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A threshold concentration of anti-merozoite antibodies is required for protection from clinical episodes of malaria☆

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ABSTRACT

Antibodies to selected *Plasmodium falciparum* merozoite antigens are often reported to be associated with protection from malaria in one epidemiological cohort, but not in another. Here, we sought to understand this paradox by exploring the hypothesis that a threshold concentration of antibodies is necessary for protection. We analyzed data from two independent cohorts along the Kenyan coast, one in which antibodies to AMA1, MSP-2 and MSP-3 were associated with protection from malaria (Chonyi) and another in which this association was not observed (Junju). We used a malaria reference reagent to standardize antibody measurements across both cohorts, and applied statistical methods to derive the threshold concentration of antibodies against each antigen that best correlated with a reduced risk of malaria (the protective threshold), in the Chonyi cohort. We then tested whether antibodies in Junju reached the protective threshold concentrations observed in the Chonyi cohort. Except for children under 3 years, the age-matched proportions of children achieving protective threshold concentrations of antibodies against AMA1 and MSP-2 were significantly lower in Junju compared to Chonyi (Fishers exact test, $P < 0.01$). For MSP-3, this difference was significant only among 4–5 year olds. We conclude that although antibodies are commonly detected in malaria endemic populations, they may be present in concentrations that are insufficient for protection. Our results have implications for the analysis and interpretation of similar data from immuno-epidemiological studies.

1. Introduction

Antibodies play an important role in mediating protection against clinical malaria. Purified total IgG obtained from malaria-immune African adults was successfully used to treat children and adults hospitalized with malaria, while control sera from adults not exposed to malaria had no protective effect [1–3]. Identifying the target(s) of these "protective" antibodies continues to be a priority for malaria vaccine development. Immuno-epidemiological studies are widely used to assess the potential protective efficacy of antibodies against *Plasmodium falciparum* antigens in humans. However, such studies have often yielded inconsistent results with some studies demonstrating a protective role for antibodies to a specific antigen, while others do not [4]. One important reason for this may be the lack of a standardized approach to the reporting of antibody concentrations, and the methods used for their analysis [4,5]. While there is reasonable agreement that high levels of antibodies are better indicators of protection than sero-positivity, the definition of "high" varies considerably between studies [5–10], making it difficult to compare findings from different sites.

Here, we investigated why antibodies appeared to be protective in some settings but not in others. Specifically, we tested the hypothesis that a threshold concentration of antibody was required for protection and that in some settings, although antibodies were present, their concentrations were below the thresholds required for protection. Quantitative correlates of protection have been reported for vaccine-induced antibodies against many infectious diseases [11]. For malaria, although antibodies to several specific antigens have been shown to correlate with protection from clinical episodes of malaria [4], similar quantitative correlates have not

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yet been defined. In one study, the concept of an antigen-specific threshold concentration of antibodies that correlated best with protection against *P. falciparum* infection was explored [5] but not applied in subsequent studies [12,13]. In the study reported here, we develop this concept by using data from one cohort to identify "protective thresholds", defined as the antibody concentrations against specific antigens that best correlate with protection from clinical episodes of malaria. We subsequently tested the validity of these thresholds in an independent cohort.

Our previous studies have shown that antibodies to specific merozoite antigens were associated with protection from clinical episodes of malaria in the Chonyi cohort [6,14–16]. In subsequent studies conducted in the same geographical area along the Kenyan coast, but during a period of moderate transmission, antibodies to the same panel of merozoite antigens were not associated with protection (data presented here). We used a purified IgG preparation as a reference reagent to standardize the measurement of antibody concentrations in both cohorts, and statistical methods to determine the relative IgG concentrations against each antigen that best correlated with protection in the Chonyi cohort. We show that antibody concentrations in the moderate transmission cohort were below the thresholds required for protection.

2. Materials and methods

2.1. Study populations

2.1.1. Chonyi cohort

The Chonyi cohort in Kilifi, Kenya, has been extensively studied [6,14–22]. The parasite prevalence rate in children aged 2–10 years ($PfPR_{2-10}$) [23] was 44% at the time of the study. For this report we analyzed 286 serum samples collected in October 2000 at the start of a malaria transmission season from children aged 0–10 years. These children were subsequently followed up for 6 months for clinical episodes of malaria. In this area, the age-specific criteria for defining clinical episodes of malaria are established and are as follows: for children <1 year old, a temperature of >37.5 °C plus any parasitaemia; for children >1 year old, a temperature of >37.5 °C plus a parasitaemia of >2500/μl [20]. Clinical episodes of malaria were monitored by both active and passive case detection. Trained field workers visited the participants every week whereby children with fever (axilliary temperature >37.5 °C) had a blood slide taken. Children with a positive test result were treated with antimalarial drugs. In addition, parents were advised to report to a dedicated outpatient clinic at Kilifi District Hospital if their child developed symptoms of disease at any time.

2.1.2. Junju cohort

Children aged 1–6 years in a second independent group, the Junju cohort, were originally recruited in 2005 [24] and have been followed up as above, for clinical episodes of malaria [20,24]. In addition, trained field workers were available in the village to conduct passive surveillance. Children born into study households are continuously recruited into the cohort. Peak malaria transmission occurs during the rainy months of May–July and November to December. Blood samples are collected annually during a cross-sectional survey conducted at the beginning of the malaria transmission season in May. The $PfPR_{2-10}$ in Junju was 29% at the time of sampling. Participants in the Junju cohort area live approximately 25 km away from those described above in the Chonyi cohort. We analyzed 304 serum samples collected in May 2008 from children aged 1–12 years. Data on 6 months of follow up in the subsequent malaria transmission season are presented here.

The Kenyan national scientific and ethics committees reviewed and approved the studies.

2.2. Recombinant P. falciparum merozoite antigens

All antigens are based on *P. falciparum* and include the 19 Kilo-Dalton C-terminal fragment of merozoite surface protein (MSP)-1 of the Wellcome parasite line [25], full-length recombinant apical membrane antigen (AMA)-1 of the HB3 parasite line [26], MSP-2 of the Dd2 parasite line [27] and MSP-3 of the 3D7 allelic type [6,28]. Responses to two fragments of Glutamate-rich protein (GLURP) representing the N-terminal non-repeat region (GLURP-R0) and C-terminal repeat region (GLURP-R2) were also analyzed [29].

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum IgG responses to individual antigens were measured using a well-established standard ELISA protocol [6,14–16]. Additionally, a purified IgG standard was incorporated into the assay and allowed for the extrapolation of relative antibody concentrations. Eleven two-fold serial dilutions of a reference Malaria Immune Globulin (MIG) reagent (Central Laboratory Blood Transfusion Service SRC, Switzerland) [30] were included for every antigen to generate a standard ELISA curve. This preparation contains 50 mg/ml of immunoglobulins (98% IgG) purified from a pool of healthy Malawian adult plasma and was originally manufactured to test its potential use as an adjunct therapy to quinine in the treatment of cerebral malaria [30]. The four-parameter logistic function was used to fit the standard curve in GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). ELISA OD values of test samples were converted into relative antibody concentrations using parameters estimated from the standard curve, assuming the purified IgG preparation contained 50 arbitrary units (AU) of antigen-specific antibodies. A pool of sera from Kilifi adults was included on every plate as a positive control. Sera from twenty Europeans served as negative controls to determine cutoff values for seropositivity, defined as mean optical density plus three standard deviations. Responses to AMA1, MSP-2, and MSP-3 were measured in both cohorts whereas those to $MSP\text{-}1_{19}$ and GLURP were measured in the Chonyi cohort only.

2.4. Statistical analysis

Data analysis was performed using Stata 11 (StatCorp, TX). A modified Poisson regression model was used as previously described [6] to examine the effects of individual antibodies on the outcome, defined as a clinical episode of malaria during 6 months of follow-up. Age and reactivity to parasite schizont extract were fitted as covariates in multivariate analyses, to minimize confounding by parasite exposure. Age was fitted as a categorical variable (age bands of 0–3, 4–5, 6–7, 8–12 years), while reactivity to schizont was fitted as a continuous variable. This model was used for all the analyses described below.

We used data from all children in the Chonyi cohort to estimate the relative concentration of antibodies that best correlated with clinical protection for each antigen as follows: (i) different antibody concentrations were applied as cutoffs for high versus low responders over a range of increasing concentrations up to the maximum concentration recorded against each antigen, (ii) a modified Poisson regression model was used to calculate the risk ratio at each cutoff value, (iii) the best fitting model was selected using the log pseudolikelihood [31]. The antibody concentrations that resulted in the best fitting models were designated as "protective thresholds". The protective thresholds were then used in two ways (i) to compare age-matched antibody levels in the Chonyi versus Junju cohorts and (ii) as cutoffs, comparing the clinical outcome of children with levels above, versus below the threshold, for each antigen in the Junju cohort.