Abstract. The present study was initiated to characterize antibody responses to repetitive epitopes of the circumsporozoite protein (CSP), liver stage antigen-1 (LSA-1), and merozoite surface protein-2 (MSP-2) of *Plasmodium falciparum* in infants residing in a *Plasmodium falciparum*-hyperendemic area of western Kenya. In this study, development and maintenance of these antibody responses in 28 infants were studied longitudinally by use of monthly serum samples collected from birth to age 1 year. Mother plasma and infant umbilical cord plasma were also tested to assess the transplacental transfer of maternal antibodies. Results showed that antibodies passively transferred from mothers were detectable for CSP, LSA-1, and MSP-2 repeat epitopes. Infants were able to mount and transferred a strong antibody response against LSA-1 in their first year of life. Infants often responded to CSP repeats, but with a much lower antibody titer. Antibody responses in infants against Fc27 and 3D7 repeats of MSP-2 were low throughout their first year. In addition, 51 infants whose first detected infection occurred at > 4 months of age were selected to determine antibody responses to the antigens tested upon their first and second detected infections. Antibody responses to LSA-1 and, to a lesser degree, CSP increased in positivity rates and titer upon second infection. Antibody responses to Fc27-type and 3D7-type repeats of MSP-2 were low upon both infections. There was no association between maternally transferred anti-LSA-1, anti-CSP, or anti-MSP-2 antibodies and an infant’s first detected infection. No significant correlation was found between an infant’s antibody responses to the 4 antigen repetitive epitopes and protection against malarial parasitemia during the first year of life.

INTRODUCTION

*Plasmodium falciparum* is a major cause of morbidity and mortality in children in tropical countries. Protection of the children born to immune mothers has been attributed to various mechanisms, but mainly to passively transferred specific antibodies. Infants as young as 4 months, however, are at high risk of clinical attack. The high rate of clinical diseases in children in comparison with adults might be because of their history of lower exposure to malaria antigens, poor ability to mount immune responses, or other age-related factors. The mechanisms involved in the development of naturally acquired immunity against malaria parasites are still not clear. The identification of parasite antigens and epitopes that induce protective antibody responses, and the elucidation of immune responses to malaria parasites in infants would be important steps toward the understanding of naturally acquired immunity to malaria.

The presence of extensive repetitive regions is a feature of many *P. falciparum* proteins. Antibody responses induced by parasites infection are in large part directed against these repetitive epitopes. Antibody responses to T-cell–independent antigens are usually mounted against epitopes with a highly repetitive structure, which contributes to the immunodominance of repetitive epitopes. Many studies have identified the presence of B-cell epitopes in the repeats of circumsporozoite protein (CSP), liver stage antigen-1 (LSA-1), and merozoite surface protein-2 (MSP-2) of *P. falciparum*. These antigens are also vaccine candidate antigens against sporozoite, liver, and blood stage parasites, respectively. *In vitro* studies have revealed that antibodies directed against the CSP repeats can inhibit sporozoite invasion into cultured liver cells and administration of such antibodies can completely protect mice and monkeys against sporozoite-induced malaria. There is also in vivo evidence suggesting the presence of protective antibodies induced by the antigen repeats. Antibody responses to CSP repeats of *P. vivax* are protective in Saimiri monkeys. Mice immunized with a peptide (EQQSDLEQERLAKEKLQ) from LSA-1 repeat of *P. falciparum* are protected against challenge with *P. berghei* sporozoites. Monoclonal antibody against the epitope of Ser-Thr-Asn-Ser (STNS) from the MSP-2 Fc27 repeat region (ADTIASGSQRSTNSASTSTTNNGESQTTTPTA) inhibit the growth of the asexual blood stages of *P. falciparum in vitro*. The repetitive epitopes, on the other hand, may play a role in immunosuppression. It has been suggested that they serve as a smokescreen by interfering with the maturation of an effective immune response to the nonrepetitive region of the antigens. This suggestion has led to the hypothesis that the repetitive antigens are not protective, and that the immune response to the repetitive antigens interferes with the development of immune responses to other antigens. Immunoepidemiologic studies of repetitive antigens have yielded contradictory results. For instance, in the case of the CSP, some studies have shown that the parasite rate was significantly higher in anti-Asn-Ala-Asn-Pro (NANP)_6 seropositive children, which reflected a recent exposure to malaria. Conversely, other studies suggest that the CSP is poorly immunogenic in children because anti-(NANP)_3 titer remains very low throughout the first year of life.

The purpose of the present study was to characterize antibody responses to CSP, LSA-1, and MSP-2 repetitive epitopes and to investigate the development and maintenance of these responses in naturally exposed young children. These data could be useful in the understanding of naturally acquired immunity to malaria and in the development of a vaccine against malaria.
MATERIALS AND METHODS

Study design and participants. This study was part of a prospective malaria project, the Asembo Bay cohort project, in a rural region of western Kenya hyperendemic for malaria. Malaria transmission was high throughout the year (2–10 infected bites per person per month), with the highest transmission in May–July. In the cohort study, pregnant women were enrolled in their last trimester of pregnancy. The pregnant women, and then the women and their delivered infants, were prospectively followed every other week. Blood was collected every month, or more often if illness was detected or reported. The blood was examined by microscopy for Plasmodium parasites and sera were stored at −70°C. If a participant had asexual parasites > 5,000/μL or a temperature ≥ 37.5°C with any parasitemia, they were treated with sulfadoxine-pyrimethamine. The term “detected parasitemia” refers to any slide positivity for falciparum parasites in microscopy.

From the cohort of mothers and children, we selected participants for this study in 2 ways. First, to study the antibody response dynamics of infants during their first year, we selected 28 infants who were followed monthly from birth to age 1 year. Second, to determine if children could respond to the antigens determined to be present in their first and second detected infection, we selected 51 infants whose first detected infection occurred at ≥ 4 months of age. Children < 4 months of age were excluded to minimize the detection of maternal antibodies. To control for malaria transmission, we only studied children born during January–March 1993. Because the children were followed at least every other week, and because treatments were documented, we could use the detection of asexual parasitemia by microscopy as an indication of an infection that resulted in asexual parasitemia. The seroprevalence of the repetitive epitopes of CSP, LSA-1, and MSP-2 in the general population in the study area was examined by use of serum samples from 30 volunteers aged 17–60 years.

Peptides. A 20-mer peptide containing 5 copies of tandem repeating domain (NANP) of the CSP, a 34-mer peptide containing 2 copies of tandem repeating domain (AKEKLQEGQSDLEQERL) of LSA-1, a 32-mer peptide (ADTIASGSGSRTSSSTTNNGESQTTTPA) representing one copy of 32 amino acid repeat of MSP-2 FC27, and a 20-mer peptide (GAGGTAGGSAGGSAGGSAGG) containing 3 copies of MSP-2 3D7-type repeat were synthesized at the Biotechnology Core Facility, the National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia.

Enzyme-linked immunosorbent assay (ELISA). Antibodies to CSP, LSA-1, and MSP-2 repeats were tested via ELISA. The tests were performed in flat-bottom microtiter plates (Dynex Technologies, Chantilly, VA). Synthetic peptides were coated to the well surface by incubating 100 μL of individual peptide (10 μg/mL in 0.01 M phosphate-buffered saline [PBS] pH 7.2) overnight at 4°C. The plates were then washed 4 times with PBS plus 0.05% Tween-20 (PBS-Tween) and blocked at 37°C for 1 hr with 5% nonfat milk in PBS. Plasma samples were initially tested for the presence or absence of anti-repeat antibodies at dilution of 1:100. The plasma samples that were positive for CSP, LSA-1, or MSP-2 repeats were titrated subsequently with 2-fold serial dilutions up to 1:20,4800. After incubation at 37°C for 1 hr, unbound material was washed away with PBS-Tween. Horseradish peroxidase-conjugated goat anti-human immunoglobulin (Ig) G (Fisher Scientific, Pittsburgh, PA) diluted 1:6000 was added to each well. After incubation for 1 hr at 37°C, the wells were washed, and 150 μL of the peroxidase substrate, 3′,3′,5′,5′-tetramethylbenzidine (Kirkgead and Perry Laboratories, Gaithersburg, MD) was added to each well. Fifteen minutes later, the reaction was stopped by adding 50 μL of 1 M phosphoric acid. The plates were read at an absorbency of 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). Cutoff points were set at mean optical density value plus 3 times the standard deviation generated with plasma from 15 people from North America with no history of exposure to malaria.

Data analysis. Statistical analysis was conducted with SAS software (SAS Institute, Cary, NC). All data were normalized by logarithmic transformation before the analysis. Spearman’s correlation test was used to assess the relationship between antibody level and parasitemia. Wilcoxon matched pairs signed-rank (mWPR) test was used to compare antibody levels between first and second infections. McNemar’s test or Fisher’s exact test were used to compare all proportions or rates of antibody responders; P ≤ 0.05 or mWPR Z ≤ 0.05 was considered statistically significant.

RESULTS

Antibody responses against the repetitive epitopes of CSP, LSA-1, and MSP-2 in the general population of the study area. To test the validity of the use of repetitive epitopes in examining immune response to CSP, LSA-1, and MSP-2, antibody responses to overlapping peptides from these antigens were assessed in 30 people living in this study area. The prevalence of IgG antibody was 90% for CSP repeat, 90% for LSA-1 repeat, 80% for MSP-2 FC27, and 93% for MSP-2 3D7 repeats.

Maternal antibodies against the repetitive epitopes of CSP, LSA-1, and MSP-2. In this study, mother’s plasma was available for only 14 and cord plasma available for only 20 of 28 infants used to characterize longitudinal antibody responses. Mothers had strong antibody responses to CSP, LSA-1, and MSP-2 3D7 repetitive epitopes, with highest responses to CSP repeat and lowest responses to MSP-2 FC27 repeat (Table 1). There was no positive or negative association in maternal antibody responses to one antigen with another (P > 0.05). A high level of transplacental transfer of maternal anti-CSP, LSA-1, and MSP-2 antibodies occurred in these mothers as judged by the prevalence and titer of antibodies in cord blood. The titer of antibodies to LSA-1- and 3D7-type MSP-2 in cord blood were strongly correlated to the titer of antibodies in the peripheral blood of mothers (P = 0.002 and P = 0.0001, respectively), whereas antibody titer to CSP and FC27-type MSP-2 were not significantly correlated (P = 0.058 and P = 0.10, respectively). Passively transferred maternal antibodies waned quickly in infants at 1 month, as evidenced by a stepwise decline in antibody titer to 0 in successive weeks when the infant’s first infection occurred after age 3 months. This was especially notable for MSP-2 repeats (geometric mean titer, 271.1–
Antibody responses of infants at first and second infections against the repetitive epitopes of CSP, LSA-1, and MSP-2. We selected 51 infants whose first detected infection occurred after 4 months of age to test antibody responses at the first and second infection. Table 1 shows the geometric mean antibody titer and positivity rates for the 4 antigen epitopes. At the first infection, the positivity rate of anti-CSP repeat antibodies was 64%, which was higher than the positivity rate for either anti–MSP-2 Fc27 type (35.5%, P = 0.011) or 3D7 type (40%, P = 0.16). The positivity rate of anti–LSA-1 repeat antibodies (67%, P = 0.004) was also higher than the positivity rate for either anti–MSP-2 Fc27 or 3D7 (P = 0.09), but not significantly different from the anti-CSP repeat antibody response (P = 0.93). The geometric mean titer of antibodies to CSP and MSP-2 repeats was low, whereas the titer to LSA-1 was moderate (Table 1). Compared with the antibody response at the first detected infection, there was a significant increase in anti–LSA-1 repeat antibody response at the second infection for both positivity (67% versus 93%, P = 0.003) and titer (123.3 versus 684.0, mWPR Z = 0.006). Antibody response to CSP increased slightly in both positivity rate and titer at second infection. Antibody responses to Fc27-type and 3D7-type repeats of MSP-2 were low at the first and second infections. There was also no obvious difference between antibody responses against the peptides representing 2 allele families of MSP-2 at the first and second infections.

Antibody responses throughout the first year of life. Antibody responses against CSP, LSA-1, and MSP-2 Fc27 repetitive epitopes were tested monthly in 28 infants throughout their first year of life. Positivity rate of antibodies against CSP and LSA-1 repetitive epitopes were high (60%) during the first year of life. In contrast, positivity rates of antibodies against MSP-2 Fc27 repeat decreased steadily during the first 6 months. Afterward, anti–MSP-2 Fc27 antibody prevalence remained low (Figure 1). Figure 2 summarizes antibody titer by antigen epitope group and the parasite density by month of
age. Titer of IgG to LSA-1 repeats increased with the age of infants, whereas the titer of IgG to CSP and MSP-2 repeats remained low during the first year of life.

To further characterize the antibody responses in an infant’s first year of life in the context of parasitemia, we categorized each child’s antibody responses for each of the antigens as one of the following: (1) positive (consistently positive following 1–2 detected parasitemias); (2) low (consistently positive with antibody titer <400); SL (short-lived antibody responses temporal with parasitemia); NEG (consistently negative). The number of infants in each category having less than 1–2 parasitemias was important in the development of antibody responses, as nearly half (42–54%) of the infants did not have antibody response to either allelic form of the MSP-2 repeat peptides, however, their antibody titer was low. Antibody positivity rates to CSP were at very low level throughout the first year of life, positivity rate to CSP repeat was as high as to LSA-1 repeat. During the children’s first year of life, positivity rate to CSP repeat was as high as to LSA-1 repeat. The antibody titer was low. Antibody positivity rates and titer to MSP-2 Fc27 repeats were at very low level throughout the first year of life. Thus, infants can mount a better antibody response to LSA-1 repeats and maintain this response at high levels than to CSP and MSP-2 repeats.

Correlation between the antibody responses. We investigated whether there was a correlation between the antibody responses against all 4 repeats detected in each sample of infants. The antibody titer to the different antigens was not correlated ($P > 0.05$). However, there were associations between the antibody positivity rates of each of the antigens. When considering all of the samples, including samples that had maternal antibody present, there was a positive association between an antibody response to the MSP-2 Fc27 antigen and the MSP-2 3D7 antigen. However, after excluding sample points that contained maternally transferred antibody, there was no association between antibody positivity to the 2 different MSP-2 alleles: 45% were negative to both alleles, 25% were positive to Fc27, 12% were positive to 3D7, and 17% were positive to both alleles. Similarly, the anti–LSA-1 and anti-CSP responses were not correlated ($P > 0.05$).

**Table 2**

<table>
<thead>
<tr>
<th>Antibody response</th>
<th>CSP</th>
<th>LSA-1</th>
<th>MSP-2</th>
<th>MSP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total of infants</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>POS, constant (%)</td>
<td>7 (25)</td>
<td>19 (68)</td>
<td>7 (25)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>No. with &lt;2 Parasitemias</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>LOW, constant (%)</td>
<td>14 (50)</td>
<td>4 (14)</td>
<td>1 (4)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>No. with &lt;2 Parasitemias</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SL, short-lived (%)</td>
<td>3 (11)</td>
<td>1 (4)</td>
<td>5 (18)</td>
<td>ND</td>
</tr>
<tr>
<td>No. with &lt;2 Parasitemias</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NEG (%)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>15 (54)</td>
<td>8 (42)</td>
</tr>
<tr>
<td>No. with &lt;2 Parasitemias</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

Children’s antibody response histories were summarized as being: POS (consistently positive following 1–2 detected parasitemias); LOW (consistently positive with antibody titer <400); SL (short-lived antibody responses temporal with parasitemia); NEG (consistently negative). The number of infants in each category is less than 1–2 parasitemias detected in their first year.

**DISCUSSION**

Results of this longitudinal study indicated that antibody responses to the different antigen repetitive epitopes in infants evolved differently during the first year of life. After a drop in levels of anti–LSA-1 antibody at the first month, the IgG titer remained low until the age of 4 months, suggesting a waning of maternally acquired antibodies. From the fourth month on, IgG titer increased until the 11th month.

Exposure was important in the development of antibody responses, as evidenced by the fact that 4 of the infants who were found to be negative to all antigens tested had <2 detected parasitemias. However, only 1–2 detected parasitemias could be sufficient to cause a significant, long-lasting antibody response against LSA-1 because 8 of 19 constant responders had ≤2 detected parasitemias (Table 2).

The difference in immune responses to these antigens may be due to the different structure of repetitive epitopes. Another possible explanation is that anti-(NANP)n antibodies are unstable and short-lived. We also found that infants...
who responded to MSP-2 had short-lived or low antibody responses. Apparently, a long period of exposure (> 1 year) to malaria is required before a stable, significant anti-CSP and anti-MSP-2 response occurs. Taken together, immune responses to distinct antigens of *P. falciparum* may evolve differently and may be differentially regulated.

IgG against (NANP)n has been used as a marker for exposure to sporozoites. In the present study, antibody responses to (NANP)n remained low during the first year of life in infants despite repeated exposure to malaria infection. In contrast, antibody response to LSA-1 repeat increased dramatically after the first infection. Although the antibody response to CSP and MSP-2 repeats decreased sharply during an intermittent period of aperiodism, the antibody response to the LSA-1 repeat remained high after the first infection. The immune response to LSA-1 showed high IgG prevalence rate and gradual increase in antibody titer during the first year of life, indicating antibody responses to LSA-1 required limited exposure and persisted over time. It seems that this antibody response is a better marker of first exposure than protection.

Results of the present study also suggest that the maternal antibodies to CSP, LSA-1, and MSP-2 repeats were not associated with protection. IgG antibodies passively transferred from mothers were detectable for CSP, LSA-1, and MSP-2 repetitive epitopes. IgG titer, however, declined during the first few months from the relatively high values, indicating a fast waning of maternal antibodies against those repetitive epitopes. We did not find a correlation between levels of maternal antibodies and time of first infection for any of the antigen repeats. Maternal antibodies transferred across the placenta are considered the main contributor for protection against malaria in very young infants in highly endemic areas.

Our previous study showed that maternal and infant merozoite surface protein-1 (MSP-1) 19kDa antibodies are associated with protection against malaria infection in the same population. In the present study, antibody responses to the tested antigenic determinants were not associated with protection. This further indicated that the immune responses to conserved epitopes and repetitive epitopes might be differentially regulated. It is also possible that the high level of maternal antibodies against CSP, LSA-1, and MSP-2 repeats might be protective for the newborn, but because maternal antibodies waned quickly, a positive correlation between antibody titer and protection against the first infection could not be detected. The protective mechanism of passive immunity against malaria in infants is not well known. Some previous studies have shown conflicting results on the protective role of maternally transferred antibodies and many other factors may also involve in resistance to malaria in infants.

Several previous studies failed to show a protection against malaria by natural or vaccine-induced antibodies against CSP repeats either because of lack of positive antibody or because of lack of protective function of antibodies. In this study, antibodies to CSP and MSP-2 repeats remained at very low levels through the first year of life, preventing the assessment of protective effect of these antibodies. After the waning of maternal antibodies, the titer of IgG antibody to LSA-1 repeat increased with age of infants, but no significant correlation was found between the level of antibodies and levels of malaria parasitemia. A negative association between antibody response to MSP-2 non-repeat regions and risk of malarial fever and anemia was observed in a seroepidemiologic study in older children and adults of Papua New Guinea, suggesting a possible protective role for anti-MSP-2 antibodies in natural infection. However, the protective role of antibodies against the repeat regions of MSP-2 has not been documented and was not found in this study. For the nonrepeat regions of other malaria antigens, an infant’s antibody responses to MSP-1 19-kDa antigen have been associated with protection in this study population.

The present study was an initial step in the characterization of the pattern of antibody responses to malaria repetitive epitopes in infants who live in hyperendemic areas in western Kenya. It provides useful profiles on antibody response to CSP, LSA-1, and MSP-2 in the population that may be useful in monitoring the effects of a malaria vaccine in the future.

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