The variant surface antigens (VSAs) of *Plasmodium falciparum*-infected red blood cells are potentially important targets of naturally acquired immunity to malaria. Natural infections induce agglutinating antibodies specific to the VSA variants expressed by the infecting parasites. Previously, when different parasite isolates were tested against a panel of heterologous plasma from Kenyan children, the proportion of plasma that agglutinated the parasites (the agglutination frequency [AF]) was highly variable among isolates, suggesting the existence of rare and prevalent variants. Here, the AF of 115 isolates from Kenyan children were compared. The results show that the AF of isolates causing severe malaria were significantly higher than those of isolates causing mild malaria; and AF decreased significantly with the increasing age of the infected child. We propose that parasites causing severe disease tend to express a subset of VSA variants that are preferentially associated with infections of children with low immunity.

The fact that children become naturally immune to malaria during the first 5 years of life supports the feasibility of developing an effective malaria vaccine. Although naturally acquired immunity to malaria takes many years of exposure to develop, epidemiological data suggest that the risk of severe manifestations of the disease are diminished after only a very few clinical episodes [1]. Identification of the targets of this immunity is clearly a high priority.

The *Plasmodium falciparum* variant surface antigens (VSAs) meet many of the criteria of potential immune targets. First, through coordinated parasite gene expression, VSA undergoes clonal antigenic variation in a manner analogous to surface antigens expressed by trypanosomes and *Borrelia* and *Neisseria* species [2]. Until recently, VSA was thought to be made up exclusively of *P. falciparum* erythrocyte surface protein–1, the name given to a large family of parasite adhesion molecules expressed by the var genes [3–5]. Recently, we described an even larger family of clonally variant surface molecules called rifins; however, their role in the agglutination of field isolates is still unknown [6, 7]. Second, individual VSAs can bind to different combinations of microvasculature endothelial receptors, including thrombospondin, CD36, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule, E-selectin, CD31, P-selectin, chondroitin sulfate A, and αvβ3 integrin [8–10]. Such interactions are thought to play an important role in the distinctive pathology of falciparum malaria, in particular the sequestration of infected red blood cells (iRBC) in the microvasculature of the brain [11–14]. Finally, VSAs are highly immunogenic, and we have previously shown that naturally occurring anti-VSA antibodies provide variant-specific protection against malaria [15, 16].

Despite the prominence and immunogenicity of VSA, their extreme diversity, revealed both through the analysis of expressed var genes from field isolates [17, 18] and through comparisons of the specificities of naturally occurring anti-VSA antibodies [19–26], may be thought to limit the usefulness of these molecules in a vaccine. However, at least some degree of antibody cross-reactivity is detectable toward the epitopes expressed by different parasite isolates [20, 21, 24, 27–29]. Furthermore, children's immune systems learn to recognize most local isolates during the period when naturally acquired immunity develops [16, 25, 30], and the immune systems of adults are capable of mounting responses that recognize infected erythrocytes from different continents, suggesting that the global diversity of VSA is finite [31].

We have suggested previously that the cytoadherence function of VSA may restrict the diversity of distinct antigenic phenotypes that parasites can display to the host immune system [24]. Consistent with this idea, in vitro studies [32, 33] show that affinity selection of parasites on purified endothelial receptors result in the purification of limited subsets of variants.
The extent to which affinity selection on endothelial receptors in vivo could restrict the overall diversity of epitopes found in the parasite population is unknown. However, the VSAs of parasites sequestered in the placenta during pregnancy have recently been shown to be distinctive in both their adhesive and antigenic properties [34, 35], suggesting that the epitope regions and cytoadherence ligands of at least some VSA subsets are closely associated.

Several previous field studies have searched among established infected cell cytoadherence phenotypes for markers of parasite virulence [36]. Here, we have taken a novel approach that acknowledges the potential relationship between the cytoadherent and immunomethological properties of VSA variants. We showed, in a previous study of parasites taken from young children, that isolates differ considerably in their ability to be agglutinated by naturally occurring antibodies from different individuals (here referred to as the agglutination frequency [AF] of the parasite isolate), suggesting that some VSA epitopes may be more prevalent in the community than others. In addition, we found that isolates from children with severe malaria often had a surprisingly high AF [24].

To explore whether high AF could be a general characteristic of isolates causing severe disease, we developed a procedure, using a panel of plasma from Kenyan children, to compare the AF of parasite isolates from children attending Kilifi district hospital in Kenya. The results show that parasites from children with severe malaria had a significantly higher AF than those from children with mild disease. In addition, among children <7 years old, there was a significant reduction in AF with increasing age of the infected child, consistent with selection of low AF variants by the developing naturally acquired immune response.

Materials and Methods

**Study site.** The study was carried out at Kilifi district hospital, situated 50 km north of Mombasa on the Kenyan coast. The hospital has an outpatient department, a pediatric ward, and a high-dependency ward for children with life-threatening illness.

**Clinical definition of severe disease.** Children coming to the hospital with a primary diagnosis of malaria were divided into 3 categories. Those who came to the outpatient department but were not admitted were defined as mildly affected. Those who were admitted to the general pediatric ward were defined as moderately affected. Those who were prostrated or had signs of respiratory distress were admitted to the high-dependency ward and were defined as severely affected [37].

**Parasite isolates.** Children were recruited for the study if they were blood group O or A, had a primary diagnosis of malaria, and had >1 trophozoites per 100 erythrocytes. Isolates were collected, and lymphocytes and phagocytes were removed, as described elsewhere [24]. For each isolate, a sample of acute-phase plasma was stored at −20°C (homologous acute-phase plasma; see below). The study was divided into 2 parts. First, between January 1997 and August 1997, parasites from all admitted patients meeting the above criteria were collected to test the effect of host age on the AF of the infecting parasites. Second, between January 1998 and March 1999, parasites from mildly and severely affected patients were collected to study the relationship between host disease severity and parasite AF.

**Cell culture and dilution.** Parasites were cultured until they were mid- to late trophozoites, as described elsewhere [24]. After culture, the total parasitemia, including immature or dead parasites, was calculated from a count of trophozoites per 1000 erythrocytes. One hundred infected erythrocytes were counted, and parasitized erythrocytes were categorized as mature trophozoites, gametocytes, or dead, pyknotic parasites. Because early gametocytes are often formed in culture and observed in agglutinates (P.C.B., unpublished data), the effective parasitemia was calculated from the combined parasitemias of pigmentated trophozoites and gametocytes (early-stage I–IIa gametocytes have recently been shown to express P. falciparum erythrocyte surface protein–1 [38]).

**Agglutination assays.** Parasite isolates were tested against a panel of 15 Kenyan plasma samples, positive controls, a pool of Kenyan plasma, the homologous acute-phase plasma, and a negative control.

Plasma samples drawn from 15 Kenyan children formed the panel used to assess the AF of each isolate. To ensure that the measurements of AF did not merely reflect the prevalence of VSA variants at the time that parasites were collected, the plasma panel (with the exception of sample 3108-a) was sampled well before the beginning of the study. Plasma samples were collected between May and September 1995, except numbers 1058-a (November 1994), 1800-k (December 1995), and 3108-o (August 1996; see figure 1 for a full list of plasma numbers). All were acute-phase samples from children with moderate malaria. The children were 24–36 months old and had blood group A. The AF of each parasite isolate was defined as the total number of the panel that promoted infected cell agglutination of that isolate.
To detect antibodies that were common to many children and to dilute away those that were rare, a pool of plasma (blood groups were mixed) was prepared from 100 children who were admitted to the pediatric ward in Kilifi and were between the ages of 24 and 36 months. The following dilutions were made in European serum: 1:1, 1:5, and 1:25. With respect to each individual plasma sample within the pool, this corresponded to the lower 3 dilutions of the adult plasma sample (1:100, 1:500, and 1:2500).

Homologous acute-phase plasma was sampled at the time of disease from the child infected by each parasite isolate. A negative control was assessed to control for autoagglutination: each isolate was tested against a nonimmune European serum specimen.

Plasma samples were tested for their ability to agglutinate iRBC. Reactions were performed in microtiter plates (Falcon, Franklin Lakes, NJ) at 4% hematocrit in RPMI in a 12.5-µL total assay volume and were rotated for 1 h, as described elsewhere [24]. Agglutination assays have traditionally been scored immediately after rotation to avoid iRBC rupture [40]. This method limits throughput and makes complete blinding of the assay for clinical status almost impossible. We therefore developed a novel method of preserving agglutinated iRBCs so that they could be stored and studied in a blinded fashion before each set of assays was scored. The entire agglutination assay volume was transferred onto a glass slide, and the cells were spread gently in a circle 1–1.5 cm in diameter with the plastic tip of a Gilson P-200 pipette, using the meniscus formed between the pipette and the slide. Cells were dried for 10 min at room temperature, fixed with methanol, and stored at room temperature. Before being read, slides were stained, as described elsewhere [41], using 13 µL acridine orange (5 µg/mL). Parasites were visualized using a fluorescence microscope with a ×10 objective lens.

Scoring of agglutination. Each slide was scanned for agglutinates. Agglutinates were categorized by size as follows: size A, 3–5 cells; size B, 6–20 cells; size C, 20–100 cells; and size D, >100 cells. Assays were scored as either positive or negative. They were scored as positive if >30 cells were in agglutinates containing >6 parasites, after adjusting for any agglutination observed in the European reference slide. For each slide, the size of the largest agglutinate observed was recorded.

Measurement of iRBC-specific IgG. IgG specific to the surface of iRBC was measured in each of the panel of 15 Kenyan plasma samples by indirect immunofluorescence using a method modified from Marsh et al. [40]. Cultured trophozoites were combined at 5% hematocrit with each test plasma sample (20% v/v) in PBS/0.1% bovine serum albumin (BSA). Cells were incubated 30 min at room temperature and washed in PBS/0.1% BSA. To fluorescently label IgG that was bound to cells, fluorescein isothiocyanate–conjugated goat anti–human IgG (Sigma Immunochemicals, St. Louis) was added at 1:50 dilution in PBS/1% BSA. Infected erythrocytes were stained by adding 10 µg/mL ethidium bromide. Cells were incubated at room temperature for 30 min, and after washing they were resuspended in 50 µL PBS plus 0.1% BSA. To determine the proportion of iRBC recognized by IgG in each plasma sample, 100,000 erythrocytes were analyzed using a fluorescence-activated cell sorter (EPICS/XL; Coulter Electronics, Luton, UK). The proportion of iRBC recognized by each plasma sample was calculated by counting the proportion of iRBC fluorescently labeled after correcting for the background fluorescence of
uninfected erythrocytes. The mean proportion of iRBC recognized by each plasma sample was determined among 7 clinical parasite isolates from Kilifi collected in 1999.

Data storage and analysis. Parasite data were stored and analyzed using Stata version 5.0 (Stata, College Station, TX). The AF exhibited a distribution that was highly skewed toward zero. Univariate analysis was therefore carried out using the following nonparametric tests: when 1 variable was binary, the Mann-Whitney U test (z) was used; otherwise Spearman’s rank correlation (r) was used. The Wilcoxon signed rank test was used to compare paired titers of antibody in an immune Kenyan adult with pooled plasma from Kenyan children. Multivariate analysis using logistic regression was used to correct for autoagglutination, culture parasitemia, and rosetting in the relationships between AF and host age and between AF and disease severity. For logistic regression, because of the skewed distribution of AF, it was necessary to convert AF into a binary variable by defining isolates with an AF >1 as high (AFH) and the remainder as low (AFL).

Estimation of multiplicity of infections. Polymerase chain reaction was performed, as described elsewhere [42], using primers for merozoite surface protein (MSP)-1 and MSP2, and the polymerase chain reaction products were fractionated by agarose gel electrophoresis. For both MSP1 and MSP2, the total number of bands amplified was determined. The multiplicity of infection was estimated as the largest number of bands observed between the 2 sets of amplification reactions.

Results

Between January 1997 and February 1999, 213 parasite isolates were collected and cultured. Of these, 37 were from mildly affected patients, 96 were from moderately infected patients, and 80 were from severely affected patients. Isolates were excluded for the following reasons: failure to grow (5%, 7%, and 13% of mildly, moderately, and severely affected patients, respectively); parasitemia <1% after culture (14%, 27%, and 14%); high autoagglutination (11%, 11%, and 18%); giant rosetting (0%, 4%, and 4%); and parasites bursting out of erythrocytes (0%, 0%, and 3%).

After exclusion of these isolates, AF was determined for 70 isolates from children admitted to hospital with malaria for the first part of the study; 49 from moderate cases (median age, 14 months; interquartile [IQ] range, 9–13 months) and 21 from severe cases (median, 21; IQ range, 9–41). In part 2 of the study, AF was determined for 45 isolates: 24 from mild cases (median age, 36 months; IQ range, 16–48) and 21 from severe cases (median, 40; IQ range, 24–59).

There was a large variation in AF between isolates, and the agglutination profiles were diverse (figure 1). Various parasite parameters were tested to determine their association with AF. There was no evidence for an association between the parasitemia of the culture and AF of the isolates (Spearman’s rank correlation, r = −.05, P = .60), nor was there any evidence for an association between AF and the proportion of parasites that died in culture (r = −.04, P = .70) or the proportion that formed early gametocytes (r = .004, P = .97). Analysis of these data suggests that AFH was not simply the result of cell stress during culture. Although autoagglutination and rosetting exhibited weak association with AF (r = .19, P = .047 and r = .16, P = .083, respectively), formation of agglutinates in homologous acute-phase plasma was strongly associated with AFH (Mann-Whitney, z = −3.17, P = .0015). Similarly, the titers of agglutinating antibodies in the pool of Kenyan children’s plasma and in immune adult plasma were strongly associated with AF (r = .72, P < .0001 and r = .60, P < .0001, respectively). Consistent with the immune adult donor having been exposed to repeated infection, the antibody titer was consistently higher in immune adult plasma than in the children’s plasma pool (Wilcoxon signed rank test, z = 6.6, P < .0001).

We considered the possibility that AFH isolates merely expressed more VSA variants, increasing the likelihood that any one of them would be recognized by the plasma panel. For each isolate, we recorded the size of the biggest agglutinate observed in either children’s plasma or the plasma of immune adults (all but 2 isolates were agglutinated in ≥1 of these samples). Because each agglutinate should only contain single variants, maximum agglutinate size in assays carried out at constant parasitemia should be inversely related to the diversity of the VSA variants present in each isolate. Thus, we predicted that AFH isolates would have smaller agglutinates if they contained a more complex mix of VSA variants. However, such an association was not observed. Maximum agglutinate size observed for each isolate was positively associated with AF (r = .39, P < .0001). Thus, AFH isolates showed no evidence of being more complex. Consistent with this, the genotype complexity of each isolate, as estimated by counting the number of MSP1 and MSP2 alleles, showed no significant association with AF (r = −.14, P = .15).

Heterogeneity of the 15-plasma panel. Despite being sampled from children of a similar age, the agglutinating reactivity of the plasma panel used in this study was highly variable (figure 1). Such variability in plasma reactivity was noted in a study in Sudan [27]. To confirm that the reactivity of each plasma sample was related to the presence of antibodies against the surface of the infected erythrocyte, each of the 15 plasma samples was tested as follows. First, to assess the ability to enhance nonspecific agglutination, each plasma sample was tested for its ability to agglutinate a cloned laboratory isolate ITO/C10 that exhibits autoagglutination in nonimmune European serum [39]. The AF of ITO/C10 was 0. There was no evidence of any enhancement of agglutination by any of the panel plasma samples, as compared with agglutination in European serum. Second, each plasma sample was tested for iRBC-specific IgG against 7 parasite isolates, using indirect immunofluorescence and flow cytometry. The mean percentage of iRBC immunofluorescently labeled in the 7 isolates by each plasma sample was calculated. The reactivity of each plasma sample as assessed by immunofluorescence was strongly associated with its reac-
Figure 2. Host age–dependent variation in agglutination frequency of 70 parasite isolates. Each point represents a single parasite isolate. The agglutination frequency of each isolate is plotted against the age of the child from whom the isolate was drawn.

Activity as measured by agglutination ($r = 0.72$, $P = 0.0024$). This, together with the diversity of the agglutination profiles obtained for the 115 parasite isolates (figure 1), is consistent with agglutination being mediated by variant specific antibodies.

**AF decreases with increase in host age.** To determine whether AF decreased during the age range over which naturally acquired immunity to malaria develops, we tested 70 isolates from children 2–82 months old who were admitted to hospital with malaria between January and August 1997. Among these isolates, there was a significant negative association between AF and host age ($r = -0.26$, $P = 0.033$; figure 2). There was no significant association between host age and the parasitemia of infection ($r = 0.11$, $P = 0.35$), parasite autoagglutination ($r = -0.03$, $P = 0.78$), or parasite rosetting ($r = -0.01$, $P = 0.91$). Similarly, there was no evidence for a negative association between host age and the titer of antibodies to each isolate in the plasma pool and adult plasma ($r = -0.14$, $P = 0.29$ and $r = -0.15$, $P = 0.23$, respectively). Finally, there was no significant association between host age and the ability of each isolate to be agglutinated by the homologous acute-phase plasma sampled at the time of infection ($z = -0.79$, $P = 0.43$). Thus, the only feature of the isolates that appeared to be related to host age was the AF in heterologous plasma.

To confirm that AF independently decreased with host age, the association was analyzed by logistic regression. For this analysis, those isolates with an AF $>1$ were defined as AF$^H$, and the remainder were defined as AF$^L$ (see Materials and Methods). After adjusting for rosetting, autoagglutination, and infection parasitemia, the odds ratio for being infected with an AF$^H$ isolate was 0.56 per year increase in host age (95% CI, 0.34–0.92, $P = 0.021$).

**Parasites causing severe disease tend to be AF$^H$.** To determine the relationship between AF and disease severity, we compared the AF of isolates from 21 severe and 24 mild cases of malaria sampled concurrently between January 1998 and March 1999. As shown in figure 3, isolates causing severe disease had a higher AF than did those causing mild disease ($z = -2.59$, $P = 0.0097$). Although the pool of children’s plasma was not tested, the titer of antibodies in adult plasma was higher against the isolates causing severe disease ($z = -1.96$, $P = 0.050$). In contrast, there was no evidence for a difference in the level of parasite giant rosetting or parasite autoagglutination between the 2 groups ($z = 0.03$, $P = 0.97$ and $z = 0.64$, $P = 0.52$, respectively). This was not surprising, because isolates that exhibited strong autoagglutination and giant rosetting were excluded from the study. Finally, an equal number of isolates from each group were agglutinated by the homologous acute-phase plasma ($z = 0.00$, $P = 1.00$; only 16 tested in each group).

Thus, the only striking difference between the 2 groups of isolates was their AF in heterologous plasma. This difference is unlikely to be the result of age differences between the groups of children, as the children’s ages were similar ($z = -0.81$, $P = 0.42$).

To test whether AF was an independent marker of severe disease, logistic regression analysis was again used to adjust for parasite autoagglutination, parasite rosetting, and infection parasitemia. The adjusted odds ratio for having severe disease when being infected by an AF$^H$ isolate was 5.8 (95% CI, 1.3–25.0, $P = 0.019$).

**Discussion**

Data gathered when malaria was used in the treatment of neurosyphilis demonstrated that repeated challenge with the same parasite isolate often led to the rapid acquisition of immunity to that isolate [43]. In light of these observations, it has...
often been suggested that the many years of exposure needed to develop naturally acquired immunity to malaria is a reflection of the diversity of crucial parasite target antigens in natural populations and the need for exposure to many different parasite genotypes [15].

Although the overall diversity of the parasite population may be enormous, analysis of recent epidemiological data suggests that substantial immunity to severe malaria may develop after only 1 or 2 clinical episodes [1]. Analysis of the data we collected from Kenya and Gambia has shown that antibody responses to VSA may be important in the development of naturally acquired immunity to malaria [15, 16]. Given the diversity of these molecules, responses to VSA could only have a role in the rapid acquisition of protection against severe disease if protective responses could be generated to conserved regions, or if a subset of VSA variants preferentially associated with infections of young children, had a role in the pathogenesis of severe malaria.

This study was carried out to test the latter possibility, on the basis of a previous observation that parasites from children with severe malaria are often surprisingly frequently agglutinated by plasma from other children (i.e., the parasites were AFH).

We first tested whether there was any evidence of immune selection against AFH parasites among a group of children <7 years old who were admitted to hospital with malaria. Second, we compared the AF of isolates from children with severe and mild malaria. The results show a significant reduction in AF with increasing host age, suggesting that immunity developed during the first 7 years of life has a measurable impact on the population of VSA variants expressed by the parasites causing disease. The AF of isolates causing severe malaria was significantly higher than the AF of those causing mild disease, supporting the idea that parasites causing severe malaria express a prevalent subset of VSA variants. Furthermore, 3 of 4 children who died in this study were infected by AFH isolates (figure 1).

A possible explanation for these results, outlined elsewhere [24], is that during infections parasites are under 2 opposing selection pressures, the first favoring parasites expressing VSA variants with optimal cytoadherence characteristics (functional selection) and the second favoring parasites displaying novel VSA epitopes that are not recognized by preexisting antibody responses (immune selection). If it is accepted that cytoadherence is essential to the life cycle of *P. falciparum*, then within this scenario the relative importance of immune selection over functional selection in shaping the infecting parasite population would depend on the immune status of the host. In young children, functional selection of the parasite population may be more important than immune selection. VSA variants with optimal cytoadherence characteristics may tend to expand rapidly and dominate the infections. In older children, the relative importance of the immune selection may be greater, leading to the displacement of optimally cytoadherent VSA variants by those with novel epitopes. If only a subset of VSA variants were capable of optimal binding but a very large number of variants were capable of being novel, these 2 types of selection pressure could potentially result in a spectrum of VSA with different seroprevalence in the community. Thus, the predominance of AFH isolates in young children and those with severe malaria may be the result of functional selection having a dominant role in shaping the parasite population that causes these infections.

If VSA variants expressed in severe disease are prevalent, most exposures to these variants clearly do not lead to severe disease; otherwise, severe disease would be much more prevalent. It is possible that a subset of VSA variants in every parasite genotype has a tendency to dominate severe infections in a manner analogous to the antigenically distinct populations of metastatic somatic cells in cancer [44]. Alternatively, the entire VSA repertoire of certain parasite genotypes may be adapted to optimize cytoadherence at the expense of epitope novelty. Such genotypes may correspond to the highly transmissible parasite strains discussed elsewhere [1, 45]. In either case, what events trigger the onset of severe disease at the individual level is still an open question.

In summary, we have found that high parasite AF is associated with severe disease and young host age. We have proposed the existence of a subset of VSA variants associated with infections of young children that may play a role in the pathogenesis of severe malaria. Such a subset of variants could potentially explain the relatively rapid onset of immunity to severe malaria.

Functional and epidemiological characterization of the epitopes expressed by AFH isolates may prove to be a useful new approach to identifying important targets of naturally acquired immunity to malaria. Further studies are needed to test the association of AFH isolates with specific disease syndromes, relate the age-specific reduction in AF to specific immune responses, identify the epitopes involved, and determine their prevalence in the parasite population over space and time. While characterizing the epitopes in AFH isolates, particular attention needs to be paid to their level of expression in relation to molecules such as the cytoadherence-linked asexual gene [46].

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References


