Age-related prevalence of antibody response against three different, defined *Plasmodium falciparum* antigens in children from the Haut-Ogooué province in Gabon.

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Abstract

The kinetics of the humoral response to defined *Plasmodium falciparum* antigens was studied in 543 children, 1 month to 15 years old, living in an area endemic for malaria. The antigens used for enzyme-linked immunosorbent assay were (i) the synthetic peptide $(NANP)_{40}$ representing the immunodominant repeated region of the circumsporozoite protein, and (ii) the fusion peptide 31.1, representing the N-terminal portion of the 83 kDa polypeptide expressed at the surface of merozoites which is a processed product of the 190–200 kDa glycoprotein. In addition, glutaraldehyde-fixed infected red blood cells (RBC) were used to detect ring-infected erythrocyte surface antigen (RESA) and unfixed infected RBC to detect intra-erythrocytic asexual form (IEF) antigens by immunofluorescence. In the 1 to 2 months age group, 50%, 26% and 21% of the children had antibodies for IEF, $(NANP)_{40}$ and 31.1 respectively, but none had anti-RESA antibodies. The proportions of positive subjects decreased until 3 to 6 months and then increased progressively for the 4 antigens, approaching, but not reaching, adult values by the age of 15 years. Antibodies against specific antigens were acquired concomitantly. Children born from $(NANP)_{40}$-positive mothers showed enhanced anti-$(NANP)_{40}$ IgG responses.

Introduction

In regions endemic for *Plasmodium falciparum* resistance to malaria is normally acquired with age as a result of multiple repeated infections (McGREGOR, 1972). During the development of resistance, antibodies specific for different life cycle stages are produced. Purified gamma-globulins (IgG) from immune adults transferred to acutely infected children have been shown to reduce parasitaemia (COHEN *et al.*, 1961). Moreover placental transfer of IgG from immune mothers to their offspring is thought to be, at least in part, responsible for the resistance to malaria in newborns (EDOZIEN *et al.*, 1962).

Recently several *P. falciparum* genes have been cloned and the corresponding peptides have been produced both by genetic engineering and by chemical synthesis. These peptides are powerful tools to investigate and to dissect the immune response naturally acquired against *P. falciparum*. Moreover some of these peptides are candidates for vaccines. In particular the gene encoding the circumsporozoite (CS) protein has been cloned and the aminoacid sequence determined (ENEA *et al.*, 1984). The immunodominant epitopes of the CS protein are located in the region composed of tandem repeats of the tetrapeptide asparagine-alanine-asparagine-proline (NANP) and peptides consisting of such repeats have been used to detect *P. falciparum* sporozoite-specific antibodies (DEL GIUDICE *et al.*, 1987a). Both synthetic and recombinant NANP peptides have been used in clinical trials and induce partial protection in humans against infectious anopheline bites (HERRINGTON *et al.*, 1987; BALLOU *et al.*, 1987). Similarly, the gene encoding the 190–200 kDa schizont glycoprotein has been sequenced (HOLDER *et al.*, 1985). The fusion peptide 31.1 has been shown to contain part of the 190–200 kDa glycoprotein and some of the epitopes expressed by its 83 kDa processed product, which is the major surface component of *P. falciparum* merozoites (CHEUNG *et al.*, 1985). Monkeys immunized with synthetic peptides, representing different parts of the 31.1 fusion protein, were partially protected when challenged by experimental *P. falciparum* infection (CHEUNG *et al.*, 1986). The 31.1 peptide has been used to detect specific antibodies in subjects living in an endemic area (GABRA *et al.*, 1986).

This paper reports a study of the presence of antibodies specific for the $(NANP)_{40}$ and the 31.1 peptides in 543 children and 387 mothers living in a region mesoendemic for malaria. Antibodies to these 2 peptides were detected using 2 distinct micro-enzyme-linked immunosorbent assays (ELISAs). In addition we used a modified immunofluorescence assay (IFA) to detect ring-infected erythrocyte surface antigen (RESA), mainly the 155 kDa component of *P. falciparum* (PERLMANN *et al.*, 1984), which has also been proposed as a candidate vaccine, and a classical immunofluorescence assay (IFA) to detect antibodies against intra-erythrocytic asexual forms (IEF). Relationships between the specific antibodies were investigated as well as relationships between sero-positive mothers and their offspring.

Material and Methods

Subjects

Blood donors were 543 children (1 month to 15 years old) referred to the paediatric unit of the General Hospital of Franceville (Haut-Ogooué province, Gabon); 57% were males. The primary reasons for consultation were rubella (33%), pulmonary pathology (17%), fever of undefined origin (12%), sickle cell anaemia (11%), diarrhoea (7%), acute malaria (4%) and other pathologies (16%). Blood was

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also obtained from 387 healthy mothers (15–55 years old) who were accompanying their children. Sera were kept at −70°C until used. Healthy European sera, used as controls, were kindly provided by Dr Luc Perrin (Blood Transfusion Centre, University Hospital, Geneva, Switzerland).

Study area

Patients referred to the hospital came from the entire province, which comprises savanna in the south and rain forest in the north. Although exhaustive entomological, parasitological and epidemiological studies have not yet been performed, recent surveys, conducted in May 1986 and January 1987, identified this region as a mesoendemic area for malaria, where *P. falciparum* accounted for more than 95% of positive smears (Dr M. Merlin, OCEAC, personal communication). However, differences in endemicity may exist between the savanna and the forest subregions.

Indirect immunofluorescence assay

The SGE2 *P. falciparum* strain, cultured by the standard method (JENSEN & TRAGER, 1977; PERRIN *et al.*, 1984), was used for IFA assays. Anti-intraerythrocytic asexual form (IEF) antibodies were detected by applying different serum dilutions to monolayers of air-dried, unfixed, infected red blood cells. Antibody to RESA was detected similarly using monolayers of glutaraldehyde-fixed, air-dried infected red blood cells (PERLMANN *et al.*, 1984). The slides were then stained with fluorescein-conjugated anti-human immunoglobulin antiserum (Cappel, Cochranville, Pennsylvania, USA). Bright, distinct parasite-associated immunofluorescence was recorded as a positive result.

Peptides

The $(NANP)_{40}$ synthetic peptides, representing an average of 40 repeats of the *P. falciparum* CS protein, were prepared by Eniricerche (Monterotondo, Italy). The 31.1 fusion protein, purified as described by GABRA *et al.* (1986), was kindly supplied by Dr L. Perrin. Anti-$(NANP)_{40}$ and anti-31.1 antibodies were detected by ELISAs as described by CHIZZOLINI *et al.* (1988). Briefly, 96-well plates were coated overnight with antigens and then saturated with phosphate-buffered saline (PBS) containing 5% skimmed milk. Sera diluted 1:200 were applied to duplicate wells for 1 h, washed, and then a 1:1000 dilution of affinity-purified anti-human IgG antiserum conjugated with alkaline phosphatase was added for 3 h. After a final wash, 100 µl of *p*-nitrophenylphosphate, diluted in the enzyme substrate buffer, were added and the plates were incubated for 90 min at 37°C in the dark. The absorbance was measured by recording the optical density (OD) at 405 nm wavelength. In each assay uncoated plates were processed in the same way as coated plates. Results were expressed as the difference between the ODs obtained with coated and uncoated plates. The mean OD value ± standard deviation (SD) of 84 healthy European sera, used as controls, was 0·066±0·080 for anti-$(NANP)_{40}$ IgG and 0·066±0·083 for anti-31.1 IgG. The mean+3SD was chosen as the upper limit of the normal range (OD=0·300) for both antigens. Pools of positive sera were included for each micro-ELISA plate processed. The inter-test variability was 11·3% for anti-$(NANP)_{40}$ IgG and 8% for anti-31.1.

Statistical analysis of results

Relationships between values were investigated by the Spearman rank correlation method. The χ^2 test with Yates' correction for continuity was used to analyse frequency data. P values $<0·01$ were considered significant.

Results and Discussion

The proportions of subjects with antibodies specific for IEF, $(NANP)_{40}$ and 31.1 in the 1–2 months age group were 50%, 26% and 21% respectively (Figure, a, b, c). These proportions decreased until 3–5 months (31.1) or 6 months [IEF and $(NANP)_{40}$], and thereafter increased progressively with age. Transfer across the plancenta of maternal specific antibodies probably accounted for the positive samples observed in newborns. The clearance rate varied for different specific antibodies. This variability may have been due to the titre of maternal antibodies at delivery: high maternal titres were probably reflected in long persistence of specific antibodies in newborns. There are several reports in the literature of transplacental passage of antibodies specific for *P. falciparum* sporozoites (NARDIN *et al.*, 1981) and for IEF (EDOZIEN *et al.*, 1962; COLLINS *et al.*, 1977; CAMPBELL, *et al.*, 1980). Our results confirm and extend such findings. In fact, by using defined antigens, we were able to detect antibodies directed against a single, or a few, epitope(s) carried on sporozoites [$(NANP)_{40}$] or on merozoites (31.1) and to document their congenital transfer and their clearance within the first few months of life. In contrast, anti-RESA antibodies were not detected until the age of 7 months (Figure, d). This result is also supported by our finding that only 3 out of 71 cord sera, collected in the same hospital, were found to contain anti-RESA antibodies, and then only at low titres (data not shown).

By the age of 6–7 months an increase in the proportion of samples containing antibodies against the various antigens tested was observed (Figure). However, the rate of increase varied for each antigen. Thus 93% of 6–10 years old children had anti-IEF antibodies, but by the age of 10 to 15 years only 51% had anti-$(NANP)_{40}$ IgG, 24% anti-31.1 IgG and 42% anti-RESA antibodies. The respective figures in mothers were: 98% for anti-IEF, 70% for anti-$(NANP)_{40}$, 42% for anti-31.1 and 12·5% for anti-RESA antibodies. In agreement with previous studies from hyperendemic West African areas (MCGREGOR *et al.*, 1965; CORNILLE-BROGGER *et al.*, 1978), we observed an age-dependent increase in the IFA titre of anti-IEF antibodies. The age-related increase in the frequency of children positive for $(NANP)_{40}$ and RESA specific antibodies has also been reported. However, for each age group, lower proportions of positive subjects were observed in our study compared with those reported from a hyperendemic area of Tanzania for $(NANP)_{40}$ (DEL GIUDICE *et al.*, 1987b), and from a holoendemic area of Liberia for RESA (WAHLGREN *et al.*, 1986). The lower proportions, and the slower increase in numbers with age, of positive subjects which we observed could be related to the lower parasite rate of the mesoendemic area

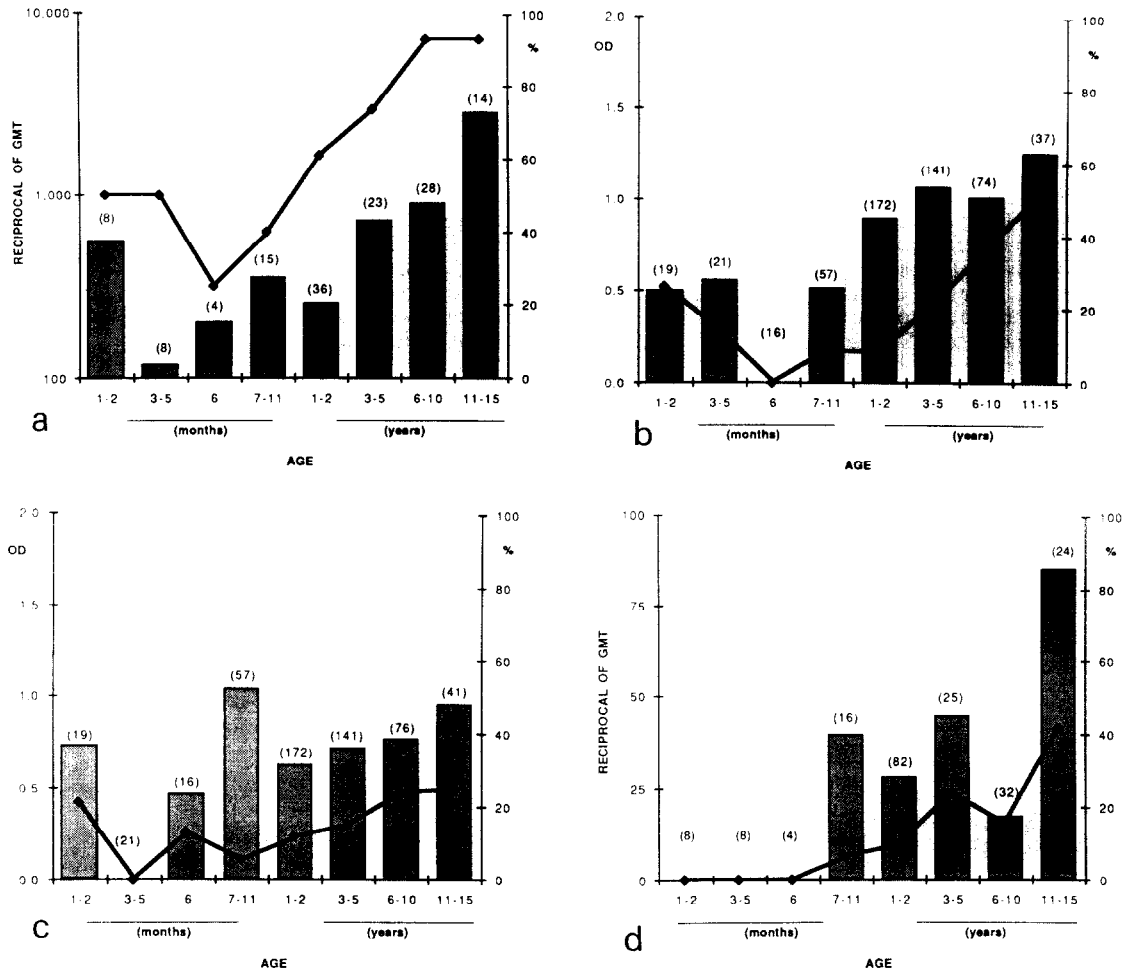

Figure. Frequency of positive subjects (♦——♦) and geometric mean of optical density (b and c) or reciprocal of geometric mean titre (GMT) (a and d) of specific antibodies in positive samples (solid columns) to various *Plasmodium falciparum* antigens in children 1 month to 15 years old living in the Haut-Ogooué province (number of samples tested in parentheses). a, Anti-intraerythrocytic asexual blood form antibodies (note that GMT is shown on a logarithmic scale). b, Anti-sporozoite IgG $(NANP)_{40}$ antigen. c, Anti-Merozoite IgG 31.1 antigen. d, Anti-RESA antibodies.

studied. Finally, our data on 31.1 antibodies did not confirm a previous study from The Gambia which showed a very high proportion of positive children, almost all of them having anti-31.1 antibodies by the age of 5 years (GABRA *et al.*, 1986). A possible explanation for this difference is the particular structure of the 31.1 fusion protein, which has been determined by cloning and transforming complementary deoxyribonucleic acid (cDNA) derived from the SGE2 isolate of *P. falciparum* (CHEUNG *et al.*, 1985). The 31.1 fusion protein is composed of a constant polypeptide sequence and 2 stretches of tandem repeats which are variable in different *P. falciparum* strains and are immunodominant (CHEUNG *et al.*, 1986; GENTZ *et al.*, 1988). Thus the 31.1-ELISA will preferentially detect antibodies directed against repeated sequences resembling those of SGE2. Considering that such sequences may be evenly distributed amongst wild *P. falciparum* strains, high parasite rates observed in regions of high endemicity (e.g., The Gambia during the wet season), in contrast with low parasite rates observed in regions of lower endemicity (e.g., Haut-Ogooué), may increase an individual's chances of being infected by wild strains carrying repeated sequences resembling those of SGE 2, which would give a positive result in the 31.1-ELISA.

As reported above and shown in Table 1 a positive correlation was observed between age and increasing frequency of subjects positive for specific antibodies. In order to know whether specific antibodies were acquired concomitantly, the relationships between them were studied. In children aged between 7 months and 15 years, a significant low order correlation was apparent (Table 2). However, this result should be interpreted with caution because at least 2 factors might have influenced the analysis. One was a general age-related increase in positivity, so that older children had higher prevalences of all the antibodies. The second was the possible difference in the

endemicity level in different sub-regions (rain forest versus savanna) of Haut-Ogooué province, which might have resulted in some samples of the population having higher infection rates than others. In fact, in a study conducted in 3 forest villages which had homogeneous environmental conditions, no relationship was found between specific antibodies (CHIZZOLINI *et al.*, 1988).

In order to study whether the presence of specific antibodies in mothers could influence the humoral response in their offspring, we compared the frequency of positive and negative children born from positive or negative mothers. When the anti-$(NANP)_{40}$ antibody response was studied in children aged 7 months to 15 years, significantly higher numbers of positive children were found in the group born from positive mothers (Table 3). The same analysis performed for 31.1 and RESA specific antibodies did not show differences between the groups. Experimental immunization of mice with peptides containing the NANP repeated epitope have shown that, in the absence of heterologous carrier proteins, the T and B cell response to NANP was linked to a specific major histocompatibility complex (MHC) haplotype (GOOD *et al.*, 1986; DEL GIUDICE *et al.*, 1986; TOGNA *et al.*, 1986). Whether the immune response to the tandemly repeated region of the CS protein of living sporozoites is genetically restricted in humans is not known. The data here presented are suggestive of an enhanced anti-NANP response in children born from positive mothers, which might be linked to a particular genetic background. However, we cannot exclude that mothers and children with higher frequencies of anti-$(NANP)_{40}$ IgG had higher infectious biting rates.

In conclusion, in this paper we have presented evidence that, in Haut-Ogooué province, antibodies specific for the tandemly repeated NANP region of CS protein and for the N-terminal portion of the 190–200 kDa glycoprotein (31.1 antigen), in addition to antibodies against the IEF of *P. falciparum*, were transferred from mothers to newborns. The frequency of sero-positive infants decreased until month 5 or 6 of life. By the 6th–7th months, newly synthesized antibodies specific for $(NANP)_{40}$, 31.1 and RESA were detected and the frequency of positive children increased with age approaching, but not reaching, adult levels by the age of 15 years. The humoral response to these specific antigens occurred concurrently. Moreover, children born from $(NANP)_{40}$-positive mothers showed enhanced anti-$(NANP)_{40}$ responses.

Table 1. Relationship between specific antibodies and age in children, 7 months to 15 years old, living in the Haut-Ogooué province

Antibodies	*n*	*r* [a]	*P*
$(NANP)_{40}$	497	0·346	<0·001
31.1	503	0·197	<0·01
RESA[b]	180	0·471	<0·001
IEF[c]	117	0·507	<0·001

[a]The correlation coefficient *r* was computed by Spearman's rank correlation method.
[b]Ring-infected erythrocyte surface antigen.
[c]Intraerythrocytic asexual form antigen.

Table 2. Relationships between specific antibodies in children, 7 months to 15 years old, living in the Haut-Ogooué province[a]

Antibodies	*n*	*r*	*P*
$(NANP)_{40}$ and 31.1	497	0·327	<0·001
$(NANP)_{40}$ and RESA	180	0·396	<0·001
RESA and 31.1	180	0·363	<0·001
IEF and $(NANP)_{40}$	117	0·529	<0·001
IEF and 31.1	117	0·275	<0·01
IEF and RESA	117	0·363	<0·001

[a]Footnotes as in Table 1.

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Table 3. Antibody response to antigens in children over 6 months old born from mothers with or without specific antibodies

		Anti-$(NANP)_{40}$ IgG[a]		Anti 31.1 IgG[b]		Anti-RESA Ig[c]	
		+	−	+	−	+	−
Mothers	+	47	186	23	116	3	17
	−	7	93	22	172	17	82

[a]$\chi^2=8·935$, $P<0·005$.
[b]$\chi^2=1·878$, not significant.
[c]$\chi^2=0·008$, not significant.

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