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Immunity in malaria: II. Heterophile and malarial antibodies in acute *Plasmodium falciparum* infection

O. A. O. AKINWOLERE* AND A. I. O. WILLIAMS†
Institute of Child Health and †Sub-Department of Immunology, College of Medicine, University of Ibadan, Nigeria

Summary

The sera of 55 Nigerian children (30 malarious and 25 healthy) were analysed for heterophile antibodies against normal sheep erythrocytes by the passive haemagglutination technique. Fluorescent antibodies to *Plasmodium falciparum* were quantified by the indirect immunofluorescence method while the three major immunoglobulins (IgG, IgA and IgM) were estimated by the radial immunodiffusion technique. An increasing age gradient was demonstrated in the heterophile antibody titres within the malarious and control groups, but there was no significant difference in the levels of immunoglobulins and malarial antibodies between the two groups. An indication of higher malarial antibody titre was only observed in the malarious group, particularly in late childhood. These results show an increasing level of heterophile antibodies with age. It is concluded that malarial antigens may play a contributory, but not a dominant role in the acquisition of heterophile antibodies. There is also a need to define the exact serum factors (antibody or non-antibody) which are associated with clinical immunity to malaria in Nigerian children.

Résumé

Les anticorps hétérophiles contre les globules rouges de mouton ont été estimées chez 55 enfants nigérians (30 paludéens et 25 témoins) en utilisant une technique d'agglutination passive. Les anticorps fluorescents contre la malaria (*Plasmodium falciparum*) et les immunoglobulines G, A et M ont été aussi estimées. Les anticorps hétérophiles montrent une inclinaison de l'âge chez les enfants paludéens et les témoins. Néanmoins, les anticorps contre la malaria et les immunoglobulines n'ont pas démontré une différence d'importance. Les anticorps du paludisme indiquent une augmentation chez les enfants plus âgés. On conclue que les antigènes de la malaria, peut-être, ne joue q'un rôle accessoire dans la formation des anticorps hétérophiles. Il est nécessaire de mettre au point les facteurs (anticorps ou non-anticorps) qui sont associées avec l'immunité clinique chez les enfants nigérians.

*To whom correspondence should be addressed.

Introduction

Two different types of agglutinins against sheep erythrocytes have been described in man. One of them agglutinates tanned erythrocytes and has been found in many Nigerians, from infancy to adulthood [1]. The other, a heterophile antibody which was first studied by Oliver-Gonzalez [2], agglutinates normal sheep red cells. Absorption of antibodies raised in rabbit against sheep tanned red cells indicated that the two may be different [3]. However, the former has been characterized as a macroglobulin by the action of the reducing agent mercaptoethanol and therefore suggests the similarity to the heterophile antibodies described by Oliver-Gonzalez [2]. However, it has been shown that activity to sheep tanned red cells can be removed by absorption in fresh sheep erythrocytes [4,5] confirming that both types of heterophile antibodies earlier described are one and the same.

The heterophile antibodies are commonly found in the sera of healthy Nigerian children and have been used as an index of established humoral responses [6]. Various organisms have

been implicated and proved to be responsible for stimulating the production of these antibodies *in vivo*, particularly those with substances immunologically related to the group A substances of human blood [7]. The malaria parasite, among others, has also been suggested to play an important role in the production of these heterophile antibodies [8].

This study investigates the possibility of a relationship between acute *Plasmodium falciparum* malaria infection and levels of heterophile antibodies to normal sheep red cells. The role played by malarial antibodies in clinical immunity to malaria infection is also examined.

Patients and methods

Selection of patients

The children in this study were the same 55 used in a previous communication [9] in which delayed-type hypersensitivity (DTH) was tested in both acute *P. falciparum* infection and normal healthy children. Briefly, the patients comprised children aged 6 months to 12 years who were recruited consecutively during the rainy season from the General out-patient (GOP) clinics of the University College Hospital (UCH), Ibadan. The criteria for selection included attendance at the GOP for the first time with fever; weight for age (WFA) of between the 3rd and 97th percentile of standards in Nigerian children [10,11] to exclude the effect of malnutrition; and a parasitaemia of *P. falciparum* of at least 6% in blood films. This selection was to ensure that only cases of severe falciparum malaria were included in the study group [12] since cases of asymptomatic parasitaemia are also common in children of this part of the world [13]. The healthy children were free from fever, malnutrition or any form of parasitaemia and these were recruited within the same age range from the Institute of Child Health (ICH), UCH, Ibadan.

Sera for tests

About 5 ml of blood were obtained from each subject by a carefully performed venepuncture (from the femoral vein in younger children and from the ante-cubital vein in the older ones) and allowed to stand in a universal glass bottle. The procedures were explained to each mother and her consent obtained before blood collection from the child was undertaken. The serum was separated, divided into aliquots in three separate bijou bottles and kept at −20°C until ready for use. The following in-vitro tests were carried out, making use of serum in one bijou bottle for each test at a time.

Assay of immunoglobulins

Three major immunoglobulin classes (IgG, IgM and IgA) were quantified in all the sera using the radial immunodiffusion method of Mancini *et al.* [14] essentially as modified by Fahey and Mckelvey [15] and as previously applied in our laboratory [16–18].

Malarial antibody titres

The indirect fluorescent antibody (IFA) technique of Kuvin *et al.* [19] and Voller [20] was used for determining the malarial fluorescent antibody titres, using heavily parasitized blood from *P. falciparum*-infected children attending the GOP clinic of the UCH, Ibadan. Culturing of these parasitized cells was done according to the candle jar method of Trager and Jensen [21] in order to obtain schizonts containing between 12 and 16 merozoites dominating the culture. The antigen slides were then made from this culture and stored at −70°C in jars containing calcium chloride until used for analysis. This method has been successfully used in our laboratory [18].

Heterophile antibody titres

Haemagglutination reactions were carried out on inactivated sera (at 56°C for 30 min) using normal sheep red blood cells (SRBC). Fresh SRBC were collected aseptically in equal volumes of Alsever's solution [22,23] and allowed to age for 4 days at 4°C prior to use. Twofold serial dilutions of serum were prepared in 'Perspex' agglutination trays so that each well contained 0.1 ml of diluted serum. Normal saline was used as the diluent and negative control. To each well of the diluted serum was added 0.1 ml of cells. The tray was

shaken to suspend the cells and kept at room temperature for 1 h after which an initial reading was made. It was then kept at 4°C for 18 h and assessed by the settling pattern formed by agglutinated cells on the bottom of the well as described by Stavitsky [24]. The positive reaction consisted of a smooth mat at the centre of the plate with somewhat ragged edges. Negative reaction showed a discrete red button of cells with smooth edges in the centre of the well. The end point was shown by the last well with positive reaction along a row.

Results

Immunoglobulin

The mean values of IgG obtained for malarious and healthy children were 7.159 ± 1.48 g/l and 7.206 ± 1.67 g/l respectively. IgA mean values were 1.086 ± 0.413 g/l and 1.306 ± 0.581 g/l for malarious and healthy children respectively, while 0.895 ± 0.252 g/l and 0.842 ± 0.294 g/l were obtained for IgM in malarious and healthy children respectively. The difference in results between the malarious and healthy children were not statistically significant in any of the three immunoglobulin classes (Table 1).

Malarial antibody titres

The mean titres of malarial antibodies were not significantly different in the two groups (Table 1). However, there is an indication of higher titres in the malarious group above the age of 10 years (Table 2).

Heterophile antibodies

There was no statistical difference between the mean heterophile antibody titres of both malarious (6.5 ± 2.3 (s.d.)) and healthy children (7.7 ± 2.6) (Table 1). However, an increasing age gradient was demonstrated in both the malarious and healthy group (Fig. 1).

Discussion

The relationship between heterophile agglutinins and tropical parasitic infections was investigated by Houba and Allison [25]. They found that 85% of sera from patients infected with *Trypanosoma rhodesiense* had reciprocal titres from a non-sensitized sheep red cell agglutination test > 300, in contrast to sera from *T. gambiense*-infected patients most of whom had a reciprocal titre < 50. Malarious

Table 1. Mean immunoglobulin levels, malarial antibody titres (IFA) and heterophile antibody titres in both malarious and healthy groups

	Malarious (n = 30)	Healthy (n = 25)	P value
Mean IgG (g/l)	7.159 (30)	7.206 (25)	> 0.10 (n.s.)
s.d.	1.48	1.67	
Mean IgA (g/l)	1.086 (30)	1.306 (25)	> 0.05 (n.s.)
s.d.	0.413	0.581	
Mean IgM (g/l)	0.895 (30)	0.842 (25)	> 0.10 (n.s.)
s.d.	0.252	0.294	
Mean malarial IFA titre*	318 (30)	230.4 (25)	> 0.10 (n.s.)
s.d.	623.9	215.8	
Mean heterophile antibody titres*	6.5 (30)	7.7 (25)	> 0.05 (n.s.)
s.d.	2.3	2.6	

*Antibody titre expressed as a reciprocal.
n.s. Not significant.

Table 2. Mean malarial antibody titres in different age groups in malarious and healthy children

	Malarious			Healthy		
Age groups	Mean	s.d.	*n*	Mean	s.d.	*n*
0–11 (months)	36.7	37.0	3	80.0	—	1
1–4 (years)	171.0	142.7	20	206.7	206.0	12
5–9 (years)	575.0	980.5	6	251.4	267.1	7
10–14 (years)	2560.0	—	1	288.0	208.6	5
Overall mean malarial antibody titre	318.0			230.4		
s.d.	623.9			215.8		
t	0.71					
d.f.	53					
P	> 0.10					

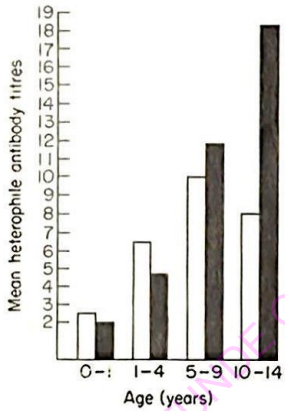

Fig. 1. Bar diagram of age gradient of heterophile antibody titres in malarious (□) and healthy (■) children.

patients were, however, not included in their study. AdeniyiJones [1] later investigated a factor capable of agglutinating sheep tanned erythrocytes in sera of healthy Nigerians including children from birth up to the age of 15. She further demonstrated a rising age gradient in the titres. Greenwood *et al.* [6] investigated heterophile antibodies against sheep red cells in children with acute malaria. They found that there was no significant difference in the heterophile antibody titres between the malarious and healthy children during the pre-immunization (with tetanus toxoid and *Salmonella typhi* vaccines) and post-immunization periods. These periods corresponded with the time of acute malaria and the 16th day after starting antimalarial treatment respectively. The results presented in our study confirm that there is no significant difference between the heterophile antibody titres of children with acute malaria and those without the infection matched for age. However, an increasing age gradient was demonstrated for the heterophile antibodies both in malarious and healthy children. The small number of patients at the extremes of childhood probably reflects adequate immunity to malaria infection in infancy and above 10 years of age. However, it was difficult to obtain consent from mothers of healthy infants for venepuncture, a procedure which many of them preferred to be postponed until their children were older. The inability to show any significant increase in titres in the malarious children over the healthy ones would suggest that malaria antigen may not play a dominant role, if any, in the acquisition of heterophile antibodies.

Immunoglobulins in malaria have been investigated in the adult or mixed populations [26–31], pointing to an elevation of immunoglobulins in malaria. Immunoglobulin studies in childhood malaria are however, very scanty. Our study, which is confined only to the paediatric age group, has not shown a statistically significant elevation in the three major immunoglobulin concentrations. Malaria antibodies are also observed not to increase significantly in the two groups. The large proportion

of children under 5 years in this study (83%) may contribute to the relatively low levels of malarial antibodies in the malarious group. The under-fives constitute the age group most susceptible to malaria. *Plasmodium falciparum* accounts for about 93% of infections and *P. malariae* for the remaining 7% [32]. Infections with *P. malariae*, *P. vivax* and *P. ovale* are therefore not expected to contribute significantly to the levels of immunoglobulins or antibodies in the Nigerian childhood population. There is also the possibility that the humoral immune responses to malaria infection have not been fully expanded during early childhood and thus reflect negatively on the specific immunoglobulin production. However, it is remarkable that in both malarious and healthy children, the antibody titres increase with age.

Humoral immunity to malaria in some environments, including Nigeria, may not be absolutely dependent on the level of fluorescent malarial antibodies. For instance, increased titres of alpha-type interferon have been found in Nigerian children with *P. falciparum* infection [33] while the activity of a non-immunoglobulin substance — the crisis-form factor (CFF) — has been shown to predominate over merozoite blocking antibodies in the protective immunity against malaria amongst the Sudanese population [34], unlike the case in Indonesian sera [35] and The Gambia [36]. Our results on the immunoglobulin levels and malarial antibodies tend to support the observations in Sudan where Jensen *et al.* [37] found no relationship between immunoglobulins and malarial antibody titres on one hand and clinical immunity on the other. They demonstrated that parasite inhibition produced crisis forms which were strongly associated with clinical immunity, and this was a consistent property of fractions of immune serum remaining after immunoglobulin removal. There is therefore a need to define the exact serum factor(s) (antibody or non-antibody) which are associated with clinical immunity to malaria in Nigerian populations. Such factor(s) would assist in identifying the relevant corresponding antigens that may help in the development of suitable malaria vaccines.

In conclusion, we have demonstrated that the titres of heterophile antibodies increase with age. The malaria parasite does not, however, seem to contribute significantly to the production of these antibodies in children as the antibody titres have not been shown to be higher in malarious patients. Malarial antigens may therefore play a contributory, but not a dominant role, in the production of heterophile antibodies. It is also suggested that clinical immunity to malaria in the childhood population in Nigeria may not be absolutely dependent on malarial antibodies but also on non-antibody serum factors which need to be defined.

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