on the specificity of the antibody in ensuring avid recognition to the epitopes of the antigen, rather than the high quantity or titres of the antibody an individual may produced. Many vaccines are formulated in adjuvants to boost specific immune responses as it is the case with the CSP based vaccines, RTS,S/AS02A (Alonso *et al*., 2005; Olotu *et al*., 2011) and RTS,S/AS01$_E$ (Asante *et al*., 2011).

One study demonstrated that sterile protection against malaria parasites could be achieved in the absence of specific immune responses to the CSP and questioned the favoured reliance on immunodominant parasite antigens for malaria vaccine development (Gruner *et al*., 2007). However, the steady progress in protective immunity that has been achieved with the CSP based vaccines at phase 2 trials (Alonso *et al*., 2005; Olotu *et al*., 2011; Asante *et al*., 2011) suggests that specific immunity is involved which hitherto is not fully elucidated. The need to continue to investigate the variant CSP antigens and determine how immune responses to the variant forms of the antigen may have a specific role to play in achieving sterile protection against malaria is still there. This is in order to support the huge body of existing knowledge needed to improve malaria vaccine development.

The finding that IgG1 and IgG3 were the predominant anti-CSP antibodies is in agreement with John *et al.* (2003) who also reported that IgG1 and IgG3 were the primary IgG subclasses that were produced against the $(NANP)_5$ repeat peptide of the CSP (Chougnet *et al*., 1991). In other pre-erythrocytic and blood stage malaria parasite antigens that have been evaluated, IgG1 and IgG3 have also been identified as the most dominant IgG subclass produced against those antigens (Bouharoun-Taoun and Druilhe, 1992; Dubois *et al*., 1993; John *et al*., 2003). Normal IgG subclass distribution suggests the order: IgG1, 66%; IgG2, 23%; IgG3, 7% and IgG4, 4% (Yount *et al*., 1970). However, the elevation of IgG3 over IgG2 during malaria infection may imply that IgG3 has a critical immune role to play in an attempt to contain the threat posed by antigens from malaria parasites. In blood stage malaria antigens *in vitro* studies have demonstrated that IgG1 and IgG3 are active participants in arming monocytes for antibody dependent cellular inhibition of malaria parasites (Khusmith and Druilhe, 1983; Groux and Gysin, 1990). It could be that a similar mechanism takes place between cytophilic IgG subclasses and hepatocyte cells during sporozoite invasion of the hepatocytes and probable inhibition in the case of anti-CSP antibodies which may not have been elucidated. Moreover, the association of cytophilic IgG subclasses and positive recognition of the CSP antigen also supports a specific role for IgG1 and IgG3 response to the malaria antigen. However, it is not very clear why some people are immuned against malaria disease why others are not in spite of similar defence mechanisms that may be mounted in both classes of individuals.

Some investigators had argued a role of competitive binding of non cytophilic IgG2 to epitopes of the parasite antigen which may predominate in non protected subjects (Bouharoun-Taoun and Druilhe, 1992). However, in the present case such interaction was lacking. The ratio of anti-CSP specific cytophilic to non cytophilic IgG subclass was 3:1. Anti-CSP specific non cytophilic IgG subclass antibodies were not the preeminent antibodies and had little affinity to the CSP antigen. The fact that these children were non immune subjects from malaria attack could therefore not be attributed to the competitive binding of the latter group of antibodies to the CSP antigen.

CONCLUSION

This study shows that at least some 34.78% of the children did not produce any IgG subclass that recognized the CSP antigen. Considering the age of the

children studied and the fact that they had been living in a holoendemic area of malaria transmission, it cannot be argued that they had not been exposed to sporizoite inoculation by mosquitoes for a sufficient duration and therefore could not produce anti-CSP specific antibodies. In adults Nigerians living within the same locality, Nwagwu *et al*. (1998) also observed that some adults of unknown immune status were non responders to apparent sporozoite inoculation even though immune adults in the same area responded differently during evaluation with the CSP antigen. It may appear that there exist a certain group of people that may frequently fail to produce anti-CSP specific antibodies. In this group of people, the CSP antigen would always perhaps remain a poorly immunogenic molecule. It would be interesting to characterize the immune response in such group of people with the current CSP based RTS, S/AS vaccine candidate molecules that have been formulated in adjuvant to boost immune responses.

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