INTRODUCTION

Adhesion of Plasmodium falciparum-infected erythrocytes (IE) in the microvasculature is implicated in the pathogenesis of severe malaria.1,2 Accumulating evidence points to the importance of antibody responses with specificity for variant surface antigens (VSA) expressed on IE in protection against malaria, and the development of immunity to severe malaria may be related to the relative dominance of a subset of VSA variants.3,4 Nevertheless, the individual factors that lead to the development of severe malaria remain to be elucidated, and very few studies have addressed this question in the context of the profile of VSA-specific antibodies. A trend toward a reduced anti-VSA antibody profile in Kenyan children with severe malaria has been reported, but no such trend was evident in an earlier study in Gambia.5,7

VSA are thought to comprise single members of a family of variable parasite antigens, collectively referred to as P. falciparum erythrocyte membrane protein (PfEMP)-1, which are expressed on the surface of individual trophozoite-infected erythrocytes.8 PfEMP-1 proteins are encoded by members of a multigene family, designated var, and are thought to be involved in rosette formation and adhesion to endothelial cells lining postcapillary venules via interactions with a number of host receptors, including the leukocyte differentiation antigens CD36 and CD54, as well as other host adhesive ligands.9-17 A second family of variable parasite antigens, the rifins, has recently been described, and they also may contribute to the constellation of antibody-inducing VSA expressed on IE.18,19 Naturally acquired antibodies agglutinate parasite-infected erythrocytes in a variant-specific manner, and switches in agglutinating phenotype correlate with switches in cytoadherent phenotype and var gene expression.4,20-22 Sera that agglutinate P. falciparum-infected erythrocytes also inhibit binding to CD36 and immunoprecipitate a protein equivalent in size to PfEMP-1.8 These observations point to PfEMP-1 as the likely principal target of naturally acquired agglutinating antibodies in humans.16,23-25 Numerous studies have demonstrated the wide diversity of VSA.5,6,20,26—33 Nevertheless, VSA do contain regions that are either identical or antigenically cross-reactive, and conserved epitopes are present in PfEMP-1.34,35 More-recent studies have confirmed that VSA contain cross-reactive epitopes, lending support to the idea that these molecules could be used in the design of antiadhesive therapeutics.36,37

In the study described here, we attempted to assess the relationship between VSA-specific antibodies and the severity of the outcome of P. falciparum infection in a cohort of Gabonese children admitted to a hospital with either mild or severe malaria. The study design included long-term post-treatment follow-up of the children, who are predicted to be in the process of acquiring immunity to malaria, which allowed a prospective assessment of the role these antibodies may play in preventing subsequent malaria episodes. Thus, we used flow cytometric methods with a panel of heterologous parasite isolates collected from the study area to assess anti-VSA antibody responses at admission (pretreatment), one month later (convalescence), and at least six months later when the children were healthy and parasite-free. Since our own published work has shown that the children who presented with severe malaria were reinfected significantly earlier and had significantly higher malaria attack rates than their counterparts with mild malaria, we specifically sought correlations between the profiles of anti-VSA antibody responses and reinfection profiles.38,39

PATIENTS AND METHODS

Study area and plasma sample collection. The study was conducted at Albert Schweitzer Hospital in Lambaréné, Gabon, which is situated in the equatorial belt of Central Africa. Malaria in this area is hyperendemic, perenially transmitted, and predominantly caused by P. falciparum, with an estimated entomologic inoculation rate of ~50.40,41 Plasma samples for the study were collected from 1995 to 1997 from a total of 200 children taking part in a matched-pair, case-control study of severe versus mild malaria. Individuals were included if they had P. falciparum malaria with a parasitemia greater than 1,000 parasites/μL, were older than six months, and were not homozygous for hemoglobin S. Severe malaria was defined as severe anemia (hemoglobin < 50 g/L) and/or
hyperparasitemia (> 250,000 parasite/μL, corresponding to > 10% infected erythrocytes) with or without other signs of severe malaria. The matched pair control for each severe malaria case was chosen from among patients of the same gender, age, and provenance but presenting with mild malaria, recruited as soon as possible after inclusion of the severe case. Mild malaria was defined as *P. falciparum* parasitemia of 1,000–50,000 parasites/μL of blood with a hemoglobin > 8 g/dL, glycemia > 50 mg/dL, and no signs of severe malaria. Participants ranged in age from 6 months to 11 years, with a mean of 44 months. Subjects with either concurrent acute infection or a previous medical history of hospital admission, or who admitted intake of antimalarials within the preceding week, were excluded. Subjects with chronic diseases or malnutrition also were excluded from the study. All participants were treated with standard courses of antimalarials.

Plasma was isolated from undiluted whole blood taken on three occasions: 1) on the day of admission to the hospital just before administration of antimalarial chemotherapy, here referred to as the acute phase sample; 2) one month post-treatment, referred to as the convalescent phase sample; and 3) at least six months post-treatment when the children had no clinically obvious infection and had been aparasitemic for the preceding six weeks, as determined during the active every-two-week surveillance undertaken in the participants’ homes after discharge from the hospital, here referred to as the healthy phase sample. The active in-home surveillance referred to above allowed identification of reinfections (new infections/malaria episodes) through examination of routinely prepared and Giemsa-stained blood smears. Children diagnosed with malaria (defined as any *P. falciparum* parasitemia with a rectal temperature > 38°C or clinical symptoms) during the follow-up were given standard antimalarial treatment (sulfadoxine-pyrimethamine). The time to first reinfection was defined as the time from admission until detection of the first thick blood smear containing parasites. Incidence density rates (IDR), i.e., annual reinfection or malaria attack rates, were estimated by calculating the ratio between the number of reinfections detected and the follow-up observation in years, corresponding to a total of four years’ surveillance for each individual.

**Ethical permission.** Informed consent for participation in the study was obtained before inclusion from the parents of each subject. Ethical clearance for the study was obtained from the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Lambéré.

**Parasite isolates.** The three *P. falciparum* isolates used in this study (reference names cys007, cys028, and cym033) were selected from a panel of parasites obtained from patients recruited in a separate outpatient study carried out from May to December 1997 at Albert Schweitzer Hospital. The cys007 and cys028 isolates were obtained from children with severe *P. falciparum* malaria who were 38 and 12 months old, respectively. The cym033 isolate was obtained from a 23-month-old child with mild malaria. All were confirmed monoinfections with *P. falciparum* that were shown by routine standardized merozoite surface antigen-based polymerase chain reaction genotyping techniques to be polyclonal, with at least two strains (AE Tebo, unpublished observations). Peripheral blood from these patients was centrifuged, and the erythrocytes obtained were spin-washed twice with complete medium comprising RPMI1640 culture medium (Seromed, Biochrom, Germany) buffered with 25 mM Hepes (Sigma, Deisenhofen, Germany), and supplemented with 25 mM sodium bicarbonate, 2 mM L-glutamine (Life Technologies, Eggenstein, Germany), 300 mM hypoxanthine, and 10 μg/mL gentamicin (Life Technologies). Pellets containing infected erythrocytes were then cryopreserved in liquid nitrogen for subsequent *in vitro* adaptation. Primary isolates were adapted to *in vitro* culture according to the method of Trager and Jensen (1977).

**Measurement of *P. falciparum*-infected erythrocyte-specific IgG responses.** Detection of IgG with specificity for the surface of *P. falciparum* trophozoite-infected erythrocytes (T-IE) was performed using a flow cytometric assay described in detail elsewhere. For this purpose, a hyperimmune plasma pool (HIP) was made from samples collected from semi-immune Gabonese adults living in the study area, and a nonimmune plasma pool (NIP) from samples from nonmalarial-exposed European adults (UHTüb). All plasma aliquots were stored frozen at −70°C. Frozen aliquots of parasites were thawed and cultured in the presence of 10% prescreened nonimmune AB+ serum (from the blood bank of University Hospital, Tübingen, Germany [UHTüb]), and were then incubated in an atmosphere of 5% CO2, 5% O2, and 90% N2. Fresh O+ erythrocytes depleted of lymphocytes (UHTüb) were periodically added. Isolates were initially expanded over a short period of 8–10 multiplication (48-hour) cycles, after which identical stabiles of cultures containing mostly asexual ring forms were cryopreserved for later use in cytometric assays. (See below.)
Samples were segregated into infected (T-IE) and noninfected (E) erythrocytes using forward and side scatter parameters, and a gate defining fluorescing (ethidium bromide-stained) cells further segregated parasite-infected cells. Using mean fluorescence intensity (MFI) values, the amount of IgG specifically bound to the surface of T-IE was estimated by application of the following formula: MFI specifically bound to the surface of T-IE was estimated by mean fluorescence intensity (MFI) values, the amount of IgG stained) cells further segregated parasite-infected cells. Using MFI E test) - (MFI T-IE NIP - MFI E NIP). A threshold value of positivity was established for each isolate using a panel of 50 individual plasma samples from nonexposed Europeans (UHTüb), such that test samples were considered anti-VSA IgG responders when the MFI calculated with the above equation was greater than the mean plus two standard deviations of the values obtained with these control samples. The MFI thresholds established in this way for the three isolates were cys007: 11.3; cys028: 12.0; and cym033: 22.4.

**Statistical analysis.** Data were analyzed using the Statview software program. For paired and unpaired comparisons of continuous variables, the nonparametric Wilcoxon sign rank, Kruskal-Wallis, and Mann-Whitney U-tests were used. Contingency tables with continuity corrections were used to compare proportions within and among groups. Correlations between continuous variables were assessed with the nonparametric Spearman rank test. The level of significance in all cases was set at a two-tailed \( P < 0.05 \). Lack of sufficient plasma, losses to follow-up, and exclusion of data from children receiving transfusions resulted in the following minimum sample sizes used for pairwise comparisons of antibody responses of the mild and severe malaria groups, respectively: acute phase, 95 pairs; convalescence, 36 pairs; healthy phase, 27 pairs. Sample sizes for other comparisons are given where appropriate in figure legends.

**RESULTS**

We wished to determine whether children presenting with radically different clinical forms of *P. falciparum* malaria also exhibited differences in their profile of heterologous anti-VSA antibody activity. We analyzed antibody data in two ways: 1) qualitatively with respect to the presence or absence of antibody directed to VSA of the isolates; and 2) quantitatively with respect to the amount of antibody directed to VSA, represented by measurements of the MFI.

**Anti-VSA antibody activity and clinical outcome.** At the qualitative level, the proportion of individuals with IgG specific for VSA of > 1 isolate increased significantly from the acute to the healthy phase in both groups (mild: 15% versus 61%; severe: 7% versus 36%; \( P < 0.001 \) in both cases; see Figure 1). Comparison of the two groups showed that a significantly higher proportion of those who presented with mild malaria had IgG specific for VSA of > 1 isolate in both the convalescent and healthy phases (Figure 1). At the latter time point, furthermore, more than half of those who presented with mild malaria had IgG specific for the VSA expressed by all three isolates, a significantly greater proportion compared with those who presented with severe malaria (55% versus 31%, \( P = 0.025 \)).

To assess individuals’ overall anti-VSA IgG response for quantitative assessments, we used the mean MFI of pooled responses to the three heterologous isolates. As illustrated in Figure 2, those with mild malaria had significantly higher responses than their severe malaria counterparts at all time points. The anti-VSA response increased significantly over time but only in those who presented with mild malaria (median [interquartile range] MFI: 25 [28] versus 31 [27], acute versus healthy, \( P = 0.039 \), Figure 2). In the group that presented with severe malaria, after exclusion of those who had received blood transfusions (28 individuals), convalescent phase responses were significantly lower than those recorded.
in either the acute or healthy phase ($P < 0.001$ for both comparisons).

**Anti-VSA antibody activity and reinfections.** In earlier reports concerning these study participants, we documented the significant differences in the delay to the first post-treatment reinfection as well as in the IDR of reinfection, equivalent to annual malaria attack rates, in the groups that presented with either mild or severe malaria. By these criteria, we consider those with severe malaria to be more susceptible to infection as judged by the significantly shorter delays to the occurrence of the first post-treatment reinfections as well as the 2–3-fold higher IDR observed in this group. Since the prospective part of the study, in terms of the active follow-up surveillance of *P. falciparum* reinfections post-treatment, was initiated when the convalescent phase sample was collected, analyses concerning anti-VSA IgG responses were applied only to data relating to the convalescent and healthy phases.

At the quantitative level, considering the whole cohort together after exclusion of transfused individuals, convalescent phase responses showed a significant positive association with the delay to first reinfection ($\rho = 0.262, P = 0.009$) and a

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**FIGURE 3.** The association between incidence density rates (IDR) and either (a) convalescent ($N = 93$) or (b) healthy-phase ($N = 100$) antivariant surface antigens responses, measured as the mean fluorescent intensities (MFI) of pooled data for all three isolates, with corresponding correlation coefficient ($\rho$) and statistical significance of the association ($P$), assessed by the Spearman rank correlation test. Note: Data from individuals with severe malaria who received blood transfusions are excluded.

**FIGURE 4.** The antivariant surface antigens IgG responses in healthy-phase samples, expressed as mean fluorescent intensities (MFI), in (a) mild or (b) severe groups segregated according to their incidence density rates (IDR) of malaria attacks. Box-plots represent medians, with 25th and 75th percentiles and error bars for 10th and 90th percentiles, of mean MFI of pooled data for all three isolates combined. Differences between groups were assessed by the nonparametric Mann-Whitney $U$-test. Note: Data from individuals with severe malaria who received blood transfusions are excluded from these analyses; sample sizes for comparison: mild group, 14 high vs 13 low; severe group, 13 high vs 15 low. Dashed line indicates MFI of a pool of semi-immune Gabonese adult sera for reference.

**FIGURE 5.** The malaria attack rates (annual reinfection rates/incidence density rates) of groups segregated according to their content of IgG in healthy phase samples with specificity for variant surface antigens of different numbers ($0, 1, > 1$) of heterologous isolates. Box-plots represent medians, with 25th and 75th percentiles and error bars for 10th and 90th percentiles, of annual reinfection rates. (See Patients and Methods section for details of calculation.) Differences between groups were assessed by the nonparametric Mann-Whitney $U$-test. Note: Data from individuals with severe malaria who received blood transfusions are excluded from these analyses; group sizes ($0, 1, > 1$) for comparison: 6 vs 43 vs 52.
significant inverse correlation with IDR, an observation also found with healthy phase samples (Figure 3). Segregation according to both clinical presentation and higher or lower IDR, defined according to the 75th (high) and 25th (low) percentiles, demonstrates that the association between healthy-phase anti-VSA antibody levels and protection from malaria is restricted to those who presented with mild malaria (Figure 4). At the qualitative level, comparison of IDR according to the presence or absence of healthy-phase anti-VSA IgG showed a recognizable trend for decreasing malaria attack rates associated with increasing recognition of heterologous isolates (Figure 5).

Anti-VSA antibody activity in the context of age, hemocrit, parasitemia, blood group, and hemoglobin phenotype. Children recruited into the study had a mean age of 44 ± 23 months. The convalescent phase anti-VSA IgG response showed a weak but significant inverse correlation with age (p = -0.264, P = 0.005). No such trend was seen in either the acute or healthy phase. Separate assessments of either hemocrit or parasitemia at admission, as well as of hemoglobin phenotype (HbAA versus HbAS), revealed no associations with either the presence or absence of anti-VSA IgG responses or with their magnitude at any time point in either group of children. Segregation according to ABO blood group type revealed a significantly higher proportion of anti-VSA IgG responders to the cys007 isolate among those with group O compared with non-O but only in the acute phase (78% versus 61%, O versus non-O, P = 0.016), and a similar but nonsignificant trend for responses to isolate cym033 (62% versus 49%, P = 0.079) but no difference for cys028 (19% versus 25%). The magnitude of either isolate-specific or overall mean anti-VSA IgG response was not associated with ABO blood group type (data not shown).

DISCUSSION

Although designed with similar aims, this study differs in several aspects from others that have investigated anti-VSA antibody responses in the context of clinical outcome. First, assessments of these immunologic parameters in such a case-control, age- gender- and residence-matched pair study of severe versus mild malaria have not been reported before. Second, although others have assessed anti-VSA antibody responses at different time points in carefully selected groups presenting with different clinical forms of P. falciparum malaria, none has made the same systematic, prospective, longitudinal investigations in a cohort of individuals who were actively acquiring immunity to P. falciparum for the duration of the study. Last, it is also important to note that all reinfec-
tions detected during the 4-year active follow-up surveillance period considered here corresponded to clinical episodes, i.e., P. falciparum parasitemia with fever and/or symptoms.

Compared with their matched counterparts with mild malaria, we found that children with severe malaria had a narrower range of anti-VSA antibody responses at all times. Notwithstanding these differences, in the acute and convalescent phases, the profile of isolate recognition changed little: The majority in both groups had antibodies specific for the VSA of only one of the three heterologous isolates. These findings are consistent with studies conducted in an area of Kenya with transmission characteristics equivalent to those in our study area, which showed that anti-VSA antibodies in young children are similar in the acute and convalescent phases, and that they recognize approximately one-third of all heterologous isolates. Under the much less intense conditions of transmission prevalent in other areas, these profiles clearly differ.

As discussed earlier, we believe that the participants in this study were of an age at which active acquisition of immunity to P. falciparum malaria is occurring. The prospective part of the study allowed us to examine associations between preexisting antibody responses and subsequent incidence of attacks in a way similar to the recently reported study of Dodoo and colleagues, the findings of which are in good accordance with our own. Thus, we were able to demonstrate that higher anti-VSA IgG levels are associated both with longer delays to the first post-treatment malaria episode, and, in the mild malaria group at least, with lower annual malaria attack rates. In addition, our data show that recognition of a greater number of heterologous isolates per se is associated with a lower rate of malaria attacks. Where our results differ from those of Dodoo and colleagues is primarily in that the associations between anti-VSA antibody responses and apparent protection that we observed were detectable in a group of children with a mean age of 44 months, i.e., in a comparatively younger group of children. We speculate that this simply reflects the 2–3-fold higher estimated transmission rate of P. falciparum in our study area which leads to earlier acquisition of immunity in children there.

The most striking of our findings concerns the profile of anti-VSA IgG responses seen with samples taken when the children were healthy and free of parasites. Perhaps surprisingly, and notwithstanding the fact that they experienced on average two to three times more malaria episodes than their matched counterparts who presented with mild malaria, only a minority of those who presented with severe malaria had developed antibodies recognizing all three isolates, while the majority persisted with antibodies specific for only one isolate, as was seen at the earlier time points. In distinct contrast, healthy phase samples from most of those who presented with mild malaria contained antibodies recognizing all three isolates. These profiles are in accordance with our own observations, in the same cohort of children, showing that antibody hyporesponsiveness to a crude preparation of sexual stage antigens of P. falciparum is a feature of those who presented with severe malaria. Individuals with persistently low heterologous anti-VSA IgG responses have been reported by others, although a specific association with the severity of clinical outcome has not been identified and their frequency at the population level is not known. Heterologous anti-VSA IgG responses in those with severe malaria also may have a significantly shorter half-life than the equivalent responses in children with mild malaria if, for example, they are dominated by short-lived IgG3 responses. Where parasite-antigen-specific responses have been examined in detail, however, IgG1 has been shown to be the predominant anti-VSA isotype, while severe malaria per se has been associated with a specific absence of antiparasitic IgG3 responses. We are performing experiments designed to elucidate the isotype-specific profile of anti-VSA IgG responses in this cohort.

Regardless of the cause or causes of the different profiles of anti-VSA IgG responses in the two groups of children in our
study, the findings indicate that children who have experienced severe malaria persist not only with a significantly lower amount of these isolate-specific antibodies but also with a significantly greater number of potential “gaps” in their anti-VSA antibody repertoire compared with age-matched counterparts who have had only mild malaria. As others have convincingly shown, such “gaps” in recognition are associated with malaria episodes in individuals with low immunity and also with *P. falciparum* infections in pregnant women. The level of anti-VSA antibodies, furthermore, has been shown to correlate with adhesion inhibition, itself a surrogate marker of protection against *P. falciparum* infection. Thus, we believe the data presented here provide further evidence for an immunologic basis to the explanation of the radically different susceptibility to malaria in the two groups of children in our study. We showed, in an earlier nation of the study, the findings indicate that children who have experienced severe malaria persist not only with a significantly lower amount of these isolate-specific antibodies but also with a significantly greater number of potential “gaps” in their anti-VSA antibody repertoire compared with age-matched counterparts who have had only mild malaria. As others have convincingly shown, such “gaps” in recognition are associated with malaria episodes in individuals with low immunity and also with *P. falciparum* infections in pregnant women. The level of anti-VSA antibodies, furthermore, has been shown to correlate with adhesion inhibition, itself a surrogate marker of protection against *P. falciparum* infection. Thus, we believe the data presented here provide further evidence for an immunologic basis to the explanation of the radically different susceptibility to malaria in the two groups of children in our study. We showed, in an earlier assessment of cell-mediated immune responses in these same children, that parasite antigen-specific interferon-γ responses differed significantly between the groups with either mild or severe malaria. Clarifying whether the differing profiles of anti-VSA antibody responses we have reported here reflect similar differences in the ability to develop and sustain appropriate VSA-specific CD4+ T-cell responses is the subject of ongoing studies in our group.

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