EFFECTS OF PERMETHRIN-TREATED BED NETS ON IMMUNITY TO MALARIA IN WESTERN KENYA. ANTIBODY RESPONSES IN YOUNG CHILDREN IN AN AREA OF INTENSE MALARIA TRANSMISSION

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Abstract. As part of a large community-based trial on the impact of insecticide (permethrin)-treated bed nets (ITNs) on childhood morbidity and mortality in an area of intense perennial malaria transmission in western Kenya, we assessed the effects of ITNs on malaria-specific humoral responses in young children. The IgG responses to Plasmodium falciparum pre-erythrocytic antigens circumsporozoite protein (CSP) and liver stage antigen-1 (LSA-1) and the blood stage antigen merozoite surface protein-1 (MSP-119 kD) in children less than three years old were investigated during a series of cross-sectional surveys. At 14 and 22 months after the introduction of ITNs, the frequencies and levels of IgG to CSP and LSA-1 were significantly lower in children from ITN villages than in children from control villages ($P < 0.001$). In contrast, the prevalence of IgG to MSP-1 was significantly higher in children from ITN villages at 14 months ($P = 0.0069$), but not at 22 months. Our results show that decreased exposure by ITNs reduces IgG responses to pre-erythrocytic antigens, but there was no evidence that two years of ITN use compromises IgG responses to blood stage antigens in these young children in this malaria holoendemic area.

INTRODUCTION

Insecticide-treated bed nets (ITNs) have emerged as an efficacious and cost-effective malaria control strategy, and are a key technical element of the Roll Back Malaria strategy. We report elsewhere in this supplement that ITNs in an area with intense perennial malaria transmission in western Kenya reduce the force of infection by 74% resulting in marked reduction in malaria-associated morbidity and mortality in young children.

Concerns have been raised, however, about use of ITNs in high-transmission areas, since ITNs reduce malaria exposure, a prerequisite for the development and maintenance of malaria-specific acquired immunity. This view is supported by epidemiologic studies in malarious areas showing that parasite densities and prevalence decrease with age and that severe disease manifestations and mortality are restricted to early childhood, a stage that is critical in the development of acquired immunity. Older children and adults in these areas develop a non-sterilizing immunity that protects them from severe disease but not from infection. However, acquired protective immunity to malaria requires constant boosting through natural exposure to malaria parasites and wanes in the absence of such exposure. It is therefore hypothesized that the use of ITNs by young children in high-transmission areas could delay the development of acquired immunity to malaria, consequently placing these young children at an increased risk of developing severe malaria at an older age.

Despite these concerns, few studies have addressed the effects of ITNs on immunity to malaria. As part of a large community-based trial on the impact of ITNs on childhood morbidity and mortality in an area of intense perennial malaria transmission in western Kenya, we initiated both longitudinal and cross-sectional studies to systematically investigate the effects of ITNs on cellular and humoral immune responses to well-characterized pre-erythrocytic and erythrocytic malaria vaccine candidate antigens in young children and pregnant women. The present study describes the patterns of IgG responses to pre-erythrocytic circumsporozoite protein (CSP), liver stage antigen-1 (LSA-1), and blood stage merozoite surface protein-1 (MSP-119 kD) antigens in children less than three years old enrolled in a series of cross-sectional studies, pre- and post-ITN intervention. In this age group, severe malaria associated morbidity and mortality are high, and acquired protective immunity is actively developing.

MATERIALS AND METHODS

Study site and participants. The randomized controlled trial of ITNs was conducted between January 1997 and March 1999 in Rarieda Division (Asembo), Bondo District, in western Kenya. The design and implementation of the trial have been described in detail elsewhere. Malaria is holoendemic in the study area with year-round transmission. Two peaks of malaria transmission occur during and after the long rainy season (March–May) and during and after the short rainy season (October–December). Before the ITN trial, entomologic inoculation rates (EIRs) in this area had been reported to range from 60 to 300 infective bites per person per year. Plasmodium falciparum is the predominant malaria parasite and accounts for more than 95% of malaria infections. In this region, anemia is the major sign of severe clinical manifestation of P. falciparum infection, especially in young children. More than 95% of the residents belong to the Luo ethnic group.

The ITN and control areas were randomized by public lottery as described in detail elsewhere. Details of the design and methodology used in the cross-sectional survey are also described elsewhere. Briefly, households were used as sampling units in the cross-sectional surveys and different children were sampled at each survey. The baseline (pre-
intervention) cross-sectional survey (survey 0) was conducted between October and November 1996 during the short rainy season. The average daily rainfall in the previous six months before the survey (2.5 mm) was within the expected range for that period. The first follow-up survey (survey 1) was conducted between the short and long rainy season during February and March 1998, 14 months post-intervention. The average daily rainfall in the previous six months before this survey was close to average rainfall in the previous 10 years during this period. The second follow-up survey (survey 2) was conducted in November–December 1998, 22 months post-intervention and coincided with a dry spell that had persisted for the previous six months (average daily rainfall in the previous six months was 28% less than normal). Written informed consent for each participant was obtained from parents or legal guardians.

Ethical clearance for the ITN trial was obtained from the Ethical Review Committee of the Kenya Medical Research Institute (Nairobi, Kenya) and the Institutional Review Board of the Centers for Disease Control and Prevention (Atlanta, GA).

**Sample collection.** During each survey, 250–500 μL of blood from each child was collected by finger stick into EDTA microtainers (Becton Dickinson, Franklin Lakes, NJ). Thick and thin blood smears were also prepared on separate slides at the time of sampling. Blood samples stored at 4°C and smears were transported to the central laboratory in Kisian, 50 km from the field site. All samples and slides were labeled with unique identification numbers that could not be linked to the randomization status of the children by persons analyzing the specimens.

**Antigens.** Synthetic peptides corresponding to the repeat region of LSA-1, *Escherichia coli*–expressed full-length recombinant CSP and *Saccharomyces cerevisiae*–expressed 19-kD recombinant protein of MSP-1,19KD were used for assessment of antibody response in plasma samples. These antigens was chosen based on previous studies that have identified B cell determinants in these antigens that are recognized by most immune adults in this area, while other studies have suggested an association between immune responses to these antigens and clinical protection.20-22 In addition, antibodies to CSP have been shown in several studies to be a serologic marker of malaria transmission and exposure.23,24 The LSA-1 peptides used in this study were synthesized using F-MOC biochemistry at the Biotechnology Core Facility, National Center for Infectious Diseases, Centers for Disease Control and Prevention (Atlanta, GA). The peptides were 90% pure and used without further purification.

**Laboratory procedures.** Sample processing and hematologic and parasitologic examinations. A complete blood cell count was conducted on all the blood samples using an AcT 10 Coulter-Counter® (Coulter Corporation, Hialeah, FL). Erythrocytes were separated from plasma by centrifuging at 700 × g for five minutes. Plasma was aliquoted into sterile vials and stored at -70°C until antibodies were tested. Blood smears were stained with Giemsa and examined for the presence of malaria parasites and/or pigment with a 100× oil-immersion objective. The number of sexual and asexual forms were counted per 300 leukocytes and used to estimate parasite density (assuming a leukocyte density of 8,000/μL). At least 200 high-power fields were examined before a smear was categorized as negative.

Enzyme-linked immunosorbent assay (ELISA). The antibody response to recombinant CSP and LSA-1 peptides was determined by a standard ELISA. Briefly, flat-bottomed microtiter plates (Immunolon 2; Dynatech Laboratories, McLean, VA) were coated with 100 μL (10 μg/mL) of LSA-1 peptide or 100 μL (500 ng/mL) of recombinant CSP in PBS (0.01 M, pH 7.2). After overnight incubation at 4°C, the plates were washed twice with PBS containing 0.05% Tween-20 (PBS-T) and blocked with PBS-T containing 2.5% nonfat milk powder (PBS-T-M). Plasma samples diluted at 1:100 in PBS-T-M were added in duplicate wells and allowed to react for two hours at room temperature. Pooled plasma samples from children who tested highly positive in pilot experiments and negative control plasma from malaria-naive persons were included in each plate as positive and negative controls, respectively. Unbound antibodies were removed by washing the plates four times with PBS-T. Peroxidase-conjugated goat anti-human IgG antibody (ICN Biomedicals, Inc., Aurora, OH) at a 1:25,000 dilution in PBS-T-M was added and the reaction was revealed after one hour by the addition of 100 μL/well of tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reaction was stopped after 10 minutes with 1 M phosphoric acid and the absorbance was read at 450 nm with an ELISA reader (Dynatech Laboratories, Chantilly, VA). The IgG reactivity to recombinant MSP-1,19KD was assayed as described earlier with the following modifications: borate-buffered saline (167 mM borate buffer, 134 mM NaCl, pH 8.0) was used to suspend the recombinant MSP-1 for coating ELISA plates. The buffer used for blocking non-specific binding and diluting secondary antibodies was 0.15 M PBS, pH 7.2, 2.5% nonfat milk, 500 mM NaCl, 0.05% Tween-20. A positive control, consisting of pooled hyperimmune sera, was included in each experiment. The cut-off values for each antigen were determined by assaying samples from 40 malaria-naive persons. The samples that showed an optical density (OD) greater than the mean plus two standard deviations of the negative control plasma were scored as positive.

**Statistical analyses.** To ensure positive ELISA OD values, we added 0.001 to the lowest negative value for each antigen, and the new number was added to the corresponding antibody data. The new numbers added were 0.014, 0.064, and 0.034 for CSP, LSA-1, and MSP-1, respectively. The ELISA ODs were then log transformed to normalize the data. Analyses were performed using SUDAAN (version 8.0, SAS-callable version; Research Triangle Institute, Research Triangle Park, NC) and SAS (version 8.0; SAS Institute, Cary, NC) software. All analyses were adjusted for clustering at the village level since the randomization was village-based. The odds ratios (ORs) were obtained from the SAS Proc Genmod using Generalized Estimating Equations with a logistic regression model. All age stratified ORs for a given antibody were obtained from a single multivariable model containing a three-way interaction term. The overall ORs were adjusted for age from a single model with a two-way interaction term. Differences in antibody levels were analyzed by a t-test adjusting for clustering. Frequencies of antibody responses between ITN and control children were compared using the chi-square test in SUDAAN adjusting for clustering at the village level.
RESULTS

Study group characteristics. A total of 2,779 children less than three years old were tested for antibody responses to three *P. falciparum* antigens: 889 children in survey 0, 980 in survey 1, and 910 in survey 2 (Table 1). Age and sex in children from ITN and control areas were comparable in all surveys except in survey 2, in which the mean age of control-area children was significantly higher (*P* = 0.02), which probably reflected the reduced infant mortality in ITN villages following the introduction of the intervention. At baseline, parasite prevalence and densities were comparable between the two groups. Consistent with morbidity studies, the parasite densities and prevalence in survey 1 and survey 2 were significantly lower in children from ITN villages compared with children in the control villages (*P* < 0.01 for all). Similarly, children from bed net areas had significantly improved hemoglobin levels after the introduction of bed nets in survey 1 (*P* = 0.0001), but the difference was not significant in survey 2 (*P* = 0.19).

IgG response to recombinant CSP and LSA-1 peptides. At baseline, the prevalence and levels of IgG to CSP and LSA-1 were higher, although this was not statically significant, in children residing in villages randomized to subsequently receive bed nets compared with children from subsequent control villages (Figures 1 and 2 and Tables 2 and 3). At 14 and 22 months after the introduction of ITNs, this pattern was reversed (Figures 1 and 2 and Tables 2 and 3). This was apparent in all age groups with the exception of 0–5-month-old infants in survey 2 (Tables 2 and 3). Interestingly, when survey 1 and survey 2 were compared, the prevalence of CSP-specific IgG in control children in survey 2 was lower compared with that in survey 1 (Figure 1A), whereas the prevalence of IgG to LSA-1 was higher in both bed net and control children in survey 2 than in survey 1.

IgG response to recombinant MSP-1,19 kD antigen. At baseline, the prevalence and levels of IgG to MSP-1 were not significantly different in children residing in areas randomized to subsequently receive ITNs or controls (Figure 3 and Table 4). Surprisingly, the trend in antibody responses to MSP-1 was opposite to that for CSP and LSA-1 in survey 1. The prevalence of IgG to MSP-1 was significantly higher in children from ITN areas compared with control children (Figure 3A). Similarly, the levels of IgG to MSP-1 were higher, although the difference was not statistically significant (Figure 3B). The increased prevalence of MSP-1-specific IgG in survey 1 was evident in all the age groups (Table 4). However, in survey 2, there was no difference in IgG antibody responses to MSP-1 (Figure 3 and Table 4). Additional comparison between these two surveys showed that the prevalence of IgG to MSP-1 was similar in survey 1 and survey 2 in children from ITN villages. However, in control villages, the prevalence was lower in survey 1.

DISCUSSION

In this study, we found that antibody responses to CSP and LSA-1 were slightly higher in the subsequent ITN areas at baseline, but they were significantly lower at 14 and 22 months after the introduction of ITNs. The reduction in IgG reactivity to both CSP and LSA-1 was greater in the first follow-up survey compared with the second follow-up survey. This immunologic finding is consistent with the observed concurrent reduction in the prevalence of malaria parasitemia in these children after the introduction of ITNs. This decrease in pre-erythrocytic stage-specific IgG responses and malaria prevalence is likely caused by a reduction in human-vector contact by the use of ITNs, as shown by the 74% reduction in the force of infection in infants. Our data differ with that obtained in The Gambia, where no differences were observed in the prevalence and titers of antibodies to pre-erythrocytic the CSP repeat region (NANP)$_{60}$ between children from areas with untreated conventional bed nets and controls or between children from areas with permethrin-treated bed nets and those with untreated conventional bed nets. Several factors could explain the difference. In the study conducted in The Gambia, children 1–9 years old were studied while our study participants were children less than three years old. In addition, malaria transmission in The Gambia is low and seasonal (EIR = 1–10), whereas it is high and perennial in western Kenya (EIR = 60–300).

Contrary to the IgG responses to pre-erythrocytic CSP and LSA-1 antigens, there was no evidence that the responses to the blood stage MSP-1 antigen, which is known to be correlated with protection from malaria infection in this area, was compromised in children from ITN areas. Indeed at the 14-month survey, the prevalence of IgG to MSP-1 was signific-
TABLE 2

Effects of ITNs on the IgG response to circumsporozoite protein in different age groups*

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Cross-sectional surveys†</th>
<th>Survey 0</th>
<th>Survey 1</th>
<th>Survey 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OR (95% CI)</td>
<td>n</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>0–5</td>
<td>130</td>
<td>0.96 (0.45–2.03)</td>
<td>124</td>
<td>0.48 (0.17–1.31)</td>
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<tr>
<td>6–11</td>
<td>207</td>
<td>1.62 (0.78–3.36)</td>
<td>158</td>
<td>0.62 (0.33–1.19)</td>
</tr>
<tr>
<td>12–23</td>
<td>209</td>
<td>2.50 (1.38–4.53)</td>
<td>272</td>
<td>0.34 (0.22–0.52)</td>
</tr>
<tr>
<td>24–35</td>
<td>194</td>
<td>1.61 (0.74–3.49)</td>
<td>288</td>
<td>0.35 (0.20–0.62)</td>
</tr>
<tr>
<td>Overall‡</td>
<td>1.66 (0.94–2.92)</td>
<td>0.40 (0.13–1.30)</td>
<td>0.40 (0.30–0.54) (&lt;0.0001)$</td>
<td>0.50 (0.35–0.73) (0.0002)$</td>
</tr>
</tbody>
</table>

*ITNs = insecticide-treated bed nets; OR = odds ratio; CI = confidence interval.
†Three cross-sectional surveys were conducted: survey 0 at baseline, survey 1 at 14 months, and survey 2 at 22 months after ITN introduction.
‡Age-adjusted odds ratio.
§P value to test if the effect of ITNs in survey 1 or survey 2 is different from the effects in survey 0.
cantly higher in ITN villages, but there was no difference between ITN and control villages at the end of the two-year intervention period. These findings differ from two previous studies that evaluated antibody responses to blood-stage antigens in bed net users and nonusers. In Papua New Guinea, children and adults from areas with untreated bed nets were less likely to present with IgG to *P. falciparum* ring erythrocyte surface antigen and major merozoite surface antigen-2 than those who did not use bed nets. In Kilifi, Kenya, the prevalence of IgM to sonicated schizonts was lower in infants from areas with ITNs than control infants. The difference between our study and the Kilifi study is not likely explained by the duration of bed net usage, since in the Kilifi study, low antibody response in infants in ITN areas was observed one year after introduction of ITNs and at different time points thereafter. Various explanations are possible for the discrepancy between our study and the one conducted in Kilifi: differences in the study design, type of antigens used, antibody type measured, or level malaria transmission in Kilifi (EIR = 1–30) compared with our study site are all reasonable. Although malaria transmission in Papua New Guinea is high and perennial similar to western Kenya, differences in both study design and antigen type possibly explain differences between our results. Further assessment of this phenomenon will be addressed by longitudinal studies in children in this study area.

The increased IgG reactivity to MSP-1 but decreased response to CSP and LSA-1 in ITN users at 14-months post intervention could have occurred by chance since this phenomenon was not observed in survey 2. Another explanation is that the host immune system encounters different numbers of stage-specific parasites, resulting in different effects of ITNs on antibody response to different antigens. The asexual cycle of the malaria parasite generates larger quantities of antigens and malaria pigment in the human host compared with the pre-erythrocytic cycle. We hypothesize that, in high transmission areas such as western Kenya, the immune system, especially in young children, is overwhelmed by high-density parasitemia and more parasite diversity encountered during erythrocytic development, which results in an inefficient mounting of immune response. The use of ITNs reduces the sporozoite inoculum, resulting in lower blood-stage parasite densities and less diversity, consequently leading to more efficient mounting of immune response in children sleeping under ITNs. Although it is unclear what range of blood stage parasite density and diversity is required for efficient immune responses, this hypothesis is supported by a previous birth-
It is not clear why the antibody response to MSP-1 was higher in ITN villages than control villages at the 14-month survey, but not at the 22-month survey. One explanation could be waning of antibodies titers over time in the ITN villages due to long-term use of bed nets because acquired immunity to malaria requires certain levels of boosting through natural exposure to malaria parasites. However, there was no evidence that this occurred since the mean antibody concentrations to MSP-1 in the intervention villages were highest at the 22-month survey. Of note is that the parasite prevalence and densities, and antibody reactivity to CSP in the 22-month survey, which was conducted after a relatively dry six-month period, were all lower than in the 14-month survey, which was conducted just after the short rainy season. Thus, an alternative explanation could be that the lower transmission intensity in the months before the 22-month survey resulted in a more efficient MSP-1-specific immune response in children from both ITN and control villages compared with the earlier survey.

In conclusion, our study shows that ITNs decrease IgG responses to the pre-erythrocytic CSP and LSA-1 antigens. There was no evidence that two years of ITN use compromised the MSP-1-specific IgG responses in young children from this area with intense perennial malaria transmission. The hypothesis that ITN use may lead to more efficient antibody responses to the blood stage antigens in the peak transmission season deserves further study.

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TABLE 4

Effects of ITNs on the IgG response to merozoite surface protein-1 in different age groups*

<table>
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<tr>
<th>Age (months)</th>
<th>Survey 0</th>
<th>Cross-sectional surveys†</th>
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<th>Survey 2</th>
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<td>0–5</td>
<td>190</td>
<td>1.47 (0.80–2.69)</td>
<td>136</td>
<td>0.89 (0.41–1.92)</td>
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<td>6–11</td>
<td>238</td>
<td>1.08 (0.67–1.76)</td>
<td>140</td>
<td>2.02 (0.93–4.39)</td>
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<td>12–23</td>
<td>238</td>
<td>0.64 (0.31–1.31)</td>
<td>268</td>
<td>2.21 (1.21–4.02)</td>
</tr>
<tr>
<td>24–35</td>
<td>210</td>
<td>0.99 (0.46–2.13)</td>
<td>287</td>
<td>2.23 (1.25–3.99)</td>
</tr>
<tr>
<td>Overall†‡</td>
<td>1,030</td>
<td>1.03 (0.75–1.42)</td>
<td>1,173</td>
<td>1.83 (1.21–2.76)</td>
</tr>
</tbody>
</table>

*ITNs = insecticide-treated bed nets; OR = odds ratio; CI = confidence interval.
†Three cross-sectional surveys were conducted: survey 0 at baseline, survey 1 at 14 months, and survey 2 at 22 months after ITN introduction.
‡Age-adjusted odds ratio.
§P-value to test if the effect of ITNs in survey 1 or survey 2 is different from the effects in survey 0.

cohort study conducted in the same study area, which showed that children with the highest exposure to malaria, as measured by the cumulative number of P. falciparum episodes since birth, had the lowest antibody responses to MSP-1. In addition, it has been also shown that high-density malaria infection impairs malaria-specific immune responses, as well as loss of T cells through mechanisms such as apoptosis.

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