Protection against Clinical Malaria by Heterologous Immunoglobulin G Antibodies against Malaria-Infected Erythrocyte Variant Surface Antigens Requires Interaction with Asymptomatic Infections

Samson M. Kinyanjui,1 Tabitha Mwangi,1 Peter C. Bull,2 Christopher I. Newbold,2 and Kevin Marsh1,2
1Kenya Medical Research Institute Centre for Geographic Medicine Research Coast, Kilifi, Kenya; 2Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford, United Kingdom

Erythrocytes infected with mature stages of Plasmodium falciparum express variant surface antigens (VSAs) of parasite origin, including P. falciparum erythrocyte membrane protein 1. Anti-VSA antibodies protect against clinical malaria caused by parasites bearing VSAs to which they are specific (homologous), but their role in protecting against heterologous infection is unclear. Here, we report that, among 256 Kenyan children involved in a 1-year active case surveillance study, asymptomatic parasitemia was associated with an enlarged repertoire of anti-VSA immunoglobulin G (IgG) antibodies specific to apparently heterologous parasite isolates, as measured by flow cytometry. Together, asymptomatic infection and anti-VSA IgG were associated with reduced odds of experiencing an episode of clinical malaria during follow-up, whereas, independently, they were associated with increased susceptibility. These results support previous findings and underline the importance of considering the parasitological status of study participants when examining the role that immune responses to VSAs and other malaria antigens play.

Mature stages of Plasmodium falciparum insert, into the surface of their host erythrocytes, highly polymorphic antigens that undergo clonal antigenic variation, of which P. falciparum erythrocyte membrane protein 1 is the best characterized [1–3]. Clinical episodes of malaria are normally associated with the development of anti–variant surface antigen (VSA) antibodies specific to homologous parasites (i.e., those causing the current infection) [4–7]. On the other hand, recent studies suggest that chronic infections may be associated with induction of antibodies to heterologous VSA variants (i.e., those absent from the current infection) [8, 9]. Anti-VSA antibodies are variant specific, and there is strong evidence that they provide protection against parasites bearing homologous VSA [10, 11]. However, their role in protecting against parasites bearing heterologous VSA is less clear. Studies in areas of low malaria endemicity in Sudan and in Ghana found an association between protection against clinical malaria and heterologous anti-VSA antibodies to some isolates but not to others [12, 13]. In the study by Bull et al. cited above, anti-VSA antibodies (measured by agglutination) and chronic infection together, but not independently, were associated with protection against episodes of clinical malaria caused by parasites bearing heterologous VSA [9]. This observation suggests that, among children in Kilifi, protection against clinical malaria by heterologous parasites may require interaction between anti-VSA antibodies and asymptomatic infections. In that study, malaria episodes were monitored passively, and only severe cases of malaria were considered in the analysis. In the present study, we analyzed data from a 1-year active case surveillance study and considered cases of malaria of all levels of severity, to further ex-
plore the association between asymptomatic infections, heterologous anti-VSA antibodies, and protection against clinical malaria.

**SUBJECTS, MATERIALS, AND METHODS**

**Study site.** The present study was performed between September 1998 and September 1999 at the Kenya Medical Research Institute (KEMRI) Centre for Geographical Medicine Research Coast and the Kilifi District Hospital (KDH) on the Kenyan coast. Malaria transmission in this area is moderate and normally peaks a few weeks after the rainy seasons in April and November. Residents of this area receive 10–30 infective mosquito bites/year [14]. However, in 1998, transmission, as reflected by the number of children who were admitted to KDH with a final diagnosis of malaria, peaked in September. This was probably due to the unusually heavy rains that year, which were associated with the El Niño phenomenon.

**Study population.** A total of 256 children <10 years of age who were part of a larger longitudinal study that examined the epidemiology of clinical malaria in Kilifi (T.M., unpublished data) were involved in the present study. Written consent for the children’s participation was obtained from their parents or guardians. The present study received ethical clearance from the KEMRI Ethics Review Committee.

**Cross-sectional survey.** A cross-sectional survey was performed in September 1998, when local malaria transmission was maximal. During the survey, blood samples were obtained from children by venipuncture. Plasma was separated by centrifugation and stored at −20°C. The children were also examined for fever (axillary temperature was taken by use of an electronic thermometer), and their parasitological status was determined by use of a Giemsa-stained thin malaria smear (at least 500 red blood cells [RBCs] were counted). If <1 infected RBC/500 RBCs was seen, a thick smear was examined and parasites were counted against 200 leukocytes.

**Surveillance of clinical malaria episodes.** After the cross-sectional survey, malaria episodes among the participants were actively monitored for a year, by weekly follow-up. During follow-up, the participants’ body temperature and history of fever during the preceding 24 h were recorded. Anyone with a current fever (axillary temperature ≥37.5°C) was referred to a study clinic at KDH. At the clinic, children were examined, a malaria slide was prepared, and the observations were recorded by use of a pro forma method. For malaria parasites, either 500 RBCs on a thin smear or at least 200 leukocytes on a thick smear were counted. Appropriate treatment was provided free of charge. For individuals who had a history of fever during the preceding 24 h, a malaria smear was made in the field from a finger-prick blood sample and was taken back to the laboratory for examination. However, for the analysis reported in the present study, only those individuals with a current fever were considered to be symptomatic. To maximize the detection of malaria episodes, all participants were asked to report to the study clinic whenever they felt unwell. Individuals who were unavailable for follow-up for >3 consecutive weeks were withdrawn from the study.

**Assessment of anti-VSA antibody levels in plasma by flow cytometry.** The plasma obtained during the survey was assayed for anti-VSA IgG antibodies to a panel of 5 clinical isolates and 2 laboratory isolates: A4 and ITGIC15 [15]. The clinical isolates were obtained from children admitted to KDH with severe malaria and were cultured freshly to maturity by use of standard methods [16]. A pellet of erythrocytes bearing mature trophozoites was then obtained from culture by centrifugation at 560 g. The pellet was washed 3 times in RPMI 1640 medium (supplemented with 37.5 mmol/L HEPES, 2 mmol/L glutamine, 25 µg/mL gentamicin sulphate [GIBCO], and 20 mmol/L glucose and adjusted to a pH of 7.4 with NaOH [BDH]) diluted to 2%–4% parasitemia with fresh group O–positive RBCs and resuspended at 1% hematocrit in 0.1% bovine serum albumin/ PBS (assay buffer) (GIBCO). A total of 10 µL of the suspension was mixed with 2.5 µL of test plasma, in a round-bottomed 96-well plate. The plates were incubated for 30 min at room temperature. The cells were then washed 3 times in assay buffer and resuspended in 50 µL of assay buffer containing 10 µg/mL ethidium bromide and fluorescein isothiocyanate (FITC)–conjugated goat anti–human IgG antibodies (Binding Site) at a 1:50 dilution. After further incubation for 30 min, the cells were washed 3 times, and at least 1000 infected erythrocytes were counted on an EPIC/XL flow cytometer (Coulter Electronics). The percentage of infected cells staining positive with FITC after incubation with the test serum samples was taken as a proxy measure of the level of anti-VSA antibodies in the samples and was determined as follows:

\[
\frac{\text{COUNT}_{UL}}{\text{COUNT}_{LR} + \text{COUNT}_{UL}} - \frac{\text{COUNT}_{UL}}{\text{COUNT}_{LR} + \text{COUNT}_{UL}} \times 100,
\]

where UL, UR, LL, and LR correspond to upper left, upper right, lower left, and lower right quadrants on the flow cytometry dot plot, respectively (figure 1). The subtracted term is a correction for background staining of noninfected cells. The resulting figure was further adjusted for nonspecific antibody binding by subtracting the percentage of infected cells staining positive with FITC after incubation with a pool of nonimmune European plasma. For a given isolate, plasma samples from all 256 children were tested on the same occasion.

**Statistical analysis.** Data analysis was performed by use of STATA (version 6.0; Stata). First, we examined the association between asymptomatic infection and anti-VSA IgG responses to the test isolates. For this, a child’s response (percentage of infected cells positive for surface antibodies) to a given test
Figure 1. Example of a fluorescence-activated cell sorter plot of a test isolate (1776) reaction with plasma from a study child (no. 4). The percentage of infected red blood cells (RBCs) positive for surface-bound antibodies was calculated from the plot as \[
\frac{\text{COUNT}_{UR}/(\text{COUNT}_{UR} + \text{COUNT}_{UL}) \times 100}{\text{COUNT}_{LR}/(\text{COUNT}_{LR} + \text{COUNT}_{LL})}\]
where count is the number of cells and UL, UR, LL, and LR correspond to upper left, upper right, lower left, and lower right quadrants on the flow cytometry dot plot, respectively. EtBr, ethidium bromide; FITC, fluorescein isothiocyanate.

isolate was scored on an arbitrary scale, either as 0, if the child was negative, or 1, 2, or 3, depending on whether the level of response was in the first, second, or third tertile of all the positive responses. The scores were incorporated into an ordered logit model, to examine the association between parasitemia status at the survey and the scores. To examine the association between parasitemia status and a child’s overall response to all the test isolates, the child’s scores for all isolates were summed, and the totals were further categorized as 1, 2, or 3 on the basis of the tertiles of summed scores. We then examined the association between the categorized summed response scores and asymptomatic infection in another ordered logit model. To examine the interaction between asymptomatic infection and anti-VSA antibodies in protecting against clinical malaria during follow-up, we generated a product (interaction) variable between infection status and response scores and incorporated this variable, the constituent variables (infection status and response scores), and clinical malaria experience during follow-up in a moderated multiple-regression model. A clinical episode was defined as having a current fever in the presence of \( \geq 5000 \) parasites/\( \mu L \) of blood. Children were categorized as case patients or control subjects depending on whether they had at least 1 episode of clinical malaria during follow-up. To correct for the association between age and both the prevalence of antibodies to malaria antigens (figure 2) and the incidence of clinical episodes, categorized data on age were incorporated in both the ordered logit and the moderate multiple-regression models.

A total of 16 children <7 months of age were excluded from the analysis, to avoid the effects of maternal antibodies and other innate resistance mechanisms. A further 19 children who were febrile in addition to having parasites during the survey were also excluded, resulting in data from 221 children being analyzed.

RESULTS

Prevalence of asymptomatic infections during the cross-sectional survey. Seventy (31.7%) of 221 children had a microscopically detectable asymptomatic infection at the cross-sectional survey (here referred to as “parasitemic”; those without infection are referred to as “aparasitemic”). Compared with children between 6 and 12 months of age, children >12 months of age had odds ratios (ORs) >1 for having an asymptomatic infection. However, only among children >72 months of age were ORs significantly >1 (OR, 6.19 [95% confidence interval [CI], 1.17–34.316]; \( P = .037 \)).

Figure 2. Summed anti–variant surface antigen IgG response score in aparasitemic (A) and parasitemic (B) children, plotted against age. The dashed vertical line corresponds to 7 months, and the horizontal lines correspond to the first and second tertiles.
Association between asymptomatic infections and anti-VSA IgG responses. All except 2 children had antibodies to at least 1 isolate. Being parasitic was strongly associated with being positive for anti-VSA IgG antibody. When the summed responses to the test isolates were considered, compared with aparasitemic children, parasitic children had ORs of 4.11 (95% CI, 2.22–7.56; \( P < .001 \)) and 33.47 (95% CI, 15.56–72; \( P < .001 \)) for having category 2 and 3 summed antibody responses, respectively (category 1 is the baseline) (figure 2).

When responses to each isolate were considered individually, compared with those for aparasitemic children, for parasitic children, the ORs for having a response score of 1 ranged from 0.814 to 6.64 (for responses to isolates ITGIC15 and 4451, respectively). The ORs for having a score of 2 ranged from 3.90 to 16.94 (for responses to isolates ITGIC15 and 4451, respectively), and the ORs for having a score of 3 ranged from 12.96 to 47.94 (for responses to isolates ITGIC15 and 4451, respectively). These associations were significant (\( P < .05 \)) for scores of 2 and 3 for responses to all the test isolates. These ORs and their 95% CIs are plotted in figure 3.

Association between parasitemia status, anti-VSA IgG antibodies, and clinical malaria experience during follow-up.
Fifty-seven (26%) of 221 children had 1 episode of clinical malaria during follow-up, whereas 38 (17%) had ≥2 episodes. In the presence of a weak anti-VSA IgG response (category I summed score), having an asymptomatic infection at the survey was associated with an OR of 5.90 (95% CI, 1.36–25.50; P = .018) for becoming a case patient. Similarly, having anti-VSA IgG antibodies was independently associated with susceptibility to clinical malaria. Compared with aparasitemic children with a category 1 summed response, aparasitemic children with category 2 and 3 summed responses had ORs of 2.05 (95% CI, 0.97–4.31; P = .060) and 4.34 (95% CI, 1.43–13.20; P = .010), respectively, for becoming case patients during follow-up. On the other hand, parasitemic children with category 2 or 3 summed responses had ORs of 0.21 (95% CI, 0.02–2.24; P = .2) and 0.09 (95% CI, 0.009–0.902; P = .041), respectively, compared with parasitemic children with category 1 summed responses. The ORs for parasitemic children with category 3 summed responses becoming case patients was significantly lower (P = .02) than those for aparasitemic children with a similar level of response (figure 4A).

When responses to each isolate were examined separately, we found that, in the absence of an asymptomatic infection, the ORs for parasitemic children with anti-VSA antibodies (score >0) to a given isolate becoming case patients ranged from 0.568 to 1.126 (for responses to isolates 1776 and 4451, respectively). On the other hand, among parasitemic children, the ORs ranged from 1.198 to 1.813 (for responses to isolates 4451 and 4518, respectively). The ORs for parasitemic children with anti-VSA IgG antibodies to a particular isolate becoming case patients were significantly lower (P < .05) than those for aparasitemic children with anti-VSA IgG antibodies, for responses to all the isolates except those to isolates ITGIC15 and 4451 (table 1 and figure 4B).

**DISCUSSION**

Although there is evidence that anti-VSA antibodies protect against clinical malaria by parasites bearing homologous VSA variants [10, 11], the role that antibodies directed against heterologous variants play is less clear. We used an active case surveillance framework and flow cytometry to examine the role that anti-VSA antibodies play in protecting against clinical episodes caused by heterologous parasites in Kenyan children. We did not compare the anti-VSA antibody reactivity phenotype of test isolates with that of the parasites causing disease during follow-up. However, our experience and that of others [6, 17–19] indicate that, in an area of moderate to high malaria transmission (such as Kilifi), it is very unlikely that parasites isolated from different patients or during different clinical episodes will have the same anti-VSA antibody reactivity phenotype (i.e., bear homologous VSA). As such, we considered the test isolates to be heterologous to the isolates that caused the clinical episodes observed during follow-up, with respect to VSA.

In the present study, we found that anti-VSA IgG required the presence of an asymptomatic infection for protection against clinical disease caused by parasites bearing heterologous VSA. Independently, both heterologous anti-VSA antibodies and asymptomatic infections were associated with increased risk of experiencing a clinical episode during follow-up. These observations are consistent with those of a previous study of children in Kilifi that focused on protection against severe malaria [9] and suggest that the protective interaction between anti-VSA antibodies and asymptomatic infection is effective against clinical episodes of all levels of severity. Previous studies in The Gambia [20], Sudan [12], and Ghana [13] found an association between protection against clinical malaria caused by apparently heterologous parasites and anti-VSA antibodies to some randomly selected isolates but not to others. It is possible that the protection observed was among individuals who were parasitemic during the serological survey. However, in none of these other studies was the parasitemia status of the subjects at the time of the serological survey included in the analyses, and the interaction between anti-VSA antibodies and asymptomatic infections seen among Kilifi children may have been missed.

The observed age-related trend of prevalence of malaria par-

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**Table 1. Prevalence of cases among children with and without asymptomatic infection at the cross-sectional survey who had various scores for antibodies to the test isolates.**

<table>
<thead>
<tr>
<th>Isolate, infection status</th>
<th>Anti-VSA IgG response score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A4</td>
<td></td>
</tr>
<tr>
<td>Pos</td>
<td>17/34 (50)</td>
</tr>
<tr>
<td>ITGIC15</td>
<td>50/116 (43)</td>
</tr>
<tr>
<td>Pos</td>
<td>15/34 (44)</td>
</tr>
<tr>
<td>1509</td>
<td>52/124 (42)</td>
</tr>
<tr>
<td>Pos</td>
<td>21/41 (51)</td>
</tr>
<tr>
<td>1776</td>
<td>60/138 (43)</td>
</tr>
<tr>
<td>Pos</td>
<td>20/38 (53)</td>
</tr>
<tr>
<td>3030</td>
<td>39/97 (40)</td>
</tr>
<tr>
<td>Pos</td>
<td>7/10 (70)</td>
</tr>
<tr>
<td>4451</td>
<td>52/132 (37)</td>
</tr>
<tr>
<td>Pos</td>
<td>16/39 (41)</td>
</tr>
<tr>
<td>4518</td>
<td>55/128 (43)</td>
</tr>
<tr>
<td>Pos</td>
<td>18/46 (46)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of cases/no. of children (%). Neg, negative; pos, positive; VSA, variant surface antigen.
asites and disease incidence—the former is increasing, whereas the latter is decreasing—suggests that acquisition of immunity to clinical infections may be associated with the ability to maintain chronic infections [21, 22]. Initially, immunity might be directed against relatively conserved epitopes, such as the 19-kDa carboxy-terminal of merozoite surface protein (MSP) 1 and block 2 of MSP2. Antibodies against these epitopes have been shown to inhibit merozoite invasion into RBCs and prevent parasite growth in vitro and have been associated with protection against clinical malaria [23, 24]. Such immunity is probably acquired rapidly, as the limited diversity of these antigens allows repeated contact with a given variant. This immunity may enable a child to maintain a homologous infection chronically that, in turn, helps to sustain the same immunity through continuous boosting. However, the immunity may not provide adequate protection against clinical disease caused by heterologous superinfections [25, 26]. Such a postulate would be consistent with the observation in the present study that asymptomatic infections are not associated with protection against subsequent clinical infection. On the other hand, the accumulation of anti-VSA antibodies to a given variant is slow because of the low likelihood of a child encountering the same variant of VSA repeatedly, given the large repertoire of circulating variants [6, 17–19]. However, a child with a chronic infection may be exposed to a large number of VSA variants through antigenic variation and therefore acquire antibodies to these variants. It is possible that some of the asymptomatic infections seen in the present and a previous study [9] were chronic, which would explain the enlarged repertoire of anti-VSA antibodies observed among parasitemic children. As with the other immune mechanisms induced by chronic infections, these anti-VSA antibodies, on their own, might provide only limited cross-protection against disease caused by heterologous parasites. Only when acting in concert with other partially cross-reactive immune mechanisms sustained by chronic infection do antibodies to heterologous VSA provide complete protection against clinical malaria.

In addition, current evidence suggests that anti-VSA antibody responses are short-lived [5, 7]. Therefore, it is likely that having anti-VSA antibodies in the absence of a current asymptomatic infection reflects a recently treated acute infection and may be a marker of susceptibility to acute infections, as we have found here. On the other hand, having a narrow anti-VSA antibody repertoire, despite the presence of an asymptomatic infection, could reflect either an ongoing acute infection that was asymptomatic at the time of the survey (acute infections are associated with poor induction of heterologous anti-VSA antibodies) or poor anti-VSA responses in a child who nonetheless has a chronic infection. Such individuals, who make poor responses to VSA, have been reported in previous studies by ourselves and others [5, 7, 27]. As was suggested above, in the absence of adequate anti-VSA responses, the other partially cross-protective immunity generated by the infection, whether acute or chronic, may be insufficient to prevent clinical disease by heterologous infections, and this might help explain the negative association between asymptomatic infection alone and protection against clinical disease.

Taken together, these results show that asymptomatic infections are associated with anti-VSA IgG antibodies, as measured by flow cytometry. Together, asymptomatic infection and anti-VSA IgG antibodies are associated with protection against clinical malaria caused by apparently heterologous parasites, whereas, independently, both factors may be associated with susceptibility. Further work is required to understand the mechanisms underlying these associations. The present study has underlined the potential importance of taking into account the parasitological status of study participants when examining immune responses to VSA and other malaria antigens.

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References


