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Breadth and Magnitude of Antibody Responses to Multiple *Plasmodium falciparum* Merozoite Antigens Are Associated with Protection from Clinical Malaria[▽]†

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Individuals living in areas where malaria is endemic are repeatedly exposed to many different malaria parasite antigens. Studies on naturally acquired antibody-mediated immunity to clinical malaria have largely focused on the presence of responses to individual antigens and their associations with decreased morbidity. We hypothesized that the breadth (number of important targets to which antibodies were made) and magnitude (antibody level measured in a random serum sample) of the antibody response were important predictors of protection from clinical malaria. We analyzed naturally acquired antibodies to five leading *Plasmodium falciparum* merozoite-stage vaccine candidate antigens, and schizont extract, in Kenyan children monitored for uncomplicated malaria for 6 months ($n = 119$). Serum antibody levels to apical membrane antigen 1 (AMA1) and merozoite surface protein antigens (MSP-1 block 2, MSP-2, and MSP-3) were inversely related to the probability of developing malaria, but levels to MSP-1_{19} and erythrocyte binding antigen (EBA-175) were not. The risk of malaria was also inversely associated with increasing breadth of antibody specificities, with none of the children who simultaneously had high antibody levels to five or more antigens experiencing a clinical episode (17/119; 15%; $P = 0.0006$). Particular combinations of antibodies (AMA1, MSP-2, and MSP-3) were more strongly predictive of protection than others. The results were validated in a larger, separate case-control study whose end point was malaria severe enough to warrant hospital admission ($n = 387$). These findings suggest that under natural exposure, immunity to malaria may result from high titers antibodies to multiple antigenic targets and support the idea of testing combination blood-stage vaccines optimized to induce similar antibody profiles.

While large populations of the world are at risk of malaria (30, 62), the brunt of mortality caused by *Plasmodium falciparum* continues to be borne by children in sub-Saharan Africa. It is estimated that in this region alone, nearly 1 million children under the age of 5 years died as a direct consequence of malaria in the year 2000 (59). An effective vaccine is urgently needed but has proved challenging to obtain. In endemic areas, older children and adults develop naturally acquired immunity to severe and life-threatening malaria but remain susceptible to infection (37). Classical experiments in which passively transferred antibodies from immune adults were successfully used to treat children with severe *P. falciparum* malaria (14, 40) provide the strongest evidence that antibodies are important mediators of naturally acquired immunity. Clinical symptoms of malaria result from the asexual blood stage of the infection, in which potential antibody targets include merozoite antigens involved in invasion (18) and parasite-derived surface antigens on infected erythrocytes (8).

Studies on protective immunity to malaria involve monitoring subjects in endemic communities for variable durations of time to measure the incidence of infection or clinical disease. Associations between the presence of a specific immune response to a target antigen and an outcome determine whether an immune response to the specific antigen appears to be "protective." These immuno-epidemiological studies have often provided conflicting data, with responses to the same antigen appearing to be protective in some studies but not in others (2, 5, 10, 15, 21, 24, 31, 52, 57). Most antibody-based analyses of protection are tethered on seropositivity (usually

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defined as the mean plus 3 standard deviations of non-malaria-exposed sera) and do not take into account the continuous, quantitative nature of antibody concentrations. Furthermore, the majority of studies have concentrated on associations between responses to a single or a limited number of antigens and protection from clinical malaria, despite the fact that individuals living in endemic areas are simultaneously and repeatedly challenged with numerous malaria antigens. Few studies have examined the interactions between specific antibody responses against multiple malaria antigens (32, 41) and whether these might be synergistic, antagonistic, or neither with regards to protection.

To test whether either the number of important target antigens to which antibodies are made or the levels of such antibodies in serum are associated with protection from malaria, we analyzed naturally acquired antibodies to five leading *P. falciparum* merozoite-stage vaccine candidate antigens (apical membrane antigen 1 [AMA1], merozoite surface proteins 1, 2, and 3 [MSP-1, MSP-2, and MSP-3], and erythrocyte binding antigen [EBA-175]), as well as *P. falciparum* schizont extract, in a cohort of Kenyan children who were monitored longitudinally for mild (uncomplicated) clinical malaria (Chonyi cohort). We also examined combinations of, and interactions between, antigen-specific antibodies to determine the combination(s) that predicted the strongest protection from clinical malaria. These antigens were selected for study because of the cumulative evidence that the presence of antibodies to these antigens may be associated with protection (10, 15, 42, 50, 53–56, 65, 66), backed by evidence that polymorphisms in their sequences are maintained by natural selection (16), and their biological plausibility (3, 13, 20, 25, 36, 46, 60). The analytical approaches were developed using data from the Chonyi cohort, and the methods subsequently were validated in an independent case-control study whose end point was malaria severe enough to require admission to hospital.

MATERIALS AND METHODS

Cohort study. This study was conducted in Kilifi, a rural district along the Kenyan coast. Details of the study area and population have been published elsewhere (44), along with a description of a cohort comprising both adults and children from Chonyi village in Kilifi. This area typically experiences two seasonal peaks in malaria transmission (June to August and November to December) and has an average annual entomological inoculation rate of approximately 20 to 100 infective bites/person/year (38). Following a cross-sectional bleed at the start of a malaria transmission season in October 2000, the cohort was followed for clinical episodes of malaria in the ensuing 6 months. Asymptomatic parasitemias were not subjected to drug clearance at the beginning of the study or during follow-up. Active detection of mild clinical malaria (outcome) was achieved through weekly visits to participants' homes, where a morbidity questionnaire (investigating symptoms occurring in the preceding week) was administered and the presence or absence of fever was ascertained (axillary temperature of >37.5°C). Participants found to be unwell were referred for free treatment to a dedicated outpatient clinic at the local district hospital, where they also had open access as required at any time during the study (passive case detection). Clinical episodes of malaria were treated with sulfadoxine-pyrimethamine, as per the Kenyan national treatment guidelines at the time the study was conducted. Age-specific criteria for defining clinical episodes of malaria were developed previously for this area as follows: children under 1 year, fever plus any parasitemia; children older than 1 year, fever plus a parasitemia of greater than 2,500/μl (44). Participants were only included in the study if they were present for all but three of the weekly visits during the 6 months of follow-up. For analytical purposes, only the first clinical episode was counted, although all children continued to be monitored until the close of the study. Within the cohort, children aged 10 years and less (n = 280) accounted for nearly 90% of all the clinical episodes. The current analysis focused on children who were slide positive (they were all asymptomatic) at the time the cross-sectional blood sample was collected (n = 119; details are provided below in the description of the statistical analysis). Data on antibody responses to AMA1, MSP-2, and MSP-3 were previously published (50, 53, 54), while data on the remaining antigens (EBA-175, MSP-1_{19}, and MSP-1 block 2) are reported here (see Fig. S1 in the supplemental material).

Case-control study. The findings in the Chonyi cohort were validated in a separate, spatially and temporally distinct group of children investigated in an analysis of parasite antigens on the infected red blood cell surface that was previously conducted in Kilifi (7). Children were recruited from an area immediately surrounding the administrative town of Kilifi, with an entomological inoculation rate of approximately 1.5 to 8 bites/person/year (39). In May 1995, a cross-sectional survey was carried out during which finger prick blood samples were collected from 4,783 children (aged 1 to 5 years), from which sera were separated (stored at −70°C) and thick blood smears were prepared and examined for parasites. Over the following 8 months, children who were part of this survey and presented to hospital with malaria that was severe enough to warrant pediatric ward admission were identified (passive case detection). A total of 89 children from the survey were admitted during that period and were frequency matched, allowing for age and location of children (n = 298) who took part in the cross-sectional survey but did not present to hospital with malaria. Sera from the cross-sectional survey for cases and controls (n = 387) were analyzed for immunoglobulin G (IgG) antibodies to AMA1, MSP-2, MSP-3, EBA-175, MSP-1 block 2, MSP-1_{19}, and *P. falciparum* schizont extract. Ethical approval was granted by the Kenya National Research Ethics Committee.

Recombinant antigens. The recombinant antigens were expressed in *Escherichia coli* as glutathione *S*-transferase–fusion proteins (MSP-2_Dd2 and MSP-2_CH150/9) (66), MSP-1 block 2 (RO33, Palo Alto, 3D7, MAD20, and Wellcome) (12), and MSP-1_{19} (9), His-tagged (AMA1_3D7) (23) and EBA-175_F2_CAMP (51), or as maltose-binding protein–fusion proteins (MSP-3_K1 and MSP-3_3D7 (56). Recombinant AMA1_FVO (35) was expressed in *Pichia pastoris*, while EBA-175_F2_3D7 (19) is a baculovirus-expressed product. Details on these antigens are provided in Table S1 of the supplemental material.

Antibody assays. Enzyme-linked immunosorbent assays (ELISAs) against each recombinant antigen and against parasite schizont extract were performed according to a standard protocol as previously described (50, 53, 54). Individual wells of Dynex Immunolon 4HBX ELISA plates (Dynex Technologies Inc.) were coated with 50 ng of antigen per 100 μl of carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.3). Wells were coated with *P. falciparum* schizont extract (the A4 strain for the Chonyi cohort and Wellcome strain for the hospital cohort) in phosphate-buffered saline (PBS) according to the method of Ndungu et al. (45). Plates were incubated overnight at 4°C before washing four times in PBS-Tween (PBS–0.05% Tween 20) and blocking for 5 h at room temperature with 1% skimmed milk in PBS-Tween (blocking buffer). Following this, wells were washed again and incubated overnight at 4°C with 100 μl of test serum (1/1,000 dilution in blocking buffer). Plates were then washed four times and incubated for 3 h at room temperature with 100 μl of horseradish peroxidase-conjugated rabbit anti-human IgG (Dako Ltd.) at a 1/5,000 dilution in blocking buffer before final washing and detection with H_2O_2 and *o*-phenylenediamine (Sigma). The reaction was stopped with 25 μl of 2 M H_2SO_4 per well, and absorbance was read at 492 nm. The same positive controls (hyperimmune sera) were run in duplicate on each day of the experiment, on each plate, to allow for standardization of day-to-day and plate-to-plate variations. Single-dilution serum ELISA optical density (OD) values were used as proxies for antibody titers, as they correlate closely with full endpoint antibody titrations when used at appropriate dilutions (22, 67).

Statistical analysis. All data analyses were performed with STATA version 9.2 (StataCorp, College Station, TX). Models were first developed using data from the Chonyi cohort and subsequently validated in the case-control study with some modifications (below). The primary analysis was on the subgroup of 119 children from the Chonyi cohort (n = 280) who were asymptomatically parasitized at the time of serum collection in October 2000, because in previous analyses, *P. falciparum* parasitemia at the time of serum collection modified the effects of antibodies to both variant red blood cell surface (6) and merozoite (50, 53, 54) antigens on the risk of disease. The confounding effects of exposure on antibody responses were controlled for by adjusting both for age as well as antibody reactivity to parasite schizont extract in multifactorial analyses.

The probability of a clinical episode for each antigen (and each allelic form) for given antibody levels was estimated by logistic regression, fitting ELISA OD values for the antigen as a linear covariate and adjusting for age (in 2-year categories). The logits from these models were converted into probabilities (43) to give estimates of risk (see Fig. 1, below). These analyses established that for

TABLE 1. Protective effects[a] of high levels of antibodies to individual antigens

Antigen	% of children with high-titer response	Univariate analysis[b]		Age-adjusted analysis[b]		Age and schizont adjusted[b]	
		Risk ratio (95% CI)	P value	Risk ratio (95% CI)	P value	Risk ratio (95% CI)	P value
AMA1_FVO	49	0.45 (0.25–0.80)	0.007*	0.60 (0.33–1.08)	0.093	0.65 (0.36–1.21)	0.178
AMA1_3D7	51	0.40 (0.22–0.72)	0.002*	0.50 (0.28–0.90)	0.021*	0.54 (0.29–1.00)	0.052
MSP-2_CH150/9	54	0.32 (0.17–0.59)	0.000*	0.39 (0.21–0.70)	0.002*	0.41 (0.22–0.74)	0.004*
MSP-2_Dd2	54	0.28 (0.15–0.53)	0.000*	0.35 (0.18–0.65)	0.001*	0.36 (0.19–0.70)	0.003*
MSP-3_K1	39	0.39 (0.20–0.78)	0.008*	0.50 (0.26–0.95)	0.037*	0.52 (0.27–1.01)	0.055
MSP-3_3D7	40	0.58 (0.32–1.04)	0.072	0.67 (0.38–1.17)	0.166	0.70 (0.40–1.22)	0.216
EBA-175_F2_CAMP	34	0.72 (0.40–1.29)	0.274	1.05 (0.60–1.82)	0.858	1.25 (0.71–2.19)	0.437
EBA-175_F2_3D7	41	0.41 (0.21–0.79)	0.008*	0.53 (0.27–1.04)	0.067	0.57 (0.29–1.14)	0.114
MSP-1_B2_3D7	20	0.43 (0.12–1.12)	0.085	0.56 (0.20–1.50)	0.252	0.60 (0.22–1.64)	0.328
MSP-1_B2_PaloAlto	18	0.93 (0.47–1.83)	0.846	0.91 (0.48–1.71)	0.774	0.95 (0.50–1.81)	0.895
MSP-1_B2_Wellcome	19	0.33 (0.11–1.00)	0.051	0.50 (0.16–1.51)	0.222	0.54 (0.18–1.65)	0.286
MSP-1_B2_MAD20	23	0.48 (0.21–1.12)	0.092	0.73 (0.32–1.70)	0.478	0.76 (0.33–1.78)	0.543
MSP-1_B2_RO33	15	1.40 (0.77–2.53)	0.263	1.20 (0.74–1.93)	0.443	1.43 (0.86–2.38)	0.162
MSP-1_{19}	36	1.44 (0.87–2.38)	0.148	1.14 (0.74–1.76)	0.544	1.59 (0.93–2.74)	0.089

[a] Risk of developing clinical malaria associated with high titers compared to low/undetectable titers of antibodies to individual antigens in a subset of the Chonyi cohort (n = 119). Antigens are designated by their locus name and *P. falciparum* strain ([locus]_[strain]).

[b] Risk ratios (with 95% confidence intervals [CI]) are presented for univariate and multivariate analyses (adjusted initially for age and subsequently for both age and reactivity to *P. falciparum* parasite schizont extract as a proxy for exposure). *, $P < 0.05$.

most antigens and antibodies, higher antibody levels were associated with a lower risk of disease and that allelic versions of the same antigen (or the same allelic family for MSP-1 block 2) generally gave similar patterns of protection. The probability plots were used to define a threshold (cutoff) for high versus low/undetectable antibodies as the OD level above which the risk of disease was lower than the population's average risk of 33.6% (i.e., the risk of disease assuming no role for any antibodies) (see Fig. 1, below; see also Table S2 in the supplemental material). The suitability of the logistic model was confirmed by examining the residuals when the OD data were fitted in quintiles (data not shown). The individual effects of high levels of each antibody on the risk of disease were then reanalyzed, fitting antibody level as a factor rather than as a linear covariate (Table 1) for ease of interpretation and to facilitate analyses of the breadth and the interactions between antibodies. To avoid the lack of convergence commonly encountered in conventional binomial regression analyses, data were fitted to a modified Poisson regression model with robust error variance, which tends to provide conservative results (69).

Antibodies to different allelic forms of most antigens (AMA1, MSP-2, MSP-3, and the F2 subdomain of EBA-175) and to the main allelic types of MSP-1 block 2 (K1 and MAD20 types) were highly correlated (see Table S3 in the supplemental material). Consequently, high levels of antibodies to only one allelic form of each antigen were considered for the analysis of antibodies to multiple antigens. Antibodies to MSP-1 block 2 (MSP-1_B2) were highly correlated only within the main allelic families, and so for this antigen, antibodies to one antigen from each of the three main allelic families (*MAD20*-like, *K1*-like, and *RO33*-like) were included to give an overall MSP-1 block 2 response (any of MSP-1_B2_Wellcome, MSP-1_B2_3D7, or MSP-1_B2_RO33). The criteria used to select antibodies to a single allelic form of each antigen have been outlined (see text in the supplemental material). The antigens retained for further analyses (breadth and combined responses) were AMA1_3D7, MSP-2_Dd2, MSP-3_K1, EBA-175_3D7, MSP-1_{19}, and MSP-1 block 2 (overall response).

Breadth was analyzed in an age- and schizont extract-adjusted modified Poisson regression model that compared the risk of disease among children who had high levels of antibodies (fitted as a fixed level factor) to between one and six antigens to those who had low/undetectable antibodies to all six antigens. The combination of antibodies that was associated with the lowest risk of clinical malaria was determined by analyzing all pair-wise combinations, investigating interactions between antigens by fitting a model with two main effects and an interaction term. Interaction as presented here refers to statistical interaction where the estimate of risk obtained for antibodies to two antigens is significantly lower than expected (i.e., lower than the product of the individual risk ratios). It does not exclude biological interaction. To make certain that we were not simply measuring correlated antibodies arising from shared exposure, we separately included antibodies to all antigens in a single regression model, together with age and reactivity to schizont extract, dropping each out sequentially in decreasing order of their P values. Antigens that remained significant in this model at the $P < 0.10$ level were MSP-2, MSP-3, and AMA1.

Data on children from the hospital cohort were analyzed essentially as described above with minor modifications. Models were fitted to data from the entire hospital cohort (not only the subgroup that were parasitemic at the time of serum collection) because it appeared that frequency matching of cases and controls for location (and therefore exposure) successfully eliminated the interaction between the antibody's protective effect and parasite infection status.

RESULTS

Magnitude of antibody response and protection. The probability of developing an episode of clinical malaria for a given value of measured antibody level (OD) was estimated for each antigen. We found that the levels of serum antibodies to some, but not all, vaccine candidate antigens were inversely related to the probability of developing malaria (Fig. 1). Increasing OD levels to MSP-2, MSP-3, AMA1, and the *MAD20*-like antigens of MSP-1 block 2 (denoted Wellcome and MAD20) were associated with reduced probability of malaria morbidity, while those to MSP-1_{19}, EBA-175, and the *K1*- and *RO33*-like antigens of MSP-1 block 2 had little effect. Within these loci (and within the main allelic families for MSP-1 block 2), the patterns were similar for the different allelic forms. Increased antibody titers to whole parasite schizont extract were also associated with a reduced probability of clinical malaria.

Breadth and protective efficacy of antibody response. The probability plots (Fig. 1) were used to define a threshold (cutoff) for high versus low/undetectable antibodies for each antigen. This threshold varied both by antigen and by population (the Chonyi cohort and the case-control study), ranging from relatively low OD values for the MSP-1 block 2 antigens to high values for MSP-2 (see Table S2 in the supplemental material). Children who had high levels of antibodies to one, two, three, four, five, or six unrelated (nonallelic) antigens were compared with those who did not have high levels to any antigen to test the hypothesis that the breadth of specificities for unrelated antigens in the antibody response is important for protection. The risk of malaria was inversely associated with increasing breadth of antibody specificities in both study groups (Fig. 2). None of the children in the Chonyi cohort who had high-titer antibody responses to five or more antigens

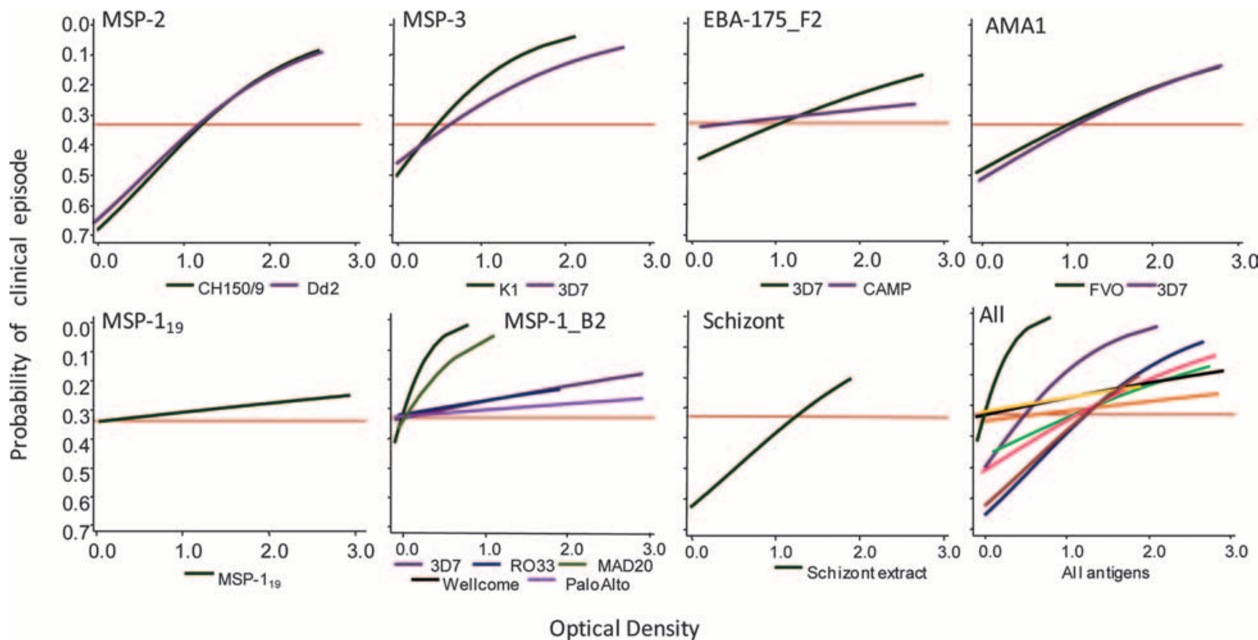

FIG. 1. The predicted probability of an episode of malaria in children decreases with increasing antibody titer for most antigens ($n = 119$). Each panel represents the allelic antigens tested at each locus, as well as parasite schizont extract. The red horizontal line represents the risk of an episode without taking antibody responses to any antigen into account. The final panel combines antibodies to one allelic form of each antigen (and one antigen from each of the three main allelic families of MSP-1 block 2). The lines, from top to bottom, represent MSP-1_B2_Wellcome, MSP-3_K1, MSP-2_Dd2, schizont extract, AMA1_3D7, EBA-175_F2_3D7, MSP-1_B2_3D7, MSP-1_B2_RO33, and MSP-1_{19}.

(17/119; 15%) experienced a clinical episode ($P = 0.0006$ by Fisher's exact two-tailed test). Similarly, in the case-control study, none of the children who had high-titer responses to five or more antigens (23/298; 7.7%) was admitted to hospital with severe malaria ($P = 0.004$, Fisher's exact two-tailed test).

Breadth of antibody specificity increases with age and concurrent parasitemia. The breadth of high-titer antigen-specific responses increased with age in both groups of children (Fig. 3). Parasite positivity at the time of serum collection significantly increased the breadth of the response. In the Chonyi cohort, nearly three times as many children who were parasitemic at the time of serum collection had high antibody titers to three or more antigens, compared to those who were aparasitemic (47% [56/119] versus 17.3% [28/161]; Pearson's chi-square, 28.67; $P < 0.001$). This difference was more marked in the case-control study, with over five times as many children who were parasitemic at serum sampling having high-titer responses to three or more antigens compared to those who were not parasitemic (57% [102/176] versus 10.4% [30/287]; Pearson's chi-square, 120.77; $P < 0.001$).

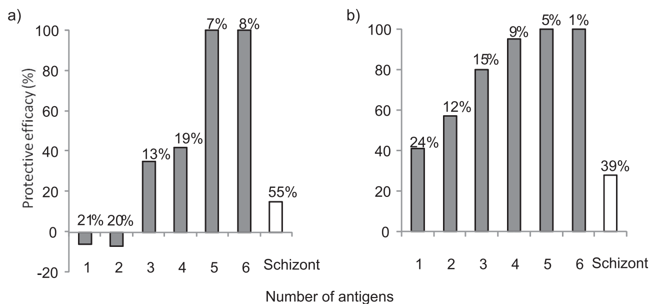

FIG. 2. Protective efficacy increased with increasing breadth of response in children from the Chonyi (parasitemic children, $n = 119$) (a) and hospital cohorts (all children, $n = 387$) (b). Each bar represents the comparison between individuals making high-titer responses to n number of antigens with those who make no responses to any antigen. Proportions above each bar are the percentage of individuals making high-titer responses to n antigens. The effect of high-titer responses to *P. falciparum* schizont extract is also shown.

Combinations of antibodies and protection. Interactions between antibodies were investigated to determine which combination(s) was associated with the lowest risk of clinical episodes in the Chonyi cohort. High levels of antibodies to combinations that included MSP-2, MSP-3, and AMA1 were associated with a lower risk of disease compared to their individual effects (Table 2). While the combined effects of antibodies were always greater than each of the individual effects, there was no statistical evidence of synergism or antagonism, i.e., more or less protection, respectively, than expected from the combination of the two antigens acting additively. The strongest protection was associated with high levels of antibodies to both MSP-2 and MSP-3. Thirty-three children (of 119) had high antibody levels to both MSP-2 and MSP-3, and none of them experienced any episodes of disease ($P < 0.001$ by Fisher's exact two-tailed test; still highly significant after a Bonferroni correction [4], allowing for multiple comparisons, $P = 0.003$). This finding was validated in the case-control study, where admission to hospital with malaria was the end point. Children who had high levels of antibodies to both MSP-2 and MSP-3 were significantly less likely to be admitted to hospital with malaria (odds ratio, 0.26; 95% confidence interval, 0.08 to 0.81; $P = 0.020$) (see Table S5 in the supplemental material).

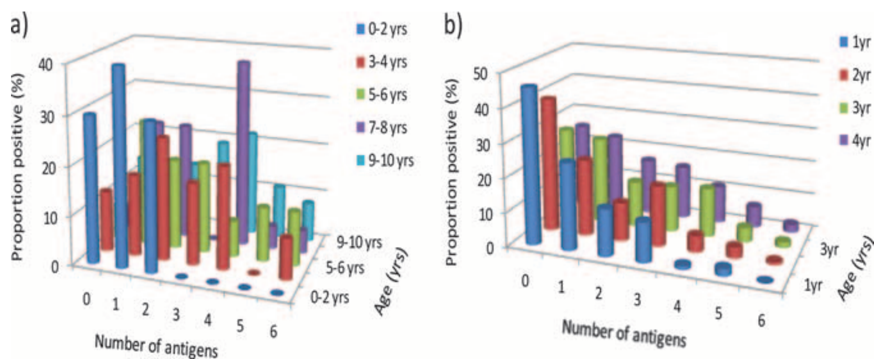

FIG. 3. The breadth of antibody specificity increased with age in both the Chonyi cohort ($n = 119$) (a) and the case-control study ($n = 387$) (b).

DISCUSSION

We found that in two independent studies conducted in both high-transmission (Chonyi cohort) and low-transmission (case-control study) settings at different times, both the breadth of specificity for distinct merozoite antigens and the magnitude of antibody responses to these antigens provide robust predictors of the immune status of children. High titers of antibodies to combinations of three merozoite antigens in particular (AMA1, MSP-2, and MSP-3) were more strongly predictive of protection from clinical episodes of malaria compared to other putative "protective" merozoite antigens (MSP-1 or EBA-175).

Out of the panel of malaria vaccine candidate antigens studied here, high levels of antibodies to combinations that included AMA1, MSP-2, and MSP-3 were the most strongly associated with protection. This is consistent with other studies in which naturally acquired antibodies to each of the three antigens individually have been associated with protection from clinical malaria in this and other populations (1, 41, 42, 53, 54, 61, 63, 65, 66). Recently, long-term clinical protection was associated with IgG3 isotype antibodies to MSP-3 in Senegalese children (58). In contrast, antibodies to MSP-1 block 2, which have been associated with protection in two cohorts in West Africa (10, 15, 55), were not similarly protective in the two cohorts we studied from Kilifi, Kenya. Antibodies to MSP-1_{19} have been associated with protection from clinical malaria in some studies, but not in others (5, 10, 15, 21, 24, 31, 52, 57). This may be explained in part by the finding that the fine specificity of anti-MSP-1_{19} antibodies appears to be more important with regards to protection (17, 48). A separate study found that individuals with high-titer anti-MSP-1_{19}-specific invasion-inhibitory antibodies were protected from infection (33) and underscored the importance of developing robust functional assays for malaria. Antibodies to the F2 subdomain of EBA-175 were not associated with protection from clinical disease in our studies, as has been found in other parts of Africa where this (49) and other subdomains of EBA-175 (32, 47, 49) have been studied. To date, only one study has reported significantly higher antibody levels to EBA-175 peptide 4 (residues 1062 to 1103, within region V) in children protected from clinical attacks of malaria compared to susceptible children (68).

The importance of allele-specific immunity was highlighted in the Combination B malaria vaccine trial in Papua New Guinea. Children who received this vaccine (containing a combination of *P. falciparum* ring-infected erythrocyte surface antigen, MSP-1, and the 3D7 allele of MSP-2) were less likely to be infected with parasites bearing the homologous allele of MSP-2 (28), suggesting (as was later confirmed) that the vaccine had induced primarily allele-specific MSP-2 antibodies (26). In the context of naturally acquired infections, while some data suggest that parasites bearing specific genotypes induce allele-specific antibodies (11, 34, 53), to our knowledge no studies have examined the protective effects of preexisting allele-specific antibodies on subsequent disease caused by parasites bearing homologous alleles. We found that for most antigens tested, responses to allelic forms of each antigen had similar effects on the probability of mild or more severe malaria, suggesting the possibility that there may be significant cross-allele protection to clinical episodes.

In a study conducted in The Gambia, Gray et al. (29) found that while antibodies to a similar panel of individual antigens were only weakly correlated with protection, those to the combinations of AMA1 and MSP-2 were significantly associated with protection from clinical malaria. There are two important differences between this Gambian study and the results reported here from Kenya. First, *k*-means clustering and phylogenetic networks were used to investigate associations between antibody reactivity profiles and clinical status in the Gambian cohort. These methods independently identified the group of children who were asymptomatic (asymptomatic parasitemia, splenomegaly, or both) at the end of the study and who had not apparently experienced clinical disease. That end point differs from that of the studies reported here, in which outcome was simply defined as mild (Chonyi cohort) or severe (case-control study) malaria during the period of observation. Second, the magnitude of responses was not taken into account, mainly because this generates increased individual differences, impairing cluster analysis. One other longitudinal study, carried out among children in Burkina Faso, examined antibodies to a different set of blood-stage malaria antigens (glutamate-rich protein, *P. falciparum* exported protein 1, and MSP-3) and, like our studies, they found that the simultaneous presence of antibodies to more than one antigen was associated with a lower frequency of malaria episodes (41). However, in a separate study on protection from malaria infection as opposed to clin-

TABLE 2. Protective effects[a] of combinations of high-titer antibody responses

Antigen	Main effects and interaction[b]		Combination effect[c]	
	Risk ratio (95% CI)[d]	*P* value	Risk ratio (95% CI)[d]	*P* value
AMA1_3D7	0.59 (0.32–1.08)	0.091	0.21 (0.05–0.88)	0.033*
MSP-3_K1	0.56 (0.29–1.08)	0.085		
AMA1_3D7 + MSP-3_K1	0.28 (0.06–1.29)	0.104		
AMA1_3D7	0.66 (0.35–1.25)	0.211	0.24 (0.09–0.64)	0.004*
MSP-2_Dd2	0.40 (0.20–0.78)	0.008*		
AMA1_3D7 + MSP-2_Dd2	0.33 (0.09–1.17)	0.087		
AMA1_3D7	0.62 (0.32–1.18)	0.147	0.61 (0.29–1.29)	0.199
EBA-175_F2_3D7	0.69 (0.33–1.42)	0.320		
AMA1_3D7 + EBA-175_F2_3D7	1.90 (0.34–10.46)	0.459		
AMA1_3D7	0.58 (0.31–1.08)	0.088	0.48 (0.17–1.38)	0.178
MSP-1_{19}	1.44 (0.82–2.54)	0.199		
AMA1_3D7 + MSP-1_{19}	0.39 (0.12–1.30)	0.129		
MSP-2_Dd2	0.40 (0.20–0.78)	0.008*	Total protection ($n = 33$)	
MSP-3_K1	0.62 (0.34–1.13)	0.121		
MSP-2_Dd2 + MSP-3_K1	Total protection ($n = 33$)			
MSP-2_Dd2	0.38 (0.20–0.73)	0.004*	0.17 (0.04–0.73)	0.017*
EBA-175_F2_3D7	0.64 (0.33–1.22)	0.178		
MSP-2_Dd2 + EBA-175_F2_3D7	0.26 (0.05–1.27)	0.098		
MSP-2_Dd2	0.36 (0.18–0.71)	0.003*	0.52 (0.21–1.30)	0.166
MSP-1_{19}	1.55 (0.92–2.61)	0.097		
MSP-2_Dd2 + MSP-1_{19}	0.62 (0.20–1.88)	0.402		
MSP-3_K1	0.57 (0.29–1.11)	0.101	0.39 (0.12–1.20)	0.103
EBA-175_F2_3D7	0.66 (0.33–1.31)	0.240		
MSP-3_K1 + EBA-175_F2_3D7	0.71 (0.17–2.97)	0.643		
MSP-3_K1	0.48 (0.25–0.93)	0.030*	0.57 (0.25–1.31)	0.191
MSP-1_{19}	1.74 (1.07–2.84)	0.024		
MSP-3_K1 + MSP-1_{19}	0.65 (0.19–2.27)	0.509		
EBA-175_F2_3D7	0.56 (0.28–1.11)	0.101	0.69 (0.26–1.78)	0.445
MSP-1_{19}	1.61 (0.97–2.68)	0.062		
EBA-175_F2_3D7 + MSP-1_{19}	0.70 (0.19–2.52)	0.586		
AMA1_3D7	0.55 (0.30–1.01)	0.055	0.66 (0.29–1.52)	0.336
MSP-1_B2	0.87 (0.51–1.50)	0.636		
AMA1_3D7 + MSP-1_B2	1.13 (0.36–3.56)	0.829		
MSP-2_Dd2	0.36 (0.18–0.70)	0.008*	0.40 (0.15–1.05)	0.064
MSP-1_B2	1.04 (0.62–1.72)	0.875		
MSP-2_Dd2 + MSP-1_B2	0.57 (0.17–1.90)	0.365		
MSP-3_K1	0.52 (0.27–1.02)	0.059	0.16 (0.02–1.13)	0.067
MSP-1_B2	0.87 (0.51–1.49)	0.634		
MSP-3_K1 + MSP-1_B2	0.15 (0.02–1.14)	0.068		
MSP-1_{19}	1.61 (0.95–2.74)	0.073	0.98 (0.48–2.01)	0.972
MSP-1_B2	0.80 (0.45–1.41)	0.456		
MSP-1_{19} + MSP-1_B2	0.83 (0.27–2.46)	0.737		
EBA-175_F2_3D7	0.58 (0.30–1.14)	0.119	0.85 (0.36–2.00)	0.720
MSP-1_B2	0.88 (0.51–1.51)	0.656		
EBA-175_F2_3D7 + MSP-1_B2	2.11 (0.56–7.94)	0.265		

[a] Risk of developing clinical malaria associated with combinations of high titers compared to low/undetectable titers of antibodies to individual antigens in a subset of the Chonyi cohort ($n = 119$). In the majority of cases, significantly more protection was obtained with high-level antibody responses to pairs of antigens, compared to single antigens (Table 1). No strong evidence of statistical interaction between pairs of antibodies was observed.

[b] The main effects of antibodies to each antigen were adjusted for each other. Interaction effects (combinations of two antigens) are those over and above the main effects.

[c] Effects of combinations of high-titer responses (combines the main effects and interaction effects).

[d] Risk ratios (with 95% confidence intervals [CI]) are from multivariate analyses (adjusted for both age and reactivity to *P. falciparum* schizont extract). *, $P < 0.05$.

ical episodes in Kenyan adults, John et al. (32) found that high antibody titers to multiple blood-stage antigens were not protective (though there was evidence of protection for responses to preerythrocytic antigens). Our data suggest that the combination of blood-stage antigens analyzed in these Kenyan adults (AMA1, EBA-175, and MSP-1_{19}) may not have been optimal. While these studies are difficult to compare directly due to differences in study design, study populations and end points, antigens tested, and analytical methodologies, the picture that nevertheless emerges clearly is that antibodies to key combinations of multiple parasite targets are more strongly associated with protection from clinical malaria than are antibodies to individual antigens.

With the completion of the *P. falciparum* genome, numerous new (and old) antigens of the parasite have been identified and are being characterized. High-throughput assays employing suspension array technology (27) or microarrays (29, 64) now allow for simultaneous analysis of antibodies to multiple antigens using minimal amounts of sera. This technology has not been matched with equivalently efficient tools for identifying protective immune responses. Robust concurrent analyses of numerous responses in relatively small studies, where children have been monitored longitudinally over a limited time period for disease episodes, remain challenging. The pair-wise analyses of combinations of high-titer antibody responses as presented here have obvious limitations when numerous antibodies are to be analyzed. Other analytical techniques, such as clustering and the use of phylogenetic networks (29), while attractive for screening of potential vaccine candidates, similarly become more complex when increasing numbers of responses are analyzed and may well obscure genuinely "protective" responses. New strategies to identify protective responses in humans are urgently needed.

Studies of associations between immune responses and clinical malaria need to take into account the possibility that any given response is merely a marker of cumulative exposure (which is itself necessary to induce immunity) or of a response to an as-yet-unidentified antigen(s) that elicits strongly protective immunity. In our study, the fact that antibodies to specific antigens were more strongly predictive of protection than those to whole-schizont extract (containing all the specific antigens and many other blood-stage antigens) (Fig. 1) suggests that specific responses do not merely reflect exposure. The finding that protective efficacy increased with increasing breadth of antibody specificity indicates that the effect of any one apparently protective response does not simply result from correlation with responses to other antigens (Fig. 2) and argues for the interpretation that these are truly protective responses. Ultimately, the critical test of any such hypotheses will be to achieve equivalent protection through vaccination. Our demonstration of strong protection against malaria associated with high antibody levels to AMA1, MSP-2, and MSP-3 lends support to the development of vaccines based on combinations of these key malaria antigens.

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