ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM*: EVOLUTION ACCORDING TO THE SEVERITY OF A PRIOR CLINICAL EPISODE AND ASSOCIATION WITH SUBSEQUENT REINFECTION

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Abstract. We measured sporozoite- and total parasite antigen-specific IgG and IgM antibodies before and after treatment in matched groups of Gabonese children who presented with either mild or severe *Plasmodium falciparum* malaria. We investigated the influence of various parameters on these antibody responses, including clinical presentation, age, and post-treatment reinfection profiles. IgG but not IgM responses were strongly influenced by both clinical and parasitological status. IgG responses to the repeat region of the circumsporozoite protein, which were low at admission, particularly so in those with severe anemia, increased after treatment but showed no association with either age or reinfection profiles. Total parasite antigen-specific IgG responses were strongly influenced by parasitological status, and also differed significantly when segregated according to clinical status at admission, age, and reinfection histories. Most notably, anti-parasite IgG responses measured when children were parasite-free were higher and a good indicator of recent reinfections in those who presented with mild rather than with severe malaria. The profile of responses in the latter group suggests some immune system dysfunction, which may reflect the induction of tolerance to parasite antigens.

INTRODUCTION

The relationship between morbidity, transmission intensity and parasite-specific antibodies in malaria due to *Plasmodium falciparum* is complex. The results of several recent studies suggest that rates of morbidity and/or levels of parasitaemia are directly related to exposure, measured as the entomological inoculation rate (EIR), although the situation under conditions of very high transmission may not be so clear.[1–4] The search for direct serological correlates of transmission intensity, especially in areas of high endemicity, has concentrated on measurements of anti-sporozoite immunoglobulin G (IgG) responses, in particular to the repeat region of the circumsporozoite protein (CSP). The prevalence and intensity of these responses show linear relationships with age, and thus, by extrapolation, with cumulated exposure, has been demonstrated in many studies.[5–13] Considerable variability has, however, been noted, with aggregation between dwellings as well as inter-individual variation, including the complete absence of a response in some persons.[13–15] In addition, anti-sporozoite antibody levels appear to have little value as an indicator either of recent infection or of transmission intensity in areas of hyperendemicity.[9,13] A more reliable measure of exposure, at least in infants, might be the prevalence of total anti-*P. falciparum* IgM antibodies, otherwise defined as the infant conversion rate.[16]

Associations between the level of parasite-specific antibodies and disease severity have been sought in a number of previous studies. An impairment of *P. falciparum*-specific antibody responses in the acute phase of a cerebral malarial attack has been reported, the most pronounced reduction being observed in those who had 'complicated' cerebral malaria or in those who died following treatment.[17–19] Lower anti-sporozoite IgG antibodies in Thai individuals with cerebral compared with uncomplicated malaria have also been reported.[20] In contrast, a comparative study of Gambian children with either severe or mild malaria failed to demonstrate a difference in either total anti-*P. falciparum* or anti-sporozoite antibody responses.[21] As part of a case-control study of severe malaria in 200 young Gabonese children we measured anti-*P. falciparum* antibody responses before and at different times after treatment in order to evaluate possible differences which might be related to the severity of the malaria attack. In addition, active longitudinal follow-up of the children generated detailed information on their reinfection histories, which differed significantly according to the severity of the initial clinical episode. Assessments of the association between anti-parasite antibody responses and reinfection thus allowed an evaluation of the potential of the former to serve as serological markers of recent exposure.

PATIENTS AND METHODS

Study design. The study was carried out at the Albert Schweitzer Hospital in Lambaréné, Gabon. Detailed descriptions of the participants, the inclusion criteria, treatment, clinical and follow-up surveillance undertaken, and hematological and biochemical methods have been given elsewhere.[22–25] Thus, 100 cases with severe malaria were matched for age (mean 44 ± 23 months), gender (61 female, 39 male), and provenance with 100 controls with mild malaria. Briefly, severe malaria was defined as either severe anemia (hemoglobin < 5g/dl or hematocrit < 15%) and/or *P. falciparum* hyperparasitemia (> 250,000 parasites/µl), while mild malaria comprised *P. falciparum* parasitemia (1,000–50,000 parasites/µl) with hemoglobin > 8 g/dl, glycemia > 50 mg/dl and no signs of severe malaria. Active follow-up of individuals every 2 weeks with examination of Giemsa-stained thick blood smears allowed detection of reinfection with *P. falciparum*. Parasitemia was assessed using a calibrated thick smear technique as previously described.[26]

During the follow-up period, all children with symptomatic reinfections (*P. falciparum* parasitemia and rectal temperature > 38°C) were given standard anti-malarial treatment. The time to first reinfection was defined as the time from the malarial attack at admission until the next *P. falciparum*-positive thick smear. Incidence density rates (IDR) of reinfections were estimated by calculating the ratio between the number of reinfections detected and the duration of follow-up observation in years for each individual. Ethical clearance for the study as well as approval of the procedure for oral informed consent was given by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné. Children were included in the study after obtaining oral informed consent from the parent or guardian.

Plasma samples. For immunological assessments, venous blood samples were taken into sterile collection tubes containing EDTA. After centrifugation of undiluted whole blood for mononuclear cell separation, plasma was drawn off and aliquots stored at −80°C until required for antibody assays. Plasma samples were collected in this way on three separate occasions corresponding to admission (pre-treatment), convalescence (one month post-treatment) and healthy (infection-free) phases. The latter sample was collected at least 6 months post-admission when the child was free of any clinically obvious intercurrent infection and had had at least three consecutive *Plasmodium*-negative thick blood smears in the surveillance period immediately preceding the sample collection.

Antibody assays. Plasma immunoglobulin M (IgM) and IgG antibody responses to two parasite antigen preparations were measured using standard enzyme linked immunosorbent assay (ELISA) procedures. We used a peptide $(NANP)_6$ corresponding to the repeat region of the *P. falciparum* circumsporozoite protein (CSP) (Bachem, Heidelberg, Germany). Secondly, we used a schizont (SCH) antigen preparation obtained by sonication of synchronised *in vitro* cultures of the Palo Alto strain of *P. falciparum*. For the ELISA, 50 μl antigen ($NANP_6$ at 1.25 μg/ml, SCH at 1.8 μg/ml, in carbonate-bicarbonate buffer, pH 9.6) was incubated overnight at 4°C in 96-well plates (Immulon IV, Dynex, Denkendorf, Germany). After a subsequent blocking step (200 μl/well of phosphate-buffered saline [PBS] with 2% bovine serum albumin [BSA, Fraction V, Sigma, Deisenhofen, Germany]) for 3 hours at room temperature, the plates were washed with PBS-0.5%Tween 20 (Sigma). Plasma samples (50 μl/well diluted 1:100 for $NANP_6$, and 1:200 for SCH) and reference positive and negative controls were then added to duplicate wells and the plates incubated overnight at 4°C. After further washing, 50 μl/well of peroxidase-conjugated goat anti-human IgM (μ-chain specific) or IgG (Fc-specific) antibodies (both Sigma) diluted in PBS-0.5% Tween 20 were added, and the plates incubated for 2 hours at room temperature. Following a final wash 100 μl/well of chromogen-containing substrate mixture (TMB, Kirkegaard & Perry, Gaithersburg, MD) was added and reactions stopped after 15 minutes by addition of 50 μl/well of 1 M H_3PO_4. Optical densities (OD) were measured using an automated Digiscan plate reader (ASYS, Vienna, Austria) running on Mikrotek software (supplied by Dunn, Thelenberg, Germany) using a 450-nm filter with 550-nm reference filter. Results are expressed in arbitrary units (AU) according to the formula originally defined by Rasheed and others:[27]

$$AU = \frac{Ln(OD \text{ test sample} - Ln(OD - ve \text{ control})}{Ln(OD + ve \text{ control}) - Ln(OD - ve \text{ control})} \times 100$$

Antibody responders were defined as those having an AU value greater than 2 standard deviations above the mean AU of 50 negative control plasma samples.

Statistical analysis. Correlation between continuous variables was assessed by the Spearman rank test corrected for ties where ρ represents the correlation coefficient. Contingency tables using Fisher's exact test were used to compare proportions within and between groups. For paired and unpaired analyses the non-parametric Wilcoxon signed rank and Mann-Whitney U tests were used to determine the significance of differences in continuous variables. The level of significance in all cases was set at a two-tailed $P < 0.05$.

RESULTS

Losses to follow-up. Individuals were lost to follow-up principally because they moved away from the study area. Therefore, from the 100 per group in the acute phase the following sample sizes were available from subsequent time-points for the serological assays: convalescent phase: 80 'mild' and 75 'severe'; healthy phase: 56 'mild' and 51 'severe'.

Clinical status and reinfection rates. As we have previously reported, the groups defined initially on the basis of clinical presentation at admission were distinguishable by the significant differences in their reinfection rates.[24,25] Thus, the median IDR in subjects with severe malaria was almost three times higher than in those with mild malaria (1.4 versus 0.5, $P < 0.001$).

Evolution of antibody responses related to clinical status and parasitaemia. The profile of IgM and IgG responses in the groups segregated according to their clinical status at admission is shown in Figure 1. IgM responses to NANP were similarly high in both groups and showed no significant change at any of the three time-points (Figure 1a). In both groups, IgG responses to NANP at admission were similarly low (median = 0 for both groups) but subsequently showed a progressive and significant ($P < 0.003$) rise to maximal levels in the healthy phase (Figure 1b). The proportion of IgG responders in the groups did not differ significantly at any time, although the median plasma concentration of anti-NANP IgG was significantly higher in both the convalescent and healthy phases in subjects with severe malaria (Figure 1b). Restricting the comparison of IgG concentrations to those who were antibody-positive revealed the same significant difference between the two groups but only in the healthy phase ($P = 0.023$, data not shown). In both groups, a similar proportion of individuals completely lacked anti-NANP IgG antibodies (11/45 and 7/32 in mild and severe malaria groups, respectively), but all had NANP-specific IgM on at least one occasion.

At admission, IgM anti-schizont antigen responses were similar in both groups (Figure 1c). In the convalescent phase, the proportion of IgM responders in the group that presented
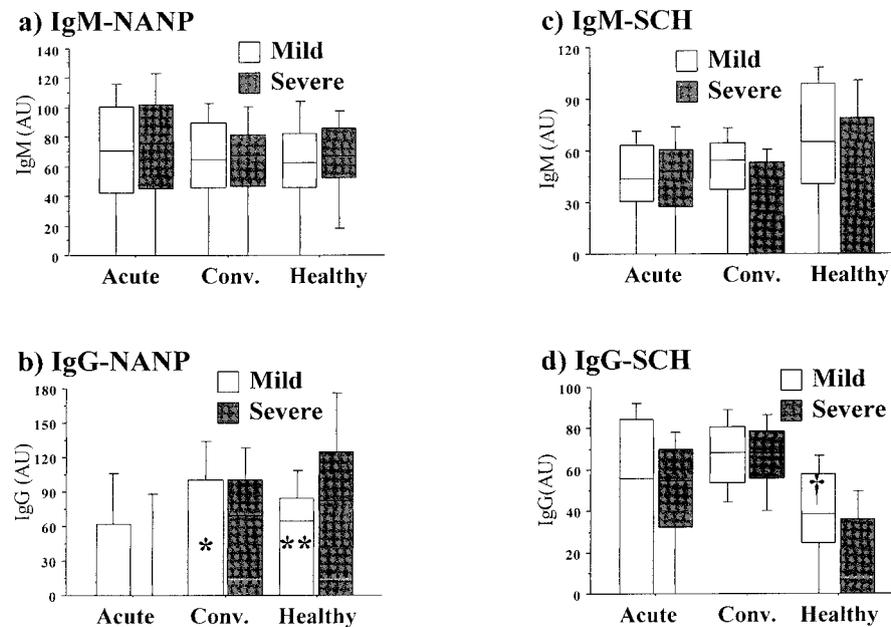
FIGURE 1. Plasma IgM and IgG antibody responses specific for the CSP repeat region (NANP; Figures 1a and 1b) and schizont antigen (SCH; Figures 1c and 1d) of children, segregated according to their clinical malaria status at admission, detected by ELISA in samples collected in acute-, convalescent- and healthy (parasite-free)-phases. Box-plots represent medians with 25th and 75th percentiles (error bars for 10th and 90th) of antibody concentrations in Arbitrary Units (AU) according to the formula originally defined by Rasheed and others:[27] *$P = 0.002$; **$P = 0.022$; †$P < 0.001$ (all by Wilcoxon signed rank test). Conv = convalescent phase.

with severe malaria was significantly lower than in those with mild malaria ($P < 0.001$) but no difference was observed when the children were healthy. The profile of IgG anti-schizont responses was similar in both groups at admission as well as in convalescence, at which time all were antibody-positive (Figure 1d). In the healthy phase, the proportion of IgG responders and the median IgG anti-schizont antigen concentration declined significantly in both groups ($P < 0.001$) from the maximal levels in convalescence, but both parameters were nevertheless significantly higher in the group that presented originally with mild rather than severe malaria (41/52 versus 24/51, $P < 0.001$; Figure 1d). Comparison only of those who were antibody positive at this stage showed a similar although statistically non-significant trend towards higher responses in the group that had had mild malaria ($P = 0.053$, data not shown).

Segregation of those with severe malaria according to the presence of severe malarial anemia had no bearing on the profile of anti-schizont IgG responses at any time-point (Figure 2a). The median anti-NANP IgG response was, however, significantly lower at admission in those with severe anemia (Figure 2b). At this time but not subsequently, the proportion of responders in this group was also significantly lower than in those without severe anemia (1/32 versus 14/58, $P < 0.007$). In the convalescent and healthy phases, median anti-NANP IgG responses were consistently lower in persons with severe anemia compared to the group without anemia ($P = 0.052$ and $P = 0.039$, respectively). No such differences were revealed for IgM responses (data not shown).

No significant differences in either anti-NANP or anti-schizont antigen IgM or IgG responses were evident in either group according to the level of parasitaemia at admission (data not shown).

Evolution of antibody responses related to age, gender, and genetic traits. The study subjects had a mean age of 44 ± 23 months. Segregation of IgG antibody responses at different times in relation to different age classes in the two groups is illustrated in Figure 3. The profiles of anti-NANP responses in both groups in terms of both proportions of responders as well as of antibody concentrations showed trends towards an age-related increase in the healthy phase, but these were not statistically significant (Figure 3a,c). There were significant positive age-related associations of anti-schizont antigen IgG responses both at admission ($\rho = 0.363$, $P < 0.001$) and in convalescence ($\rho = 0.443$, $P < 0.001$) in subjects who presented with mild malaria (Figure 3b). In persons with severe malaria these responses showed a weak age-related association at admission ($\rho = 0.227$, $P = 0.025$) but no such association subsequently (Figure 3d). In both groups, IgM responses to the two antigens showed strongly positive age-related associations at admission and in convalescence, and the proportion of IgM responders in the different age classes were similar at all times (data not shown).

The mean age of female study subjects was significantly higher than males (48 ± 25 months versus 39 ± 19 months, $P = 0.006$). There was a trend for females to have higher IgM responses to both antigens tested but no such trend for IgG responses (data not shown). Separate analyses of hemoglobin phenotype (AA versus AS) or ABO blood group type revealed no significant differences in any antibody response in any group at any time (data not shown). In addition, comparisons were made of antibody responses of groups segregated according to the presence or absence of point mutations in either the inducible nitric oxide synthase
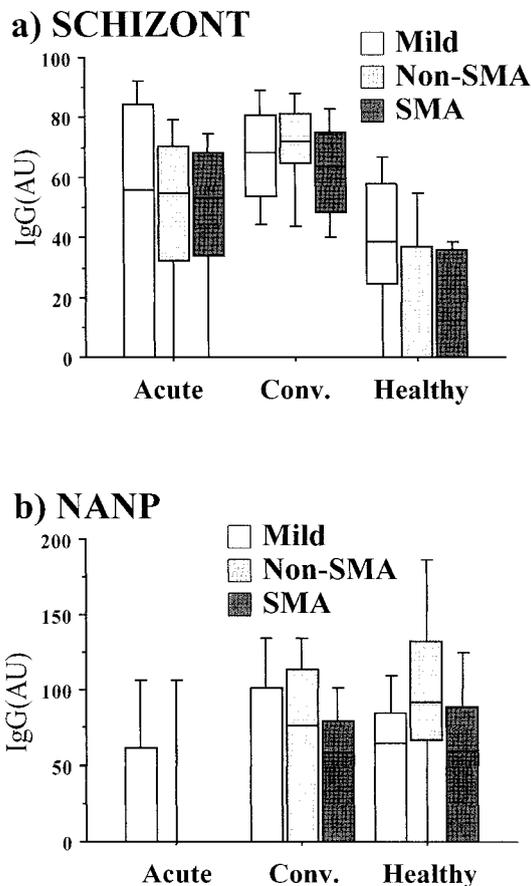
a) SCHIZONT

b) NANP

FIGURE 2. Plasma IgG responses specific for schizont antigen (2a) or NANP, the CSP repeat region (2b) detected by ELISA at different time-points pre- and post-treatment in those presenting with mild or severe malaria, the latter group segregated according to the presence or absence of severe malarial anaemia (SMA, see Study design for definition). Box-plots represent medians with 25th and 75th percentiles (error bars for the 10th and 90th) of antibody concentrations in Arbitrary Units (AU) according to the formula originally defined by Rasheed and others.[27] *P = 0.009 (Mann-Whitney U test) for comparison of SMA (median and percentiles = 0) with Mild or non-SMA groups. Conv = convalescent phase.

gene promoter[28] or in the mannose-binding lectin gene[29] but no differences were detected (data not shown).

Evolution of antibody responses related to reinfection. Since reinfection in many study subjects occurred in the interval between collection of the convalescent and healthy phase (parasite-free) plasma samples, we evaluated antibody responses specifically in the context of the reinfection data relating to this period.

The level of convalescent phase anti-schizont IgG antibodies in those with mild malaria showed a trend towards a positive association with the number of reinfections (ρ = 0.255, P = 0.061), but no such trend was evident for those with severe malaria (ρ = 0.033, P = 0.813). In the healthy phase, the level of anti-schizont IgG in the group that presented with mild malaria showed a strongly significant positive correlation with the number of reinfections (ρ = 0.58, P < 0.001), but this association was not found in those who presented with severe malaria (ρ = 0.16, P = 0.264). Table 1 illustrates this observation. It shows that in the group ad-

mitted with mild malaria the median level of anti-schizont IgG was significantly higher in persons with reinfection compared to those without reinfection. A similar trend was seen for those admitted with severe malaria but the difference was not statistically significant. No such association with reinfection was found for the levels of anti-NANP IgG in either group. Similar analyses of the levels of IgM responses specific for either antigen revealed no association with reinfection in either group (data not shown).

DISCUSSION

In this study, we determined the possible association between the severity of a malaria attack and *P. falciparum* parasite antigen-specific antibody responses in matched groups of young African children with well-defined and distinctly different clinical presentations. Secondly, we wished to follow the evolution of the responses following an acute clinical episode, both to assess the potential long-term effects of the initial attack and changes in responsiveness which could be related to subsequent reinfection history. The latter were compiled from the results of active surveillance over at least one year and differed significantly at both the individual and the group levels as previously reported.[24,25] Lastly, we wished to know if there was any serological evidence to support the idea that differences in reinfection rates may be a direct reflection of different rates of exposure in these children. For these purposes we chose to use antigen preparations to detect antibody responses which have been widely reported to be useful as indicators of exposure and/ or which illustrate an age-related increase.

Severe anemia and hyperparasitemia are the most frequent reason for hospital admission due to malaria in our own study setting, whereas organ complications such as cerebral malaria, and, perhaps as a consequence, death, are a relatively rare occurrence (only three deaths occurred in our study cohort). Comparison of our results concerning antibody responses measured at admission with those of studies with much higher fatality rates is therefore not informative. Our findings are nevertheless in broad agreement with those of a study of Gambian children,[21] although we did find that the circumsporozoite antigen-specific IgG response was significantly lower in the group with severe anemia. This impairment was not related to differences in age, level of parasitemia, or reinfection rate.

We observed little change over time in IgM antibody responses, which were similar for the two groups in this study. This may reflect persistent stimulation with T-cell-independent antigens and/or primary responses following reinfection with novel parasite strains. This unchanging pattern of IgM responses contrasted markedly with fluctuations in the profile of IgG responses. The pattern of initially low pre-treatment followed by increasing post-treatment anti-NANP IgG responses we observed is consistent with that reported in a prospective longitudinal study in Thai adults.[30] Our data further show that these particular responses persist at higher levels in the absence of infection, suggesting that lower levels of antibody found in the acute phase of infection may be directly related to the presence of blood-stage parasites. At least one protein expressed by the latter contains a sequence homologous with NANP,[31] thus raising the possibility that
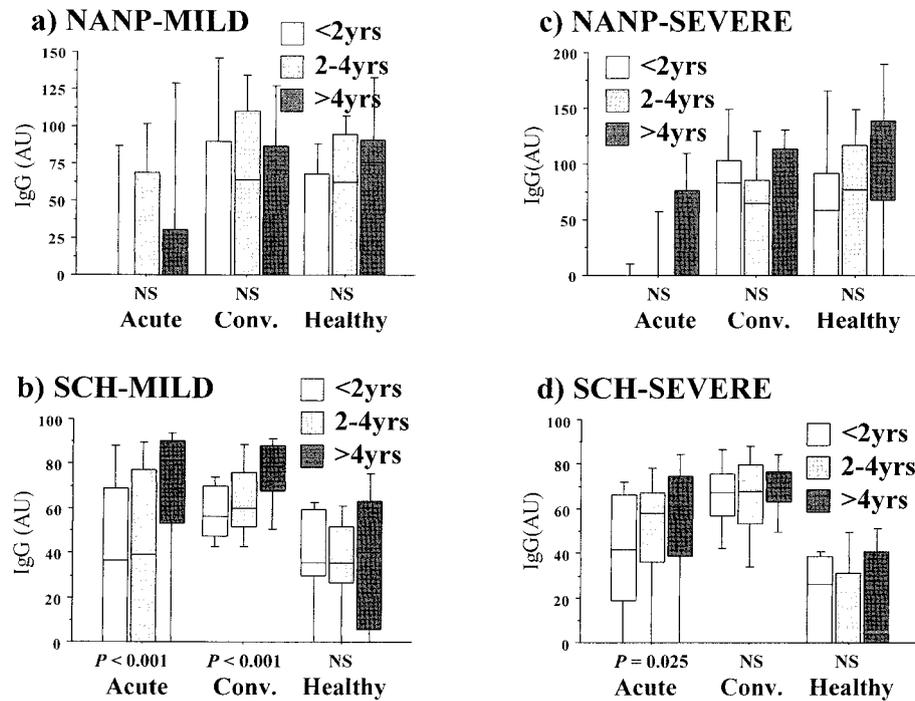
FIGURE 3.   Plasma IgM and IgG antibody responses specific for the CSP repeat region (NANP; Figures 3a and 3c) and schizont antigen (SCH; Figures 3b and 3d) detected by ELISA at different time points pre- and post-treatment in those presenting with mild or severe malaria, segregated according to the different age-classes as indicated. Box-plots represent medians with 25th and 75th percentiles (error bars for the 10th and 90th) of antibody concentrations in Arbitrary Units (AU) according to the formula originally defined by Rasheed and others:[27] Conv = convalescent phase.

the presence of such cross-reactive epitopes may result in a reduced level of detectable NANP-specific IgG antibodies in the acute stages of the infection. When the children were healthy, higher levels of these same antibodies were detected in the plasma of those who presented with severe compared with mild malaria. Interestingly, the highest levels were in the sub-group that had severe malaria but no evidence of severe anemia at admission. Reinfection rates did not correlate with these responses, and other variables, such as the age profile, did not differ between the groups. This therefore remains an unexplained observation, perhaps related to chronic persistence of antigen after a hyperparasitemic episode.

Non-responsiveness to circumsporozoite protein in a pro-

TABLE 1
The levels of plasma IgG responses according to the presence or absence of reinfections in groups segregated by their clinical presentation at admission

	IgG NANP*			IgG SCH*		
	Reinfected†	Not Reinfected	P‡	Reinfected†	Not Reinfected	P‡
Mild	68 (90)	60 (78)	NS	60 (19)	30 (39)	<0.001
	(n = 17)	(n = 29)		(n = 16)	(n = 31)	
Severe	77 (115)	89 (124)	NS	24 (39)	0 (30)	
	(n = 25)	(n = 19)		(n = 26)	(n = 22)	NS

* Plasma levels of antibodies to CSP repeat region (NANP) and schizont antigen (SCH) measured in healthy-phase samples; values are medians of antibody concentrations in AU (*interquartile ranges*), AU = arbitrary units according to the formula originally defined by Rasheed and others.[27]
† Reinfections with *P. falciparum* recorded during follow-up surveillance between convalescent- and healthy-phase sample collection.
‡ *P* values assessed by Mann-Whitney U-test.
NANP = CSP region; SCH = schizont antigen.

portion of individuals living in endemic areas is a well-documented finding.[5,10,14,15,30,32,33] Our findings both confirm and extend these observations, showing that the lack of an anti-NANP IgG response persists over time in some individuals despite the fact that they are all able to produce IgM to the same molecule. In addition, more than 50% of these non-responsive individuals were reinfected at least once prior to collection of the last plasma sample. This argues against a lower degree of exposure as the explanation for their non-responsiveness. We conclude as have others[10,14] that genetic factors are likely responsible for this phenomenon. In the study described here, NANP non-responders were also low- or non-responders to a repetitive epitope of liver stage antigen-1 of *P. falciparum* (Luty AJF and Ulbert S, unpublished data), suggesting that the mechanism involved may operate at the level of control of the recognition of or class-switching in response to T-cell-independent epitopes.

An additional conclusion from these data is that blood-stage parasites, particularly their antigens or breakdown products following drug-induced parasite clearance, leads to transient enhancement of the level of total anti-parasite IgG responses. This is corroborated by the fact that, in the absence of current or recent infection, these responses declined significantly, as others have also reported.[30] A notable aspect of the current study was the marked reduction of such responses in persons with the highest rate of reinfection, i.e., in the group that presented initially with severe malaria. We have observed the same profile of IgG responses to the 19kDa C-terminal fragment of the *P. falciparum* merozoite surface protein-1 (Luty AJF and Köhler C, unpublished data). The level of parasitemia was a principal criterion dis-

tinguishing the two groups, while differences in reinfection rates distinguished them subsequently. We have also shown that reinfection associated with both higher parasitemia levels and lower hematocrit values in those that had had severe malaria.[25] These observations point to a fundamental difference in susceptibility to infection in these two groups of children. We speculate that the significantly lower parasite-specific IgG responses observed in the healthy phase in those with severe malaria may reflect a developing state of immunological tolerance with respect to parasite antigens. This could result from the cumulative effects of repeated high level antigenemia experienced during successive infections. Antigen-specific B- and T-cell anergy could both be expected under these conditions.[34,35] T-cell-independent IgM and low-affinity IgG responses, however, would be less affected,[35] thus allowing for maintenance of at least a degree of antigen-specific antibody activity. T-cell-independent responses represent a high proportion of the anti-parasite antibodies present.[36] One outcome of such an exposure-related effect might be to abrogate age-related enhancement of responses. In support of this, our data show that the levels of anti-parasite IgG responses in those with severe malaria had only a weak age-related association at admission, which was not sustained in convalescence. This is in marked contrast to the significant age-related association of these IgG responses both at admission and in convalescence in those with mild malaria.

In summary, the principal conclusions from this longitudinal study are that age and current as well as previous clinical and parasitological status are all factors which can strongly influence the level of parasite-specific IgG responses in young African children. These findings may have important implications for the interpretation of cross-sectional as well as vaccine-related studies which incorporate antibody measurements in their design. Our data nevertheless gave no indication of a significant difference in the degree of exposure to infection experienced previously by the children who presented with either severe or mild malaria. This lends weight to our argument against an exposure-related explanation for the difference in reinfection rates. The results also provide evidence to suggest that a severe malaria attack, associated with increased susceptibility to infection with *P. falciparum* in the same individuals, may lead to a state of anergy with respect to antibody responses to parasite antigens in some children. Further longitudinal sampling in the same children will help clarify this particular aspect. Lastly, our data show that in the absence of microscopically-detectable parasitemia the level of total anti-parasite IgG antibody but not that of anti-circumsporozoite IgG can be a reliable indicator of recent infection in young children. Differences in the development of IgG subclass responses, in particular of IgG3, may be of pivotal importance in relation to the outcome of infection in those with cerebral malaria.[19,37] Whether such qualitative defects also play a role in the development of severe, but non-cerebral, malaria remain to be elucidated.

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