A LIGWOODED INVESTIGATION OF IgG AND IgM ANTIBODY RESPONSES TO THE MEROZoI PROTEIN-1 19-KILODALTON DOMAINE OF PLASMODIUM FALCIparum IN PREGNANT WOMEN AND INFANTS: ASSOCIATIONS WITH FEBRILE ILLNESS, PARASITEMIA, AND ANEMIA

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Abstract. This study was aimed at delineating characteristics of naturally acquired immunity against the merozoite surface antigen-1 (MSP-1) of Plasmodium falciparum, a candidate malaria vaccine antigen. A case/control study was performed on 75 case/control pairs of infants with febrile illness at the time of the first detected infection indicating a clinical case. The presence and level of antibodies at one month prior to the first infection and at the time of the first infection in the afebrile group was significantly higher than in the febrile group. Decreased parasite density and decreased infection-related loss of hemoglobin was seen in infants with anti-MSP-1
d IgG antibodies. In addition, mothers who were positive for the presence of these antibodies conferred protection against placental infection and infection in their infants. In this study, development of anti-MSP-1
d antibody responses in 24 infants were studied longitudinally using monthly serum samples collected from birth until approximately one year of age. In addition, umbilical cord blood sera and respective mothers' sera were analyzed. Longitudinal studies of antibody responses revealed several short-lived IgG and IgM peaks throughout an infant's first year that correlated with detection of parasitemia. The protection against parasitemia and febrile illness was observed in infants when anti-MSP-1
d antibodies were present; when infants were negative for IgG, they had a 10-times greater risk of becoming parasitemic. These data from a longitudinal and prospective study of malaria suggest a protective role for anti-MSP-1
d antibodies in infants and pregnant women.

The development of resistance by Plasmodium falciparum to antimalarial drugs and the resistance of the Anopheles mosquito to insecticides has been associated with a global resurgence in malaria.1 Because the brunt of malaria-related morbidity and mortality is borne by children less than five years of age and pregnant women, a subunit or recombinant vaccine for combating malaria should target these groups. Testing malaria vaccines in young children may be best accomplished in a population in which at least four parameters are known: the malaria-related morbidity and mortality, malaria transmission pressure, the molecular nature of the infectious agent, and the characteristics of naturally acquired immune responses that confer protection against parasitemia and clinical illness.

Merozoite surface protein-1 (MSP-1), a potential vaccine candidate antigen against malaria, is expressed on the surface of asexual blood-stage parasites. This protein is synthesized as a precursor form with an apparent molecular weight of 200 kD, and is proteolytically cleaved into fragments before schizont rupture.2 The C-terminal, glycosylphosphatidylinositol (GPI)–anchored 42-kD fragment (MSP-142K) undergoes a secondary processing leading to a GPI-anchored 19-kD fragment (MSP-119K), and is believed to be involved in the invasion of erythrocytes.3,4 The MSP-119K is highly conserved, with two epidermal growth factor–like motifs containing several cysteine residues, and antibodies of maternal-exposed people recognize these motifs.2,5

A protective role for anti-MSP-119K antibodies has been established by in vitro and animal model experimental studies. Anti-MSP-119K antibodies have been shown to block in vitro growth of P. falciparum.3,6-11 Blackman and others suggested that these antibodies mediate their protective effect by blocking proteolytic processing of MSP-119K to MSP-142K.12 In addition, active immunization with P. yoelii MSP-119K and passive transfer of antibodies specific for this protein provided protection against a lethal challenge in rodents.13,14 Finally, in a recent study with Aotus monkeys (Aotus nancymai) P. falciparum MSP-119K fragment was shown to induce protection against parasite challenge.15 Several immunoepidemiologic studies of immune responses to MSP-1 antigens have been undertaken to determine the association between antibody responses and clinical manifestations of illness. However, all of these studies investigated immune responses in older children, and none followed each subject longitudinally. The first associations between anti-MSP-1 antibodies and protection were shown by Riley and others.16 They studied the natural immune response to a recombinant MSP-119K antigen by testing children grouped by age (3, 4, 5, 6, 7, and 8 years old) and adults. They found increasing antibody responses in each age group and showed that these responses were correlated with protection.16 In another study by Tolle and others, associations between antibody and protection were not seen. However, they did not use antigens that maintained the native conformation.17 Since it has been shown that the protective epitopes of MSP-119K are disulfide bond (conformation) dependent, one could argue that an antigen with a native conformation may have yielded different results.

Compared with previous immunoepidemiologic studies, our study design allowed longitudinal investigation of the development of natural immunity against MSP-119K. This is because women were enrolled during the last trimester of
pregnancy and then, after the delivery of the baby, the mother-infant pairs were followed biweekly from delivery for the first five years of the infant’s life. It has been previously shown that 70–90% of healthy, nonparasitemic adults residing in our study area have anti-MSP-119kD IgG antibody responses.15 The comprehensive and frequent monitoring of our participants enabled us to document the development of anti-MSP-119kD immune responses, clinical parameters, and parasitologic parameters. Recently, Al-Yaman and others have longitudinally studied children’s development of MSP-1 antibodies in Papua New Guinea.16 They found associations between increasing levels of antibodies to the 42-kD domain of MSP-1 and protection against malaria illness, and suggested that the antibodies were directed to the 19-kD region of the 42-kD fragment. However, anti-MSP-119kD responses were not directly studied. Also, immune responses during first infections were not investigated and each individual’s time points were not followed as closely as we report here. In conclusion, our study design helped us to delineate the protection associated with anti-MSP-119kD from protection associated with age.

We investigated the relationship between antibodies to MSP-1 and the clinical manifestations of malaria and asked the following questions. 1) Is the presence of anti-MSP-119kD antibodies in infants at the first documented infection and one month prior to the first infection associated with level of parasitemia and the clinical manifestation of illness? 2) Is the presence of anti-MSP-119kD associated with protection against placental infection? 3) Are maternally transferred anti-MSP-119kD antibodies associated with protection against malaria infection in newborns? We also investigated the development of MSP-119kD antibodies in response to malaria exposure throughout the first year of life, and their effect on subsequent infection.

MATERIALS AND METHODS

Asembo Bay Cohort Project. This study was part of an ongoing prospective and longitudinal malaria project in a holoendemic, rural region of western Kenya near Lake Victoria (Asembo Bay). Malaria is transmitted throughout the year in this area, and peak transmission is highest during and following the long rains (March to May). In an area immediately adjacent to and similar to the study site, entomologic inoculation rates have been estimated to average 0.75 infective bites per person per day.20 Mothers in their last trimester of pregnancy and their infants from the date of delivery were enrolled and visited every two weeks to obtain their clinical history after written consent. Also, siblings <15 years of age were enrolled in the study. This study was approved by the Centers for Disease Control and Prevention and Kenyan Institutional Review Boards for the use of human subjects. A fever between scheduled visits resulted in a blood smear examination for parasitemia. Blood samples were collected once a month for parasitologic examination and serologic studies, and at any time the participants presented with febrile illness. The plasma obtained from fingerstick blood and the plasma collected from the umbilical cord was stored at ~70°C until used. Thick and thin films of the maternal-side blood in the plasma and the fingerstick blood were examined by microcopy for parasites.

A comprehensive summary of the cohort and initial epidemiologic findings has been described (Bloland PB and others, unpublished data). Briefly, during the first year of the Asembo Bay Cohort Project (June 1992–July 1994) 1,008 women were enrolled who had their delivered infant(s) followed at least once. Of the participants <15 years of age monitored throughout the first year of the Asembo Bay Cohort Project, only 34% of the examined blood smears did not contain Plasmodium parasites of any species. Clinical malaria, as defined by asexual parasitemia and an axillary temperature ≥37.5°C, was concentrated in infants and decreased dramatically after 12 months of age.

Participants who were febrile (axillary temperature ≥37.5°C) with parasitemia were considered to have a malarial illness. Afebrile participants with parasite densities <5,000/μl were not considered to have clinical malaria; however, they were closely monitored for development of fever and high parasitemia. Febrile participants were treated with sulfadoxine/pyrimethamine and monitored to confirm the clearance of parasitemia.

Study design. The clinical manifestations of a child’s first malaria infection were investigated using a case/control study design from infants born and enrolled during the first year of the Asembo Bay Cohort Project. When investigating associations between anti-MSP-119kD antibodies and morbidity, the clinical results of the first infections are subject to fewer confounding factors between infants. This is because infants may develop different levels of specific and nonspecific immunity with subsequent infections. Cases were defined as febrile first-detected parasitemias. Controls were defined as febrile first-detected parasitemias. Although all afebrile first infections were considered controls, if their first-detected parasitemia was high, they were subsequently treated. We stratified the infants based on this definition and sorted them by age at the first detected parasitemia. The probability of febrile illness with first detected infection may be associated with the age of the infants. We controlled for this in our sample selection process. We randomly matched febrile (case) infants with afebrile (control) infants by day of the documented first infection ±10 days.

The questions regarding clinical manifestations of first infection study were studied to test the antibody levels in case versus control infections and the correlation between parasite density and change in hemoglobin during infection with antibody. We first randomly selected 35 case/control pairs. The results of this study were confirmed by repeating the analysis with another 40 case/control pairs. The combined results are reported here. Some of the infants did not have adequate amounts of sera available at both one month prior to and time of first infection. Overall, 74 febrile infants and 70 afebrile infants were tested for antibody responses one month prior to the first infection, whereas 73 afebrile infants and 67 febrile infants were tested for antibody responses at the time of first infection.

Twenty-four of the first 35 case/control pairs were followed monthly from birth until approximately one year of age. Selection was based upon availability of sera. Because the infants were born between September and December 1992, each infant experienced a single high transmission sea-
son between six and nine months of age. The infants’ umbilical cord sera were used as time 0 and their mother’s sera within one month before delivery was tested. To determine the possible effects of maternal anti-MSP-1<sub>19kD</sub> IgG antibodies on the time of first infection in infants, we tested plasma from 40 additional randomly selected mothers. A total of 60 mothers were used in the maternal antibody investigation.

The MSP-1<sub>19kD</sub> recombinant antigen. The MSP-1<sub>19kD</sub> antigen, corresponding to Ugandan-PA type (E-KNG) recombinant protein was expressed in *Saccharomyces cerevisiae* and affinity purified as previously described. This recombinant protein was shown to retain the natural conformation. Although four different variant forms of this protein have been described, we used Ugandan-PA type because prior gene sequence studies of parasites in this study area revealed that the predominant genotype was E-KNG. Furthermore, serum samples from adult residents of this area recognize the Ugandan-PA type variant more frequently than the other three variants. All MSP-1<sub>19kD</sub> responses given in this paper are responses to the E-KNG variant form.

Enzyme-linked immunosorbent assay. Standard ELISA procedures were used as described elsewhere. Microtiter Immunolon 2 (Dynatech, Chantilly, VA) plates were coated with MSP-1<sub>19kD</sub> (200 ng/ml) in borate buffer solution (BBS) overnight at 4°C and then blocked with BBS containing 5% nonfat lyophilized milk. The plates were washed four times with sodium phosphate-buffered saline (PBS, pH 7.4) containing 0.5 M NaCl, 0.05% bovine serum albumin, 0.005% Tween 20, and 0.05% thimersol (PBS-T). The sera were diluted 1:100 in PBS-T containing 1.5% nonfat milk and added in triplicate to microtiter plates and incubated at room temperature for 1 hr. The unbound antibodies were removed by four washes with PBS-T. Bound antibodies were detected with peroxidase-conjugated goat anti-human antibodies (Fisher Scientific, Pittsburgh, PA) diluted 1:500. The secondary antibody was allowed to bind for 1 hr, the wells were washed with PBS-T, 100 μl of 3,3′,5,5′-tetramethylbenzidine (Microwell Peroxidase Substrate System; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added, and 15 min later the reaction was stopped with 1 M phosphoric acid. The plates were read at an absorbance of 450 nm (A<sub>450</sub>) with an ELISA reader (Molecular Devices, Menlo Park, CA). As a control, 24 serum samples from healthy individuals never exposed to malaria parasites were obtained from the Centers for Disease Control and Prevention Blood Bank. Serum samples that showed an optical density (OD) greater than the mean plus 2 SD of these control sera were scored as positive.

Statistics and data analysis. The SAS<sup>2</sup> software was used for all statistical analyses, unless otherwise noted. Type I errors (alpha) were set to 0.05. Two-tailed tests were used for all comparisons. The ELISA ODs were normalized with a logarithmic transformation for computations of geometric means, confidence intervals (CIs), and any statistical modeling. Antibody responses were categorized as positive or negative by an ELISA OD at 450 nm (OD<sub>450</sub>.

The chi-square test of independence was used to compare all proportions or rates for categorical variables. Stratified tables were analyzed with the Mantel-Haenszel test. The Wilcoxon rank sum test was used to compare distributions of continuous variables by category (e.g., antibody levels or changes in antibody levels by case/control status). The Wilcoxon matched-pair signed rank test was used to compare prior to postinfection antibody levels. General linear models (GLM) were used to model continuous variables as a function of combinations of continuous and categorical variables (e.g., change in the hemoglobin level as a function of parasite density, gender, etc.). To estimate the change in the hemoglobin level associated with the infection, we subtracted the hemoglobin level after first detection of the parasitemia from the hemoglobin level present at first detection of the parasitemia. Multiple comparison issues were minimized in linear model comparisons through the use of the Duncan multiple range procedure to detect which categories of a particular variable were significantly different. All other multiple comparisons are presented in the results.

Regression was used to model prevalence or percent as a function of both continuous and categorical variables and was used to compute the smoothed probabilities of febrile illness as a function of parasite density. Effective protection from febrile illness with the presence of antibodies was computed as (1 − odds ratio) × 100.

## RESULTS

**Anti-MSP-1<sub>19kD</sub> levels at the first documented first infections.** Infants were selected by matching afebrile with febrile infants at the time of their first detected parasitemia. The mean age of infants at their first documented infection was 97 days (CI = 89–105) and ranged from seven to 340 days. We tested anti-MSP-1<sub>19kD</sub> IgG and IgM levels at the time of their first infection and at one month prior to their first infection. Of the 138 infants tested at both time points, 59% had an IgG response one month prior to their first documented infection and 77% had an IgG response at the time of their first documented infection. The difference was significant (McNewmar *P* = 0.0030). The IgG antibody levels (ELISA ODs) to MSP-1<sub>19kD</sub> also increased with the infant’s age at the time of the first documented infection (matched pair Wilcoxon rank *P* = 0.0038). Only 13 (9%) infants had IgM to MSP-1<sub>19kD</sub> one month prior to the first documented infection, while 55 (39%) had IgM at the time of the first documented infection (McNewmar *P* = 0.0001).

**Anti-MSP-1<sub>19kD</sub> levels in cases versus controls.** No differences were noted between the febrile and afebrile groups with respect to gender or sickle cell trait (χ² = 0.796 and χ² = 0.912, respectively). Infants were selected by matching afebrile infants with febrile infants whose first infections were at the same age ± 10 days. Table 1 shows the anti-MSP-1<sub>19kD</sub> IgG responses in febrile and afebrile infants one month prior to and at the time of their first documented infection. When the IgG response rate at each time point individually was considered, the responder rate was higher in the afebrile group (controls) than it was in the febrile group (cases) at both one month prior (Mantel-Haenszel *P* = 0.0049), and at the time of first infection (Mantel-Haenszel *P* = 0.0150). The amount of protection against febrile illness was 33% (CI = 14–73%) if antibodies were present at one month prior to infection and 28% (CI = 10–28%) if antibodies were present at time of the first documented infection. At one month prior to the first infection, afebrile infants had a mean geometric IgG OD of 0.560 (CI = 0.381–
infants had a mean geometric IgG OD of 0.506 (CI of first infection cleared their infection without receiving one (4%) of the infants without an IgG response at the time group (GM different from the parasite density levels in the untreated [GM] as parasite density levels in the treated group (geometric mean

amination, became febrile, and required treatment. The parasite density levels in the untreated group (GM = 831/μl, CI = 463–1,496/μl) were not significantly different from the parasite density levels in the untreated group (GM = 832/μl, CI = 413–1,600/μl). Also, the mean ± SE age of infants who cleared their parasitemias (81 ± 8 days) was not different from that of the infants who did not clear their parasitemias (97 ± 9 days).

We compared the frequency of IgG responders in infants who cleared their untreated parasitemias versus infants who did not clear their parasitemias before requiring treatment. Only three (17%) of the 18 infants without an IgG response one month prior to first documented infections cleared their infections without receiving treatment ($x^2 P = 0.0128$). Only one (4%) of the infants without an IgG response at the time of first infection cleared their infection without receiving drug treatment ($x^2 P = 0.1211$). As shown in Figure 1, the self-cleared group had a geometric mean IgG level of 1.000 (CI = 0.545–1.836) at one month prior to infection and a geometric mean IgG level of 1.271 (CI = 0.876–1.845) at the time of the first documented infection. The group that did not clear the infection without receiving treatment had lower IgG levels both at one month prior to infection (GM = 0.317, CI = 0.183–0.548) and at the time of infection (GM = 0.896, CI = 0.681–1.179) (Wilcoxon rank P = 0.0015 and P = 0.0400, respectively). The contributions made by cleared and not cleared afebrile infants to the overall geometric mean of the afebrile infants are shown in Figure 1.

Relationship between febrile illness, parasite density, and anti-MSP-19kd antibodies. At one month prior to the first infection, infants with anti-MSP-19kd IgG had a geometric mean parasite density of 1,350/μl (CI = 874–2,088/μl). Infants without detectable anti-MSP-19kd at one month prior to the first documented infection had a geometric mean parasite density of 2,688/μl (CI = 1,609–4,491/μl). When the effects of both IgG response (positive/negative) at the time of first infection and the infants’ age at the first infection on parasite density were considered in a GLM, both variables had significant, independent positive correlations (IgG response $P = 0.0458$; age $P = 0.0443$). When considering the infants’ response rate at the time of infection

<table>
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<tr>
<th>IgG response</th>
<th>Afebrile infants</th>
<th>Febrile infants</th>
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<tbody>
<tr>
<td>One month prior</td>
<td>Time of infection</td>
<td>No. (%)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>43 (66)</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>5 (8)</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>15 (23)</td>
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<td>2 (3)</td>
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* A positive response was defined as ≥ control + 2 SD. Infants with a fever ≥37.5°C were scored as febrile. The number febrile and afebrile in each group is shown along with the percent afebrile/febrile for each response group. $\chi^2 P = 0.0010$.

![Figure 1](image-url)
and the infants’ age, only age was significantly correlated with parasite density.

Because afebrile children had a geometric mean parasite density of 891/μl (CI = 889–894/μl) and febrile infants had a much higher geometric mean parasite density of 3,467/μl (CI = 3,465–3,470/μl) (Wilcoxon rank P = 0.0001), we further examined association of the IgG response with febrile illness to determine if it was primarily due to the parasite density. Using a logistic regression model, the cumulative IgG response rate to MSP-119kD is represented as follows: infants positive for IgG both one month prior to and at the time of infection = positive-positive; infants without IgG at one month prior or at the time of infection = negative-negative; infants positive at one month prior to infection only = positive-negative; infants positive at the time of the first infection only = negative-positive. The model resulted in the estimated probabilities of febrile illness for each of these groups (Figure 2).

As shown in Figure 2, infants with a positive-positive response were most likely to be afebrile and infants with a negative-negative response were most likely to be febrile, regardless of parasite density. Infants with a positive response at one month prior to their first infection had an effective protection (1 – odds ratio) against fever at different parasite densities of 62% (CI = 17–83%) (χ² P = 0.0153). Those with a positive response at the time of first infection had an effective protection of 76% (CI = 33–91%) (χ² P = 0.0066). The IgG responses at one month prior to and at the time of infection were additive and independently reduced the probability of febrile illness with the first infection. No statistical difference was noted between the negative-positive and the positive-negative response groups.

**Anti-MSP-19kD IgG and the loss of hemoglobin.** A significant association was found with respect to IgG antibodies and a change in hemoglobin associated with the first documented parasitemia. The change in hemoglobin information was available only for 124 infants. Infants with a positive antibody response gained a mean of 1.0 g/dL (CI = 0.7–1.3 g/dL), whereas infants without IgG at one month prior to infection lost a mean of 0.8 g/dL (CI = 0.1–0.9 g/dL) (GLM P = 0.0024) The same trend was seen with IgG responses at the time of first infection, but it was not significant (GLM P = 0.1551). Infants in the febrile group did not have a significantly different change in the hemoglobin level. The days separating the tested hemoglobin levels, gender, and age were not correlated with the loss of hemoglobin. Parasite density, however, was positively correlated with the change in hemoglobin level and was included in the model (GLM P = 0.0432). The change in hemoglobin levels associated with the infants’ anti-MSP-19kD IgG responder rate at both time points is shown in Table 2. Infants without anti-MSP-19kD IgG at both time points had a much greater loss of hemoglobin (Table 2) than did infants with anti-MSP-19kD at both time points (GLM/Duncan P = 0.0208).

**Influence of maternal anti-MSP-19kD antibodies on protection against placental infection and infection in infants.** Parity was divided into three categories: first or second child (n = 20), third or fourth child (n = 22), fifth or greater child (n = 19). Of the 60 mothers studied during the last month of their pregnancies, 71% were positive and 29% were negative for anti-MSP-19kD IgG. Furthermore, the IgG level in mothers at the time of delivery was strongly correlated with the IgG levels in the cord blood (R = 0.8989, P < 0.0001). This correlation was not significantly affected either by placental infection (GLM P > 0.7) or parity (GLM P > 0.2). The cord sera was available for only 21 of the 24 infants followed for one year. The mean difference between a mother’s anti-MSP-19kD IgG level and their respective cord sera anti-MSP-19kD was 0.0758 (CI = 0.003–0.149). There was a trend for the mothers’ levels to be higher than their cord sera levels; however, the difference was not significantly different from 0 (P = 0.0550).

When considered together in a GLM model, maternal anti-MSP-19kD antibodies at the time of delivery and parity were independently correlated with protection against placental malaria (P = 0.0001 and P = 0.0213, respectively). As
shown in Table 3, the incidence and geometric mean parasite density of placental malaria were significantly lower in mothers with anti-MSP-1\textsubscript{19kD} IgG present at the time of delivery. Increasing parity also showed a protective trend. Deliveries of parity 1–2 had a geometric mean parasite density of 69.7/\mu l (CI = 13.2–367.2/\mu l). Deliveries of parity 3–4 had a geometric mean parasite density of 15.4/\mu l (CI = 3.9–61.4/\mu l). Finally, deliveries of parity > 4 had a geometric mean parasite density of 1.4/\mu l (CI = 0.7–2.7/\mu l).

To determine if maternal anti-MSP-1\textsubscript{19kD} IgG levels had any effect in delaying the timing of first infection in infants, the day of each infants’ first infection was compared with their mothers’ antibody responses. As shown in Table 3, infants born to mothers with no anti-MSP-1\textsubscript{19kD} IgG response had an earlier mean first infection day than infants born to mothers with a positive antibody response (GLM \(P = 0.0137\)). Negative mothers and positive mothers did not have statistically different dates of deliveries or dates of infants’ first documented infection. The number of infants exposed to high malaria transmission or exposed to low malaria transmission before the first documented infection was not significantly different.

**Anti-MSP-1\textsubscript{19kD} antibodies in the first year of the infant’s life.** Plasma from 24 infants, collected at monthly intervals during their first year of life, was tested for both IgM and IgG antibodies. Results for six representative cases, two with no initial levels of antibody, two with moderate levels of antibody, and two with high initial levels of antibody) are shown in Figure 3.

In the 24 infants tested for one year, there were 66 discernible episodes of anti-MSP-1\textsubscript{19kD} IgG and IgM production, as shown by peaks in the antibody level. The first IgG and IgM peaks coincided 92% of the time. Overall, the episodes of IgG production were accompanied by IgM production 83% of the time. However, there were fewer IgM peaks over the year when compared with IgG peaks (Wilcoxon rank \(P = 0.0233\)). The infants’ first IgG peaks corresponded with the infants’ first parasitemias 79% of the time. Thereafter, the infants’ parasitemias generally corresponded with increases in both IgG and IgM. The IgG antibody responses were short-lived, decreasing and increasing sharply with

<table>
<thead>
<tr>
<th>IgG at time of delivery</th>
<th>Infants’ first infection day(\dagger) Mean age in days (95% CI)</th>
<th>Placental malaria(\ddagger) Frequency</th>
<th>Geometric mean density (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>113.6 (103.5–123.7)</td>
<td>24%</td>
<td>4.7 (1.8–12.1)</td>
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<tr>
<td>–</td>
<td>69.4 (62.1–76.7)</td>
<td>81%</td>
<td>145.1 (34.1–612.4)</td>
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\(\dagger\) The mothers of 60 infants were tested <30 days prior to delivery, of which 44 responded to the antigen (73%) and 16 did not (27%). The placental malaria status of 37 mothers without IgG and 16 mothers with IgG of the 60 mothers tested were available. CI = confidence interval.

\(\ddagger\) General linear models (GLM). \(P = 0.0265\).

\(x^2\) \(P = 0.0000\) GLM placental malaria P.

**Figure 3.** Longitudinal antibody responses to merozoite surface protein-1\textsubscript{19kD} in infants during the first year of life. The geometric mean optical density (OD) (triplicate) for IgG (■) and IgM (●) are plotted against time. Six representative infants of a group of 24 tested are shown. The corresponding mother’s IgG OD is represented by an open square. An up arrow indicates febrile infection (inf.) and a down arrow indicates afebrile infection. \(Pf. = Plasmodium falciparum.\)
each parasitemia. The decrease in IgG was so sudden that our monthly time points could not be used to determine the half-life of the antibody. However, it was obvious that IgG produced by infants had a half-life of less than 60 days. The duration of antibody responses and overall antibody levels, as indicated by peaks, did not increase over time. It was shown that infants’ total antibody responses to *P. falciparum*, as tested by indirect immunofluorescent assay (IFA), was similar to mothers in level and remained consistently high throughout their first year of life.

Because we had comprehensive longitudinal data on antibody presence, parasitemia, and clinical parameters, we analyzed antibody levels and malaria incidences over time. In the 24 infants combined, there were 140 documented parasitemia infections. The 24 infants were positive for anti-MSP-1\textsubscript{19kD} IgG a total of 150 months of the cumulative 242 months tested. When the infants were positive for anti-MSP-1\textsubscript{19kD} IgG, there was an average of 0.16 infections/month (95% CI = 0.15–0.17), whereas, the negative response days averaged 1.7 infections/month (95% CI = 1.5–1.9). Therefore, there were 10 times more infections during the days without MSP-1\textsubscript{19kD} IgG. This difference was significant when tested by an analysis of variance and a GLM Dunican procedure (F = 9.0, P = 0.0045). An increased risk of infection with loss of the infants’ anti-MSP-1\textsubscript{19kD} suggests that these antibodies are playing a role in protection against malaria.

**DISCUSSION**

The brunt of malaria-related morbidity and mortality is borne by children (< 5 years old). Pregnant women often develop parasitemias in their placenta, resulting in mortality and morbidity in both the mother and neonate. Malaria vaccines will be most useful for these high-risk groups. In the last decade, several putative malaria vaccine antigens have been identified. The genes encoding these proteins have been isolated and expressed in a variety of expression systems. Some of these vaccine antigens have been tested for their protective effects in animal model systems. To complement vaccine development research, we have undertaken immunoepidemiologic studies aimed at delineating the characteristics of naturally acquired immunity to malaria infection and disease. The purpose of this study was two-fold. First, the information on characteristics of protective immune responses can be used in vaccine development. Second the information on human immune responses to vaccine antigens will be useful in assessing the efficacy of vaccines in field trials.

In this study, we have investigated the role of anti-MSP-1\textsubscript{19kD} antibodies in protective immunity against the clinical manifestations of malarial illness (anti-disease or anti-toxic immunity). We determined the antibody response in sera collected from mothers, cord blood, and at monthly intervals from infants during the first year of life. We found that anti-MSP-1\textsubscript{19kD} antibody levels in infants were associated with decreased morbidity due to malaria. This was apparent from the presence of higher levels of anti-MSP-1\textsubscript{19kD} antibodies in infants who were afebrile compared with those who were febrile. We also found that loss of hemoglobin was associated with a decreased level of anti-MSP-1\textsubscript{19kD} antibodies.

We also investigated associations between anti-MSP-1\textsubscript{19kD} IgG antibodies and anti-parasite immunity: first by examining the association between maternally transferred anti-MSP-1\textsubscript{19kD} IgG antibodies and placental malaria and infection in infants, and second by examining the association between infants’ anti-MSP-1\textsubscript{19kD} levels and the parasite density detected at and following the first detected infection. The level of anti-MSP-1\textsubscript{19kD} at the time of delivery was negatively correlated with placental malaria infection. Infants who acquired maternal anti-MSP-1\textsubscript{19kD} antibodies had a delayed first infection. Although parasite density at the time of the first detected infection negatively correlated with the infants’ antibody levels, parasite density at the time of infection may not be a good measure of immunity. More information about anti-parasite immunity was obtained by following the infections of untreated infants. Untreated infants who went on to clear their parasitemias without requiring treatment had significantly higher anti-MSP-1\textsubscript{19kD} IgG levels one month prior to their first detected infection. This observation suggests that antibodies were associated with protection against parasitemia.

The presence of anti-MSP-1\textsubscript{19kD} IgG antibody one month prior to the detection of the first documented infection appeared to have the greatest effect on clinical manifestation of illness. The time point of one month prior to the first infection was studied because it was an estimate of antibodies present at the time when an infant was exposed to the infectious bite. Protection may be exerted by preventing or decreasing the invasion of merozoites into erythrocytes. There is some circumstantial evidence to support the idea that antibodies that existed one month prior to the first infection may have been transplacentally derived maternal antibodies. In the year-long study of 24 infants, 91% of the cases had no IgM antibodies present prior to the first infection. The first IgG and IgM peaks coincided 92% of the time and this occurred only at the time when the first infection was detected. Therefore, the greater protection associated with anti-MSP-1\textsubscript{19kD} IgG present before the first documented infection may be suggesting that maternal antibodies have a greater protective ability than infant antibodies.

Obviously, the next critical issue is to determine any qualitative differences (isotype, specificity, affinity, etc.) between maternal and infant anti-MSP-1\textsubscript{19kD} antibodies. Knowing if the mothers’ antibody level at the time of delivery was a predictor of placental malaria and infants’ protection could be important for both intervention and vaccination strategies. We found that the incidence and placental parasitemia were significantly lower in mothers with anti-MSP-1\textsubscript{19kD} antibodies present at the time of delivery. These results are strengthened by the fact that a mother’s IgG level was very similar to her cord’s IgG level. We also found that increasing parity showed a protective trend against placental infection. Before the strong associations between maternal antibody and protection can be extrapolated to infant antibody level, it will be necessary to determine if the responses are qualitatively the same.

This longitudinal study enabled us to investigate the effects of the MSP-1\textsubscript{19kD} antibody responses on parasitologic and clinical aspects of malaria infection and the development of these responses in the first year of life. We found a linear decrease in antibody level followed by peaks in the anti-
MSP-119kD antibody level, which was presumed to be produced by the infant. Longitudinal monthly follow-up of each infant clearly showed that the anti-MSP-119kD IgG responses rapidly decrease, indicating that this response was short-lived. We also found multiple anti-MSP-119kD IgM responses associated with the infants’ multiple infections. It appeared that antibodies were produced as a result of infection, and when the responses were present, they were shown to be protective. However, when the levels decreased, another infection quickly occurred.

In contrast to the short-lived responses to MSP-119kD we detected in infants during the first year of their life, previous studies showed that immune adults in The Gambia had consistent levels of IgG to the 42-kD fragment of MSP-1. The IgG levels were consistent throughout both the high and low transmission seasons. These data suggested that even low, intermittent exposure was sufficient to maintain antibodies to conserved epitopes. Riley and others also showed that anti-MSP-1 antibody levels in adults were stable during changes in malaria transmission. These differences in the immune responses in adults versus infants may occur because all immunogenic determinants of the parasite antigens may not elicit memory immune responses in infants’ first few experiences with infection. Exposure-related, long-term memory has been proposed as a reason why responses to conserved epitopes are more stable than responses to dimorphic or polymorphic epitopes. Adult immune responses to the dimorphic region of MSP-1 were shown to be short-lived and temporal with parasitemia. Because of the highly conserved nature of MSP-119kD, one would expect that the anti-MSP-119kD responses in infants should become stable within one year of intense exposure. However, this is not what we observed. It is important to establish the age at which anti-MSP-119kD responses become stable with time. This may give an indication of the necessary exposures before long-term memory evolves.

Alternatively, the lack of memory response may be related to age and not exposure. It has been argued that young children may be unable to mount a memory response to this and other antigens. To address this issue, we performed blood-stage IFA analysis of antibodies from infants and their mothers. We found that infants’ total blood-stage antibody responses remained strong throughout their first year of life, and reached a level comparable with their mothers by one year of age. Therefore, the infants do not appear to be generally immunocompromised. However, it is known that infants do not develop memory responses against some antigens as would older individuals. The development of immunologic memory in infants is an area of great interest and perhaps will help us understand why short-lived responses are detected in infants.

It is possible that the short-lived responses we detected were caused by parasites binding to the antibody. Because the infants’ first documented parasitemias were frequently not detected until the initial antibody level (presumably maternally derived) decreased below a detectable level, and this decrease in initial antibody level was seen over several time points, it is doubtful that the decrease in maternal antibody was instigated by circulating parasites binding to these antibodies. Later in the infants’ first year, however, the binding of autogenous antibody to parasites could cause the appearance of peaks in infants’ antibody production. Because several time points between the peak and nadir of infants’ IgG levels were not investigated (due to the short half-life of less than one month), we could not address this issue as we did when considering maternally transferred antibody. However, of the 144 infants studied at the time of their first detected parasitemia, 77% had an anti-MSP-119kD IgG response. Also, the antibody levels of infants at the time of their first detected parasitemia were higher than at one month prior to their first detected parasitemia. This may suggest that the sudden decrease in anti-MSP-119kD antibody level was not due to a binding up of all the circulating antibodies by parasites. The only way to be more certain of this is to 1) study the infants’ responses to other antigens and 2) study the presence of parasitemia using more sensitive detection methods such as the polymerase chain reaction. This work is being undertaken in our laboratory.

The short-lived, temporal responses highlight the importance of longitudinal investigations of an individual’s natural immune responses. Antibody responses detected or not detected in cross-reactional studies of children or longitudinal age group studies may result in finding spurious associations or failing to detect associations. In addition, comparing the antibody responses of a child at one time point with their parasitologic and/or clinical manifestations at a time point greater than a few months may not be addressing the issue of antibody level-associated protection.

We must stress that associations may not represent causations, but the consistent indication of protection shown in this study is convincing. An indication that anti-MSP-119kD is involved in at least some of the protection against malaria is that, during the first year of life, loss of anti-MSP-119kD was associated with a high risk of infection. It is possible that the presence of anti-MSP-119kD antibodies may coincide with the presence of antibodies against other antigens, and that the combined effect of antibodies directed against several antigens may be exerting an anti-parasite effect. Therefore, it is essential to conduct further longitudinal studies of immune responses to other vaccine candidate antigens. This will help elucidate other antibodies protective against malaria. This is being done in our laboratory within the context of the Asembo Bay Cohort Project.

A vaccine that can induce the long-term anti-parasite responses in children similar to those found in adolescents and adults will be an important goal for future vaccine development strategies. Longitudinal immunoepidemiologic studies could identify the naturally immunogenic targets of Plasmodium falciparum antigens that may guide vaccine development efforts. Baseline data on the epidemiology of disease and immunology is important for conducting vaccine trials in this region.

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REFERENCES


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