Breadth of Anti-Merozoite Antibody Responses Is Associated With the Genetic Diversity of Asymptomatic *Plasmodium falciparum* Infections and Protection Against Clinical Malaria

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**Background.** Elucidating the mechanisms of naturally acquired immunity to *Plasmodium falciparum* infections would be highly valuable for malaria vaccine development. Asymptomatic multiclonal infections have been shown to predict protection from clinical malaria in a transmission-dependent manner, but the mechanisms underlying this are unclear. We assessed the breadth of antibody responses to several vaccine candidate merozoite antigens in relation to the infecting parasite population and clinical immunity.

**Methods.** In a cohort study in Tanzania, 320 children aged 1–16 years who were asymptomatic at baseline were included. We genotyped *P. falciparum* infections by targeting the *msp2* gene using polymerase chain reaction and capillary electrophoresis and measured antibodies to 7 merozoite antigens using a multiplex assay. We assessed the correlation between the number of clones and the breadth of the antibody response, and examined their effects on the risk of malaria during 40 weeks of follow-up using age-adjusted multivariate regression models.

**Results.** The antibody breadth was positively correlated with the number of clones (RR [risk ratio], 1.63; 95% confidence interval [CI], 1.32–2.02). Multiclonal infections were associated with a nonsignificant reduction in the risk of malaria in the absence of antibodies (RR, 0.83; 95% CI, 0.29–2.34). The breadth of the antibody response was significantly associated with a reduced risk of malaria in the absence of infections (RR, 0.25; 95% CI, 0.09–0.66). In combination, these factors were associated with a lower risk of malaria than they were individually (RR, 0.14; 95% CI, 0.04–0.48).

**Conclusions.** These data suggest that malaria vaccines mimicking naturally acquired immunity should ideally induce antibody responses that can be boosted by natural infections.

**Keywords.** malaria; immunity; multiclonal infections; merozoite surface antigens; antibodies.

Asymptomatic *Plasmodium falciparum* infections composed of multiple genetically distinct clones are common in humans living in malaria-endemic areas [1–3]. These infections are often seen in individuals who have been repeatedly infected and have gradually developed some degree of immunity against malaria. The number of clones, as characterized by polymorphisms within merozoite surface antigen genes, vary with age and...
malaria transmission intensity [4, 5]. Cohort studies in areas of high malaria transmission have shown that the number of clones at baseline in cross-sectional surveys is associated with reduced subsequent risk of malaria [4, 6–8]. Multiclonal infections have been proposed to confer protection against malaria by preventing superinfection or by mediating tolerance to infection [3]. However, the precise immunological mechanisms underlying this protective association are unknown.

Antibodies are well recognized as important components of malaria immunity [9]. In malaria-endemic areas, anti-merozoite antibody titers are higher in children with asymptomatic *P. falciparum* infections compared to aparasitemic children [10–15]. Moreover, increasing breadth of antibody responses to merozoite antigens (i.e., the number of antigens to which an individual has high antibody titers) was associated with increasing protection against malaria [11, 16]. Whereas it has been postulated that the tolerance of multiclonal *P. falciparum* infections is associated with a broad repertoire of immune responses that control parasitemia and prevent malaria [3], this hypothesis has not been empirically tested. Here, we test the hypothesis that asymptomatic multiclonal infections are associated with increasing breadth of anti-merozoite antibody responses, and that, in combination, these factors would be more strongly associated with protection against malaria than either factor individually.

In a cohort study in Tanzania that included children aged ≤16 years, we determined the number of clones in asymptomatic *P. falciparum* infections at baseline by genotyping the merozoite surface protein 2 gene (*msp2*) by fluorescent polymerase chain reaction (PCR) and capillary electrophoresis [17]. We measured antibody levels to 7 merozoite antigens using a multiplex assay. We investigated relationships between the number and types of *msp2* genotypes in asymptomatic infections and antibody responses to these merozoite antigens in relation to risk of malaria.

**MATERIALS AND METHODS**

**Study Population**

The study was conducted within a longitudinally monitored population in Nyamisati village in the Rufiji River delta, coastal Tanzania [18]. A cross-sectional survey including 890 individuals (aged 1–84 years) was conducted in March–April 1999 in which venous blood was collected in ethylenediaminetetraacetic acid and stored frozen as plasma and packed cells. Parasite prevalence was 46% by PCR, with the highest prevalence in children aged 3–5 years (74% by PCR) [7]. All participants were monitored for 40 weeks and malaria was recorded by passive case detection by researchers who operated the only health facility in the village [18]. All individuals who reported to the research clinic with fever and *P. falciparum* parasites detected by microscopy were administered free antimalarial treatment. In this study, malaria was defined as fever (axillary temperature >37.5°C or history of hot body within 24 hours) and ≥5000 parasites/μL in peripheral blood [19]. The present analysis was restricted to children aged ≤16 years who were healthy at the time of the survey (n = 320). Children with history of fever in the 4 weeks preceding or within 1 week of the survey were excluded. The project was scientifically and ethically approved by the National Institute for Medical Research in Tanzania and the Stockholm Ethical Review Board (Dnr00-084 and 2012/1151-32). Informed consent was obtained from the guardians of all participants.

**Genotyping of *P. falciparum* Infections**

DNA was extracted from packed erythrocytes using ABI6100 PrepStation (Applied Biosystems). Genotyping of the *P. falciparum* msp2 gene was performed as described previously [17]. In brief, the PCR included an initial amplification of the outer *msp2* domain, followed by nested reactions with fluorescent primers targeting the FC27 and IC-1/3D7 allelic types of *msp2*. Allelic fragments were separated by capillary electrophoresis and analyzed using GeneMapper software (Applied Biosystems). The number of *msp2* genotypes identified by this method determines the number of clones within an individual *P. falciparum* infection. Considering that PCR can amplify genomic DNA encoding the *msp2* gene from both asexual [20] and sexual [21] parasite stages, *msp2* genotypes detected could be from either stage. The *msp2* gene of a subset of *P. falciparum* infections with only 1 genotype were sequenced using BigDye terminator technology (Applied Biosystems).

**Recombinant *P. falciparum* Merozoite Antigens**

Seven recombinant antigens representing 4 malaria vaccine candidate antigens were expressed in *Escherichia coli* and purified by high-performance liquid chromatography. Two allelic forms of MSP-2, MSP-2_Dd2 and MSP-2_CH150/9 (representing the FC27 and IC-1 allelic families of *msp2* respectively), and the 19-kilodalton fragment of MSP-1 were expressed as glutathione S-transferase (GST) fusion proteins [22, 23]. Two allelic forms of MSP-3 (MSP-3_K1 and MSP-3_3D7) were expressed in *E. coli* as maltose binding protein (MBP) fusion proteins [24]. Recombinant AMA-1 from the 3D7 and FVO strains were expressed as His-tagged proteins [14]. All antigens except MSP-1_19 were expressed as full-length proteins.

**Multiplex Bead-Based Antibody Assay and Enzyme-Linked Immunosorbent Assay**

A multiplex bead-based assay was adapted to measure plasma immunoglobulin G (IgG) antibodies to the 7 antigens in plasma samples collected at the cross-sectional survey. Spectrally unique beads were coupled at 5 ng/5000 beads/well/antigen and incubated with 50 μL of 1:1000 dilutions of plasma
samples for 1 hour on a microplate shaker (rotation 500 rpm) in the dark. After 4 phosphate-buffered saline (PBS)–Tween (0.05% Tween-20 in PBS) washes, 50 µL/well of 2 µg/mL of R-phycocerythrin–conjugated, F(ab’)2, goat antihuman IgG (Jackson ImmunoResearch) was added. After 30 minutes of incubation, beads were washed 4 times with PBS-Tween and resuspended in 100 µL PBS containing 1% bovine serum albumin. Beads were analyzed on Bio-Plex200 using Bio-Plex software (Bio-Rad). Fluorescence from 100 beads/antigen was read and reported as mean fluorescence intensity (MFI) and background responses to GST and MBP tags were subtracted. Serially diluted malaria immunoglobulin [25] added to each plate as a standard positive control corrected for plate-to-plate variations and allowed for conversion of MFIs to relative anti-body concentrations in arbitrary units. Sera from P. falciparum–naive donors were added to each plate to correct for background nonspecific antibody binding. Plasma IgG to P. falciparum schizont extract of the 3D7 parasite line was measured by enzyme-linked immunosorbent assay as previously described [11].

**Data Analysis**

Statistical analysis was performed using Stata software version 11.2 and R version 2.7. Continuous variables and proportions were compared using the Mann-Whitney and χ² test, respectively. Relationships between number of clones and risk of malaria were assessed using a modified Poisson regression model [26] adjusting for age in 3 age categories; ages 1–4, 5–10, and 11–16 years. Considering that several studies have shown that high anti-merozoite antibody titers are better predictors of protection against malaria than seropositivity [11, 15, 16], we derived threshold antibody concentrations that defined high antibody titers to each antigen as previously described [27]. In brief, antibody data were fitted to a regression model, and a dichotomization point that maximized the log likelihood that antibody data would predict protection from malaria was selected and used to derive threshold antibody concentrations (Supplementary Figure 1). Antibody titers above and below these thresholds are referred to as “high” and “low” antibody titers, respectively. Relationships between antibody titers and risk of malaria was assessed using a modified Poisson regression model that compared the risk of malaria in individuals with high versus low antibody titers adjusting for age and antibody titers to schizont extract (included as a surrogate for exposure). The breadth of antibody response was defined as the number of antigens to which an individual had antibody titers above the threshold antibody concentrations (described above and illustrated in Supplementary Figure 1). Because antibody titers to allelic forms of AMA-1 and MSP-3 were highly correlated (Supplementary Table 1), responses to one allelic form of these antigens were considered in deriving breadth. Thus, the antigens considered in defining breadth were MSP-2_Dd2, MSP-2_CH150/9, AMA-1_FVO, MSP-3_3D7, and MSP-1_19. Attributable fractions were estimated using main-effects models with the R package pARTial [28]. Confidence intervals were estimated using the delta method [29]. The present analysis was restricted to children who were free from symptoms of malaria at the time of the survey, 4 weeks preceding or 1 week after the survey. The results of this analysis were unchanged when we applied a definition in which we excluded all children who developed malaria up to 4 weeks after the survey (data not shown).

**Table 1. Number of Clones in Asymptomatic Plasmodium falciparum Infections and Risk of Malaria During Follow-up**

<table>
<thead>
<tr>
<th>Age, y</th>
<th>No.</th>
<th>No. (%)</th>
<th>Unadjusted</th>
<th>Adjusted</th>
<th>Age 1–4 y</th>
<th>Age 5–10 y</th>
<th>Age 11–16 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>60</td>
<td>25 (41)</td>
<td>1 (reference group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–10</td>
<td>130</td>
<td>22 (16)</td>
<td>0.41 (.25–.66)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11–16</td>
<td>121</td>
<td>11 (9)</td>
<td>0.22 (.16–.41)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of clones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>146</td>
<td>33 (23)</td>
<td>1 (reference group)</td>
<td>1 (reference group)</td>
<td>1 (reference group)</td>
<td>1 (reference group)</td>
<td>1 (reference group)</td>
</tr>
<tr>
<td>1</td>
<td>69</td>
<td>13 (19)</td>
<td>0.83 (.47–1.48)</td>
<td>0.90 (.51–1.58)</td>
<td>0.91 (.40–2.07)</td>
<td>1.45 (.66–3.19)</td>
<td>0.23 (.02–1.78)</td>
</tr>
<tr>
<td>2–3</td>
<td>57</td>
<td>9 (16)</td>
<td>0.70 (.36–1.36)</td>
<td>0.80 (.43–1.48)</td>
<td>2.17 (1.29–3.68)</td>
<td>0.33 (.08–1.37)</td>
<td>0.35 (.05–2.67)</td>
</tr>
<tr>
<td>≥4</td>
<td>33</td>
<td>1 (3)</td>
<td>0.13 (.02–.95)</td>
<td>0.16 (.02–1.16)</td>
<td>0</td>
<td>0</td>
<td>0.52 (.07–3.92)</td>
</tr>
</tbody>
</table>

Significant associations (P ≤ .05) are shown in bold.

a All children were monitored for clinical malaria over a 40-week follow-up period.
b Adjusted for age (in 3 age groups: ages 1–4, 5–10, and 11–16 years).
c None of the individuals these categories had malaria during the 40-week follow-up.
RESULTS

Asymptomatic *P. falciparum* Infections and Subsequent Risk of Malaria

Among the 890 individuals participating in the cross-sectional survey, 320 children aged 1–16 years were asymptomatic (ie, without fever or other symptoms of malaria) at the time of the survey, 4 weeks preceding or 1 week after the survey. Samples for *msp2* genotyping were available for 305 children, of whom 159 (52.13%) were parasitemic by PCR. Among the parasitemic children, the mean number of clones was 2.24 (range, 1–7), and 90 (56.60%) of them had multiclonal infections (infections with \( \geq 2 \) clones). Among the 159 PCR-positive samples, 141 had 1 *msp2* genotype of either *msp2* allelic type, of which 100 were sequenced. Both capillary electrophoresis and sequencing identified 50 and 25 unique *msp2* alleles of the IC-1 and FC27 types, respectively (Supplementary Figure 2).

Risk of malaria during follow-up decreased with age (Table 1, Figure 1A); being parasitemic by PCR, compared to being aparasitemic, was associated with reduced risk of malaria (risk ratio [RR], 0.54; 95% confidence interval [CI], .30–.98). The number of clones increased with age up to 5–10 years and stabilized thereafter (Figure 1B). Increasing number of clones was associated with reduced risk of malaria in univariate analysis, and a similar trend was evident after age adjustment (Table 1). However, in the youngest age group, the presence of 2–3 clones was significantly associated with increased risk of malaria (Table 1).

Breadth of the Antibody Response Is Associated With a Reduced Risk of Malaria

Antibody titers to individual merozoite antigens and schizont extract increased with age (Kruskal-Wallis test, \( P < .001 \), Supplementary Figure 3). Breadth was positively correlated with age (Kruskal-Wallis test, \( P < .001 \), Figure 1C). Breadth was higher in parasitemic compared to aparasitemic children (\( \chi^2 \) test for trend, 15.95, \( P < .001 \)). There was significant reduction in the risk of malaria with increasing breadth independent of age, antibody responses to schizont extract, and number of clones (Table 2). Children with high antibody titers against individual antigens had a lower risk of malaria compared with those with low titers, but this was only significant for responses to MSP-2_Dd2, MSP-2_CH150/9, and MSP-3_3D7 in age and schizont-adjusted models, also when stratified by parasite status (Supplementary Table 2).

Multiclonal Infections, Breadth, and the Risk of Malaria

The breadth of antibody response increased as the number of clones increased (Figure 2). This association was significant in an age-adjusted model (RR, 1.63; 95% CI, 1.32–2.02). To investigate whether multiclonal infections and breadth in combination were more strongly associated with protection from malaria than they were individually, we fit an interaction term between the number of clones and the breadth of antibody responses into a modified Poisson regression model. The interaction term showed a RR of 0.87 (95% CI, .59–1.29), suggesting that there is no evidence that multiclonal infections and breadth were synergistic in predicting protection against malaria. Nonetheless, multiple
clones (≥2 clones) and breadth of antibody responses (against ≥2 antigens), in combination, were associated with a lower risk of malaria than they were individually (RR, 0.14; 95% CI, .04–.48).

Age-Dependence of the Attribution of the Protection Against Malaria to Multiclonal Infections and Breadth of Antibody Responses

The proportion of protection against malaria attributable to age was 13.09% (95% CI, 3.51%–38.41%), number of clones 2.37%, and breadth of antibody responses 14.60% (95% CI, 2.85%–49.93%), respectively (Table 3). Protection against malaria attributable to multiclonal infections and breadth of antibody responses increased and decreased with age, respectively (Table 4). In combination, multiclonal infections and breadth of antibody responses accounted for 19.57% (95% CI, 4.96%–53.12%) of the protection against malaria.

Multiple Clones of IC-1 and FC27 msp2 Types Are Associated With Increasing Antibody Titers to Antigens Representing the Corresponding Dimorphic Type of MSP-2

Among 136 parasitemic children, 50 (36.74%) and 23 (16.91%) had infections composed of clones belonging exclusively to IC-1 and FC27 types, respectively; 63 (46.32%) children were infected with clones belonging to both types. Increasing number of clones of FC27 type was associated with increasing antibody titers to both MSP-2 Chr150/9 and MSP-2 Chr150/9 (Kruskal-Wallis test, P = .0023) but not to the other MSP-2 antigen (MSP-2 Chr150/9; Kruskal-Wallis test, P = .13; Supplementary Figure 3). Infections with increasing number of IC-1 type clones were associated with increasing antibody titers to both MSP-2 Chr150/9 (IC-1 type) and MSP-2 Chr150/9 (FC27 type) (Kruskal-Wallis test, P = .0012 and P = .0006, respectively; Supplementary Figure 3). Increase in total number of clones was associated with increasing antibody titers to both MSP-2 Chr150/9 and MSP-2 Chr150/9 (Kruskal-Wallis test, P = .0077 and P = .0003, respectively; Supplementary Figure 3). Antibody titers to MSP-2 Chr150/9 and MSP-2 Chr150/9 were nonsignificantly higher among individuals whose infections comprised exclusively of clones of the corresponding msp2 type compared to individuals whose infections comprised the alternative msp2 type.
DISCUSSION

We show for the first time that asymptomatic *P. falciparum* infections with increasing number of clones are positively associated with increasing breadth of anti-merozoite antibody responses. Furthermore, we show that multiclonal *P. falciparum* infections and the breadth of anti-merozoite antibody response, in combination, are associated with a lower risk of clinical malaria than they are individually.

Asymptomatic multiclonal infections have been associated with reduced risk of malaria in previous studies in high-transmission settings [4, 6–8]. In contrast, in low-transmission settings and in children <3 years of age [30–32], as we observed here, multiclonal infections conferred an increased risk of malaria. Additionally, the number of infections acquired over time has been shown to be a major predictor of risk of malaria in children aged ≤3 years [33]. These findings suggest that in individuals who are yet to develop malaria immunity, the number of clones is a marker of exposure rather than immunity, whereas in older children, persistent multiclonal infections are a marker of immunity.

Our observation that the breadth of antibody responses is associated with a reduced risk of malaria concurs with previous studies [11, 16]. However, whether the presence of multiclonal infections leads to increased breadth, or whether breadth mediates tolerance of multiclonal infections, is difficult to resolve. The short-lived nature of anti-merozoite antibodies in absence of parasite exposure [34] argues for the importance of parasites in the maintenance of antibody responses. Conversely, the importance of breadth in mediating tolerance to asymptomatic infections is demonstrated by observations that high antibody titers to merozoite antigens are associated with protection from high-density parasitemia and malaria but not from risk of reinfection [15]. Whereas we find no evidence that multiclonal infections and breadth of anti-merozoite antibody responses are synergistically associated with reduced risk of malaria, we observe a further reduction in risk of malaria associated with these 2 factors in combination. This suggests that the dual presence of these factors is important in relation to malaria immunity.

Although premunition (immunity against clinical symptoms while chronically infected) has been described in relation to malaria [3, 35], associations between asymptomatic infections and antibody-mediated immunity remain unclear. Some studies report that asymptomatic parasitemia is neither associated with risk of malaria nor modifies the associations between antibody responses and risk of malaria [15, 16]; others have shown that anti-merozoite antibodies are associated with protection from malaria only in parasitemic children [11–14, 36]. We have shown that lack of protection in a parasitemic children may be due to suboptimal antibody concentrations [27]. Together, these reports suggest that concurrent parasitemia is important for the maintenance of anti-merozoite antibodies at high enough levels to confer protection against malaria. Our finding that the multiclonality of asymptomatic infections is positively correlated with the breadth of anti-merozoite responses supports this view. In a randomized, placebo-controlled trial...
of intermittent preventive treatment in children, multiclone infections were associated with protection against malaria only in the placebo group, suggesting that the persistent rather than the intermittent presence of multiclone infections is important for maintenance of malaria immunity [37].

We found that an increase in the number of msp2 FC27-type clones was associated with increase in antibody titers to the antigen that represents the corresponding (MSP-2_Dd2) but not the alternate (MSP-2_Ch150/9) allelic family. Conversely, increase in the number of IC-1 type clones was associated with increase in antibody titers to both MSP-2 antigens. This finding could be due to a smaller proportion of individuals with infections composed exclusively of FC27-type clones compared to IC-1-type clones, but may also suggest that the positive association between multiclone infections and breadth of antibody responses is partly allele specific [38, 39].

Together, multiclone infections and anti-merozoite antibody responses tested here accounted for approximately 20% of the protection against malaria. It is likely that antibody responses to other antigens also contribute to protective malaria immunity. Moreover, human genetics and environmental factors were shown to each account for approximately 25% of the variation in malaria incidence in children living on the Kenyan coast [40]. The age-associated increase in protection against malaria attributable to multiclone infections agrees with several studies, which show that multiclone infections are associated with increased and reduced risk of malaria in younger [30–32] and older children [4, 6–8], respectively. The age-associated reduction in the protection against malaria attributable to anti-merozoite antibody responses suggests that antibodies are most important in childhood. The gradual development of immunity against malaria might thus involve different age- and exposure-associated mechanisms.

The observed increase in protection against malaria with increasing breadth of antibody responses and genetic-diversity of *P. falciparum* infections suggests that naturally acquired immunity against malaria may be characterized by the maintenance of low-density parasitemia rather than immune-driven elimination of the parasite. This implies that malaria vaccines intended to mimic naturally acquired immunity should be designed to maintain long-lasting antibody responses to multiple antigens. The increase in protection with increasing breadth of antibody responses favors the development of multicomponent vaccines.

In summary, we found that the breadth of antibody responses against a panel of merozoite antigens was positively correlated with multiclone infections. In combination, these 2 factors were associated with greater reduction in the risk of malaria than they were individually. These data suggest that multicomponent malaria vaccines mimicking naturally acquired immunity should induce antibody responses that can be boosted by natural infections.

### Supplementary Data

**Supplementary materials** are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

### Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

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