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J Immunol 2002; 168:3444-3450; doi: 10.4049/jimmunol.168.7.3444
http://www.jimmunol.org/content/168/7/3444

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**Plasmodium falciparum** Variant Surface Antigen Expression Varies Between Isolates Causing Severe and Nonsevere Malaria and Is Modified by Acquired Immunity

Morten A. Nielsen,* Trine Staalsoe,† Jørgen A. L. Kurtzhals,‡ Bamenla Q. Goka,  † Daniel Dodoo,‡ Michael Alifrangis,* Thor G. Theander,* Bartholomew D. Akanmori,‡ and Lars Hviid*

In areas of endemic parasite transmission, protective immunity to *Plasmodium falciparum* malaria is gradually acquired after numerous disease episodes during childhood. Immuneological protection against the blood stages of the infection is mainly Ab mediated (1, 2), and acquisition of protection correlates with acquisition of a broad repertoire of agglutinating Abs to parasite-encoded, variant-specific Ags (VSA) expressed on the surface of infected erythrocytes (3, 4). In a longitudinal study in Kenya, Bull et al. (5) found that parasites isolated from malaria patients were rarely agglutinated by VSA Abs in autologous plasma samples obtained before the disease episode, whereas heterologous parasites were readily agglutinated. This finding implies that only parasites expressing VSA to which the host does not possess an adequate and specific Ab response can cause disease, and that immunity relies on the accumulation of Abs to a broad repertoire of VSA specificities. In a similar study in Ghana (6), we found that malaria episodes were associated with marked and sustained increases in VSA Abs specific for the infecting parasite isolate, whereas responses to heterologous isolates were low and transient.

Recent data have documented the existence of common and rare VSA in field isolates of *P. falciparum* parasites (7, 8). We hypothesized that the common VSA correspond to VSA that are preferred in some sense by most or all *P. falciparum* isolates, probably by maximizing the probability of their successful transmission to new hosts. One way that this can be achieved is if such VSA facilitate particularly efficacious adhesion of infected erythrocytes, thus interfering with splenic clearance. If so, acquisition of immunity may shape the repertoire of VSA expressed by parasites causing clinical disease by driving VSA expression away from such preferred VSA. To study this hypothesis we analyzed plasma samples from 96 healthy Ghanaian children for levels of IgG with specificity for VSA expressed by each of 68 clinical parasite isolates from 36 Ghanaian children with severe malaria (cerebral malaria and severe anemia) and 32 children with nonsevere malaria.

**Materials and Methods**

**Malaria patients**

Patients aged 3–11 years and admitted as inpatients to the Department of Child Health, Korle-Bu Teaching Hospital, University of Ghana Medical School (Accra, Ghana), with a diagnosis of *P. falciparum* malaria were recruited for the study. All patients were febrile at admission (≥37.5°C).
and had asexual blood stage parasitemia \(>10,000/\mu\text{L}\). Only 100 patients that could be categorized as having either severe or nonsevere malaria based on the clinical criteria listed below were considered for the study. Patients with severe disease included those with cerebral malaria (score \(\geq 5\) on the Blantyre coma scale (9)) and those with severe anemia (hemoglobin, \(<50\) g/L) in the absence of any differential diagnoses. Patients with nonsevere malaria were all fully conscious and had hemoglobin levels \(>70\) g/L. Levels of parasitemia at admission and symptom duration before admission were not statistically different between groups (10). The study was approved by the ethics and protocol review committee of the University of Ghana Medical School and by the Ghanaian Ministry of Health, and malaria patients as well as healthy children (see below) were enrolled only after signed, informed consent from parents or guardians had been obtained.

**P. falciparum isolates and parasite cultivation**

A sample of parasitized erythrocytes was collected from each of the patients included in the study and snap-frozen in liquid nitrogen as previously described (11). The cryopreserved stables were thawed and cultured in vitro as described previously (12). The degree of clonality of the isolates (multiplicity of infection (MOI)) was estimated by PCR typing of the polymorphic regions of the gene encoding merozoite surface protein-1 (msp1), the gene encoding merozoite surface protein-2 (msp2), and the gene encoding glutamate-rich protein (glurp) as described previously (13).

**Plasma samples**

We used plasma samples collected from 96 Ghanaian children living in Dodowa Town, 50 km northeast of Accra, as the source of VSA Abs. The area is characterized by hyperendemic, seasonal transmission of *P. falciparum* parasites (14). All children were healthy at the time of blood sampling. We also used a pool of plasma from healthy, parasite-exposed adults from the village of Gomoa Onyadze, 80 km west of Accra, collected as part of an earlier study (15). Plasma samples from healthy Danish adults without a history of visits to malaria-endemic areas were included as negative controls.

**Immunostaining and flow cytometry**

Erythrocytes infected by late developmental stages (hemoglobin-containing trophozoites and schizonts) were purified (to \(>75\%\) parasitemia) from culture material by exposure to a strong magnetic field (Milenyi Biotec, Bergish Gladbach, Germany) as previously described (16). Aliquots of \(2 \times 10^7\) infected erythrocytes, labeled by ethidium bromide (Sigma-Aldrich, St. Louis, MO) to allow flow cytometric exclusion of remaining uninfected erythrocytes, were sequentially exposed to 5 µL plasma, 0.4 µL goat anti-human IgG (DAKO, Glostrup, Denmark), and 4 µL FITC-conjugated rabbit anti-goat IgG (DAKO). Samples were washed twice in PBS and 2% FCS flow cytometric exclusion of remaining uninfected erythrocytes. We also used a pool of plasma from healthy, parasite-exposed adults from the village of Gomoa Onyadze, 80 km west of Accra, collected as part of an earlier study (15). Plasma samples from healthy Danish adults without a history of visits to malaria-endemic areas were included as negative controls.

**Results**

**Parasite cultivation**

To minimize the risk of unwanted changes in VSA expression due to antigenic switching after prolonged culture, only the 68/100 isolates where sufficient numbers of infected erythrocytes were available for the assays within 20 days of cultivation were used in this study. By this criterion, parasite isolates from 36 patients with severe malaria and from 32 patients with nonsevere malaria were available for analysis. Although the culture time needed to obtain sufficient parasite material varied between isolates, there was no significant correlation between time in culture, age, category of parasite donor and recognition of the isolates by plasma Abs (data not shown). Our rate of success in adapting the clinical *P. falciparum* isolates to in vitro culture in this study is comparable to that obtained in similar studies (6, 7).

**Similar levels of clonality in isolates from young vs old patients and from patients with severe vs nonsevere disease**

Minimal MOI estimates ranged from 1–5 (mean, 2.0), based on PCR genotyping on polymorphic regions of msp1, msp2, and glurp (Table I and data not shown). Contingency analysis of the relationship between MOI and donor (patient) category did not identify any significant relationships (\(p \geq 0.18\) in all cases). Apart from a difference of borderline significance (\(p = 0.04\)) in the proportion of RO33-positive isolates from patients with severe and nonsevere malaria, the proportions of different msp1 and msp2 alleles or the number of different glurp alleles were not significantly related to donor category (\(p > 0.13\) in all cases; Table I and data not shown).

**P. falciparum parasites from patients with severe malaria are better recognized by VSA-specific plasma Abs than parasites from patients with nonsevere malaria**

*P. falciparum*-parasitized erythrocytes can be agglutinated by Ab-mediated VSA cross-linking (17–19). The degree of agglutination differs between isolates, and this diversity has been related to the severity of disease in the parasite donor (i.e., the malaria patient) (8). To investigate whether VSA-specific IgG recognition of parasites isolated from patients with severe and nonsevere *P. falciparum* malaria also differed, we analyzed the levels of Abs in a...
pool of plasma from adult, parasite-exposed individuals that specifically recognized VSA expressed by the 68 *P. falciparum* isolates (Fig. 2A). Thirty-six of the isolates were from patients with severe malaria, while 32 were from patients with nonsevere malaria. Ab recognition of isolates from patients with severe malaria was consistently higher than that of isolates from patients with nonsevere malaria at all plasma dilutions (Fig. 2A). The difference in the associated regression lines of parasites from the two patient categories was highly significant (by ANCOVA, *p* < 0.0001), and the magnitude of the difference indicated that Ab recognition of VSA expressed by parasites from patients with severe disease was approximately twice that of parasites from patients with nonsevere malaria (Fig. 2A). As shown above, MOI estimates were similar for patients with severe and nonsevere malaria (Table I). Removal of the data points regarding the 8 of 36 severe malaria patients with severe anemia did not markedly affect these results (data not shown). This shows that parasites causing severe disease tended to express common/well-recognized VSA compared with those expressed by parasites involved in nonsevere *P. falciparum* malaria in semi-immune children.

**P. falciparum parasites from young children with malaria are better recognized by VSA-specific plasma Abs than parasites from older patients**

In addition to the relationship to severity described above, plasma Ab-mediated agglutination of *P. falciparum* parasite isolates has also been reported to depend on the age of the malaria patient (8). In accordance with this observation we found that the regression line of IgG recognition of VSA expressed by parasites from young patients (3–4 years of age; *n* = 26) was significantly different from that of VSA of parasites from older patients (5–11 years of age; *n* = 42; by ANCOVA, *p* < 0.0001; Fig. 2B). We did not detect significant differences in VSA-specific Ab recognition of parasites obtained from subgroups (5–6 and 7–11 years old) of children >4 years of age (data not shown). Again, Ab recognition of VSA expressed by parasite isolates from young patients was approximately twice that of parasites from older children (Fig. 2B), and this was not due to differences in MOI estimates (Table I). This shows that in this area of hyperendemic parasite transmission, the parasites obtained from young patients tended to express common/well-recognized VSA compared with those expressed by parasites from older children with *P. falciparum* malaria.

**The age dependency and severity dependency of Ab recognition of VSA occur independently of each other**

The above data, together with previously published findings, show that VSA expression by parasites infecting semi-immune children in areas of endemic parasite transmission depends on both the age of the malaria patient and the severity of the malaria episode. To corroborate this finding further, we next measured levels of Abs in plasma from 96 healthy children, aged 3–8 years, with specificity for each of the 68 parasite isolates (Fig. 3). Overall, Ab recognition of the parasite VSA differed widely among plasma donors. While plasma samples from some children contained barely detectable levels of Abs specific for VSA expressed by any of the isolates (e.g., plasma donors 15, 34, and 59; see Fig. 3), others had high levels and a broad range of VSA-specific Ab (e.g., plasma donors 14, 41, and 84; Fig. 3). By analyzing the parasite-specific sum of scores from the 96 × 68 recognition matrix, we found that recognition of VSA was independently associated with both the age of the malaria patient (3–4, 5–6, and 7–11 years; *p* = 0.005) and the clinical picture (severe or nonsevere; *p* = 0.006, by two-factor
ANOVA). There was no significant interaction between these two sources of variation ($p = 0.26$). Pairwise multiple comparison procedures (Tukey’s post-hoc test) showed that while the VSA sum of scores of parasites from the youngest patient group was significantly different from that of either of the two other age groups ($p < 0.05$), the latter two were not significantly different from each other ($p \geq 0.05$). The relationship between severity and age of the parasite donor is illustrated in Fig. 4, A and B. These results show that Ab recognition of parasite VSA was independently affected by both the age and the clinical severity of the malaria patient from whom the parasite was obtained. This is important, because disease severity is inversely correlated with age in areas of endemic parasite transmission, which in all likelihood reflects age-dependent acquisition of protective immunity (reviewed in Ref. 20). Protective immunity appears to involve acquisition of Ab responses to a broad range of VSA (5), and consistent with this observation we found that VSA Ab levels correlated with the age of the healthy plasma donors (Fig. 4, C and D). The authenticity of our finding of independent effects of patient age and disease severity is supported by the fact that the age distributions of children with severe and nonsevere disease were similar (by $t$ test, $p = 0.5$) in the present study ($5.6 \pm 0.4$ and $6.0 \pm 0.5$ years, respectively; mean $\pm$ SD), due to the exclusion of children $<3$ years of age.

Parasite isolates form distinct VSA Ab recognition clusters according to disease severity and age of the donor (malaria patient)

To further substantiate our findings and to investigate whether parasites from patients with severe *P. falciparum* malaria expressed particular VSA, we used the $96 \times 68$ recognition matrix (Fig. 3) to search for patterns of similarity in the VSA Ab recognition of the parasite isolates. Hierarchical cluster analysis identified three main clusters (data not shown). In one of these (cluster I), all but one (90%) of the isolates were from severe cases, whereas this was the case for only 5 of 20 (25%) in cluster II. The third and largest cluster (III) showed an intermediate pattern, with 21 of 38 (55%) isolates from patients with severe malaria. The proportion of isolates from severe patients in the three clusters was thus quite different (by $\chi^2$ test, $p = 0.009$). When we analyzed the age distribution of the patients donating the parasites within the clusters, cluster I was composed of parasites from young patients, whereas the other two clusters contained parasites from older patients (Fig. 5A). The cluster-specific differences in patient age composition did not quite reach conventional statistical significance (by Kruskal-Wallis test, $p = 0.06$). The distribution of the parasite-specific sum of scores among the clusters showed that cluster I was composed entirely of parasites expressing very well-recognized VSA, whereas the opposite was true for cluster II. Again, cluster III
FIGURE 4. Age dependency of Ab recognition of VSA expressed by 68 P. falciparum isolates. For each parasite/plasma combination, the Ab recognition was scored on a six-level scale, according to Ab recognition of the isolate by 2-fold dilutions of a pool of plasma from adult, parasite-exposed Ghanaians (see Materials and Methods and Fig. 1 for details). The overall Ab recognition of individual isolate was subsequently calculated as the sum of scores obtained with each of the 96 plasma samples. The dependency upon the age of the parasite donors (malaria patients) is shown in A and B, whereas the dependency upon the age of the healthy plasma donors is shown in C and D. Parasite isolates obtained from patients with severe P. falciparum malaria are shown in A and C, whereas parasites from patients with nonsevere malaria are shown in B and D. In all panels, means and 95% confidence intervals are indicated.

Discussion
In areas of intense P. falciparum transmission, parasite rates and densities as well as malaria-related morbidity and mortality are inversely correlated with age, in all likelihood reflecting acquisition of protective immunity (reviewed in Ref. 20). Although this acquisition of protective immunity is paralleled by acquisition of both cell-mediated and humoral immune responses to a long list of parasite Ags, a growing body of evidence points to an important role of Abs specific for parasite-encoded VSA expressed on the surface of infected erythrocytes (5, 21–23). The best-characterized VSA is P. falciparum erythrocyte membrane protein 1 (PfEMP1), which is encoded by the var gene family containing 40–50 members/genome. PfEMP1 can mediate adhesion of parasitized erythrocytes to a range of host receptors. This sequestration is thought to be an important factor in the evasion of host immunity by the infecting parasites and thus is a key element in the pathogenesis of P. falciparum malaria. The parasites can switch between different PfEMP1 forms, and this switching is associated with changes in antigenicity and adhesion phenotype (24–26).

The repertoire of antigenically distinct VSA is unknown, but is presumably large, and it has been speculated that the slow acquisition of protective immunity to malaria reflects the necessity to build up a broad repertoire of VSA-specific Abs. Several lines of evidence support this scenario. Thus, P. falciparum parasites causing clinical disease in semi-immune children tend to express VSA that are not well recognized by preexisting plasma Abs, and clinical episodes cause a marked increase only in VSA Abs specific for the infecting parasite isolate (5, 6). Arguably the most striking evidence in favor of a major protective role of VSA-specific Abs is the marked malaria susceptibility in otherwise clinically immune women in endemic areas during their first pregnancy and its subsequent parity-dependent reduction (23, 27–30). If clinical protection against P. falciparum malaria is indeed mediated at least in part by VSA-specific Ab, it can be expected that the VSA repertoire expressed by P. falciparum parasites causing disease in semi-immune individuals is shaped by the necessity to avoid preexisting VSA-specific immunity in the host. Recent reports of an association among VSA Ab-mediated agglutination, host age, and disease severity indicate that such modulation actually occurs (7, 8).

In this study we have used flow cytometry to measure VSA-specific IgG to provide evidence of modulation of VSA expression by acquired immunity. Our method is particularly suited to this type of analysis, as it allows unbiased and quantitative analysis of large matrices of VSA and corresponding Abs of specified isotype. We found that the level of plasma IgG recognition of VSA expressed by P. falciparum isolates obtained from patients with severe malaria was approximately twice that of VSA from nonsevere isolates (Fig. 2A). In a similar way we found that VSA Ab recognition of isolates from young patients (3–4 years of age) was ~2-fold that of isolates from older patients (5–11 years of age; Fig. 2B). In neither case was this due to differences in MOI estimates between patient categories. As such, our data suggest that the earlier findings have general validity and for the first time provide quantitative information regarding immune-mediated modulation of VSA expression.
of VSA expression by parasites causing malaria in semi-immune children.

Although the incidence of severe P. falciparum malaria generally declines with increasing age in areas of endemic parasite transmission (20), our analysis of VSA Abs in plasma samples from 96 healthy children and with specificity for each of 68 parasite isolates enabled us to demonstrate independent effects of both age and severity on VSA expression (Figs. 3 and A and B). In addition, our cluster analysis of VSA Ab recognition showed that parasites expressing common or well-recognized VSA grouped together, and that the cluster thus formed was composed of parasites from young children and from children with severe disease. A similar cluster composed of rare parasites from older patients and patients with nonsevere disease could also be identified (Fig. 5).

Our findings and the earlier studies all support the “hole in the Ab repertoire” hypothesis of susceptibility to P. falciparum malaria (5, 6). According to this hypothesis, parasites causing clinical disease in semi-immune patients express VSA that correspond to holes in the VSA Ab repertoire. Thus, only parasites expressing VSA to which there is no preexisting acquired immunity can multiply in a substantial way, leading to clinical disease. Following transmission (20), our analysis of VSA Abs in plasma samples targeting a limited subset of common and particularly virulent VSA may be a realistic goal.

Acknowledgments

We gratefully acknowledge the cooperation and support of all donors and their parents/guardians. We thank Klaus Dietz and Louis Molineaux for insights, critical comments, and encouragement; Thomas H. Scheike for statistical advice; and Kirsten Pihl and Jimmy Weng for excellent technical assistance.

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