

## Insecticide-Treated Bed Nets Reduce Plasma Antibody Levels and Limit the Repertoire of Antibodies to *Plasmodium falciparum* Variant Surface Antigens

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**The use of insecticide-treated bed nets (ITN) has been documented to reduce malaria morbidity and mortality in areas with endemic malaria, but concerns have been raised that ITN usage could affect the acquisition of malaria immunity. Several lines of evidence have indicated that antibodies against variant surface antigens (VSA) are important in the development of naturally acquired immunity to *Plasmodium falciparum* malaria and may thus be good indicators of immune status. We have compared the levels of VSA antibodies in plasma from children who have used ITN for 4 years to levels in plasma from children from a nearby village not using ITN. A total of 97 plasma samples were analyzed using 13 different *P. falciparum* isolates. We found that the children using ITN had significantly lower VSA antibody levels and recognized a smaller proportion of the VSA expressed by the tested parasite isolates than children not using ITN.**

Malaria constitutes one of the largest global health problems. Through the Roll Back Malaria Program, the World Health Organization has set ambitious targets of halving the malaria burden before 2010. One of the important strategies used to reach this target is to promote the use of insecticide-treated bed nets (ITN). ITN have been documented to reduce malaria morbidity and mortality (8). ITN reduce contact between the mosquito vector and the human host. When used widely in a community, ITN reduce the population of sporozoite-positive mosquitoes (4), which is reflected in a reduction in the plasma antibody levels to circumsporozoite protein among the bed net users (11). Even though the short- and medium-term beneficial effect of ITN are well documented, concerns have been raised about the long-term effect, since the reduction in infectious inoculations may affect both the development and the maintenance of malaria immunity (16). Malaria immunity is developed after repeated exposures to the parasite, and clinical malaria immunity is acquired at a much earlier age in areas of intense malaria transmission than in areas of more moderate transmission intensity (13).

The functional background for malaria immunity is not fully understood, but several studies have indicated that it relies on the acquisition of a repertoire of agglutinating antibodies, which recognize a broad spectrum of variant surface antigens (VSA) expressed on infected erythrocytes (2, 7, 9, 10). The best characterized of these is the molecule responsible for sequestration of parasitized erythrocytes, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) (1, 15). PfEMP1 is

encoded by *var* genes (18). Each parasite genome contains about 40 *var* genes; only one *var* gene product is expressed at a time, and through each generation some parasites will switch PfEMP1 expression phenotype (14).

This study was designed to investigate whether the plasma levels and the repertoire of VSA antibodies were affected by the use of ITN by comparing the plasma antibody levels in children living in two nearby villages in northern Tanzania. The two villages are situated on the coastal plain about 30 km from Tanga in an area characterized by perennial and very intense malaria transmission. ITN were distributed to the inhabitants of Mafere village in February 1996 as part of a comparative trial of ITN versus house spraying (4). Since then there has been annual reimpregnation of the nets with alphacypermethrin and monitoring of the malaria morbidity among the children in the villages (5, 6). In April 2000 bed nets had not yet been distributed in Kibaoni village, and only a few children were sleeping under nets in this village. Before the introduction of ITN in Mafere the entomological inoculation rates (EIR) in the two villages were similar. The EIR in a group of villages including Mafere was 540 for the year before provision of ITN and 29 for the year after (4). In 1999 and 2000 the EIR in a group of villages including Kibaoni was 397 (20). In April 2000 plasma samples were obtained from 48 children aged 2 to 6 years and 48 children aged 3 to 7 years from Mafere and Kibaoni, respectively. The children were randomly selected from a computerized census list. Only asymptomatic children who were not receiving antimalarial treatment were included. In addition, a 9-year-old child from Mafere was sampled and included in the study. All children from Mafere used bed nets on a regular basis, whereas the children from Kibaoni did not. We measured the levels of antibodies to 13 different parasite isolates by flow cytometry (17). The isolates were originally obtained from malaria patients contracting malaria in Ghana

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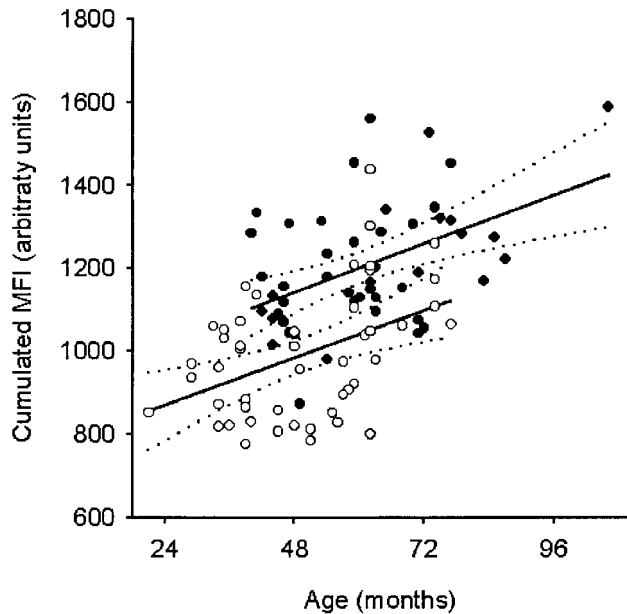


FIG. 1. VSA plasma antibody levels in relation to age among children sleeping under ITN (○) ( $n = 48$ ) and unprotected children (●) ( $n = 49$ ). The antibody levels were measured by flow cytometry, and the figure shows the cumulative MFI for 13 parasite isolates. The solid lines show linear regression, and dotted lines represent the 95% confidence interval.

( $n = 11$ ; isolates: L1127, L1051, L1156, L1114, L1094, L1070, L1093, L1106, L1047, L1052, and Q0016) or Sudan ( $n = 2$ ; isolates: G4 and G12) and were cultured according to standard procedures with slight modifications (3, 19). On the day of assay erythrocytes infected with mature blood-stage parasites were purified by exposure to a strong magnetic field, resulting in material having >75% parasitaemia (17). Aliquots of  $2 \times 10^5$  purified late-stage-infected erythrocytes labeled by ethidium bromide were sequentially exposed to  $5 \mu\text{l}$  of plasma,  $0.4 \mu\text{l}$  of goat anti-human immunoglobulin G (Dako, Glostrup, Denmark), and  $4 \mu\text{l}$  of fluorescein isothiocyanate-conjugated rabbit anti-goat immunoglobulin G (Dako). Samples were washed twice in phosphate-buffered saline between each incubation step. Twofold dilution of a hyperimmune plasma pool from adult Ghanaians and individual plasma samples from 10 Danish adults without exposure to malaria were included for each parasite isolate. For each parasite-plasma combination, data from 5,000 cells were collected on a FACScan (Becton Dickinson, Franklin Lakes, N.J.), and the mean late-stage-infected erythrocyte fluorescence index (MFI) was recorded. Nonspecific labeling was evaluated by analysis of uninfected erythrocytes. It has previously been shown that the main reactivity measured in this assay is directed against large (molecular mass, 200 to 300 kDa) polymorphic surface-expressed molecules (17) and depends on expression of PfEMP1 (12).

For each child the overall VSA antibody reactivity was calculated as the accumulated MFI of each of the 13 parasite isolates tested. Figure 1 shows the overall VSA antibody reactivity in plasma from children sleeping under ITN and unprotected children. In both groups the VSA antibody levels increased with age ( $r_s = 0.40$  and  $P < 0.005$  and  $r_s = 0.41$  and  $P < 0.004$ , for bed net users and unprotected children, respective-

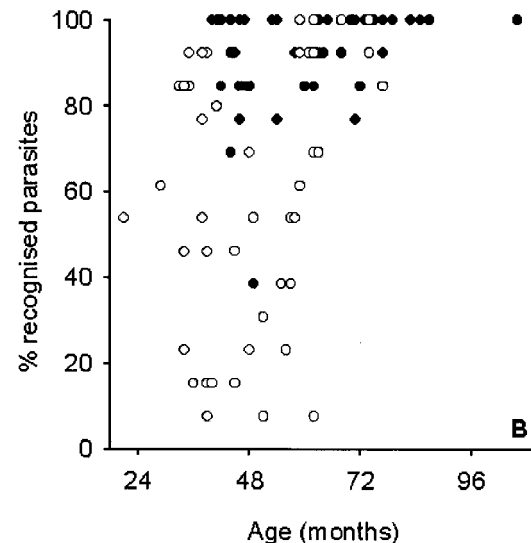
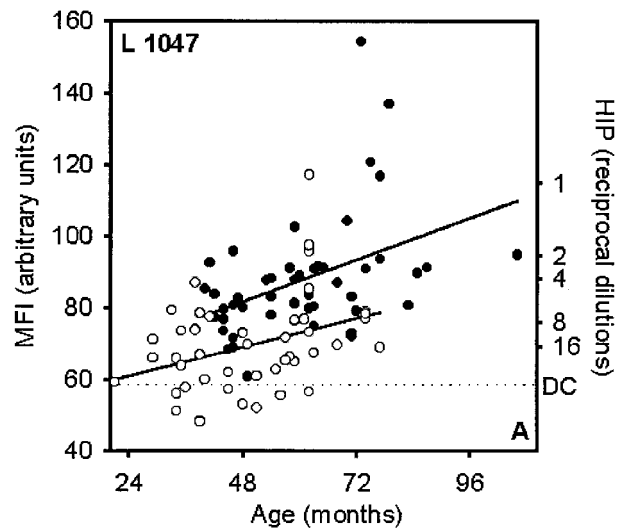


FIG. 2. VSA plasma antibody levels and repertoire in children sleeping under ITN (○) and unprotected children (●). (A) Plasma antibody levels to VSA carried by the parasite isolate L1047. The y axis at left shows the MFI measured by flow cytometry. The y axis at right shows the reactivity in dilutions of a hyperimmune plasma pool (HIP). The reactivity of Danish control plasma + 2 SD (DC) is indicated by the dotted line. The x axis shows the plasma donor age. (B) Percentage of parasite isolates recognized in relation to age, assessed by counting how many of the 13 parasite isolates the donor had plasma VSA antibody reactivity to above the cutoff level (mean reactivity of Danish controls + 2 SD).

ly). However independently of this age correlation, the level of reactivity also depended on bed net usage, with reactivity being lower in the children using ITN ( $P < 0.0001$  [analysis of covariance]). The plasma VSA antibody reactivities to the individual parasite isolates were also compared between the two groups. For all isolates the plasma VSA antibody levels were higher among unprotected children than among the bed net users ( $P < 0.002$  for each isolate). Figure 2A shows the plasma VSA antibody reactivity for a representative isolate and the corresponding values obtained using a twofold titrated hyperimmune plasma pool and Danish control plasma. For all isolates the difference between the regression lines of the two groups was between 1 and 2 dilutions of the hyperimmune plasma pool (Fig. 2A and data not shown), corresponding to two- to four-fold-lower antibody levels among bed net users than among the unprotected children. Taken together the data in Fig. 1 and 2A show that both the overall amount of antibodies to VSA and the antibody level to VSA expressed by individual parasite strains was affected by the use of ITN. To assess whether bed net usage also affected the number of parasite isolates recognized, we counted the number of parasite isolates for which each donor had plasma VSA reactivity above the cutoff level (mean reactivity of Danish controls + 2 standard deviations [SD]). Figure 2B shows that most of the unprotected children recognized VSA on all 13 parasite isolates tested, and among these children there was no association between age and the number of isolates recognized. Among the bed net users the percentage of parasites recognized was generally lower but increased with donor age ( $r_s = 0.36$ ;  $P < 0.012$ ). When comparing children 40 to 70 months old, there was a statistically significant difference between the percentage of parasites recognized between unprotected children and bed net users ( $P < 0.001$  [Mann-Whitney rank sum test]).

Our findings indicate that the use of ITN even in areas of very high malaria endemicity reduces the level of VSA antibodies and narrows the repertoire of recognized VSA. The clinical consequences of this are not known, but it could delay acquisition of malaria immunity and thereby influence the disease pattern. The reduction of the plasma VSA antibody repertoire could make bed net users more vulnerable to parasites that express rare VSA variants (2). Widespread use of ITN decreases the malaria disease burden in communities in the short run (8). Our data indicate that the immune responses to an important target of protective malaria immunity are influenced by bed net usage and suggest that ITN in the longer run could influence malaria disease patterns. However, the ongoing clinical monitoring in the mentioned villages shows that there continues to be health benefits after using ITN for 2 to 5 years, including a reduction of anemia prevalence (5, 6; Maxwell et al., unpublished data).

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