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Immunoglobulin G Antibodies to Merozoite Surface Antigens Are Associated with Recovery from Chloroquine-Resistant *Plasmodium falciparum* in Gambian Children

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We examined the hypothesis that recovery from uncomplicated malaria in patients carrying drug-resistant *Plasmodium falciparum* is a measure of acquired functional immunity and may therefore be associated with humoral responses to candidate vaccine antigens. Gambian children with malaria were treated with chloroquine in 28-day trials, and recovery was defined primarily as the absence of severe clinical malaria at any time and absence of parasitemia with fever after 3 days. Plasma samples from these children were assayed by enzyme-linked immunosorbent assay for immunoglobulin G (IgG) to recombinant merozoite antigens: apical membrane antigen 1 (AMA-1) and the 19-kDa C-terminal region of merozoite surface protein 1 (MSP-1₁₉), including antigenic variants of MSP-1₁₉, with double and triple substitutions. Antigen-specific IgG was more frequent in children who recovered, particularly that for MSP-1₁₉ (age-adjusted odds ratios: 0.32 [95% confidence interval, 0.05, 1.87; $P = 0.168$] for AMA-1, 0.19 [0.03, 1.11; $P = 0.019$] for recombinant MSP-1₁₉, 0.24 [0.04, 1.31; $P = 0.032$] for the recombinant MSP-1₁₉, double variant, and 0.18 [0.03, 0.97; $P = 0.013$] for the triple variant). IgG titers to MSP-1₁₉, and to the triple variant were higher in plasma samples taken 7 days after chloroquine treatment from children who carried resistant parasites but recovered and remained parasite free. Moreover, in children who were parasitemic on day 14 or day 28, there was an age-independent relationship between parasite density and IgG to both MSP-1₁₉ and the triple variant (coefficients of -0.550 and -0.590 and P values of 0.002 and 0.001, respectively). The results validate the use of this approach to identify antigens that are associated with protection from malaria.

The development of a malaria vaccine is increasingly important in the face of the growing worldwide threat of the disease (29, 35). Since vaccine-induced immune responses are often short-lived, many vaccine strategies aim to elicit protective immune responses that may be boosted by subsequent exposure to natural infection. Such responses are commonly identified by longitudinal cohort studies. Residents of an area where malaria is endemic are sampled for a particular immune response and then monitored for parasite infection or disease over the next few months to determine susceptibility (18, 27, 32, 33). In such studies, functional immunity may be defined as the result of a lower probability of developing clinical malaria, high parasite levels, or infection. Disadvantages of this method include the following. (i) Exposure to infection is an important confounder but is difficult to measure. (ii) The time between baseline sampling and endpoint detection allows immune responses to wax or wane, possibly depending on subclinical or microscopically subpatent infection rates. (iii) The burden of such studies is high in terms of both participant inconvenience and research costs. Alternative ways of identifying individuals with functional protective immunity could greatly improve our ability to evaluate parasite antigens as vaccine candidates.

Three studies have investigated the relationship between

antibody and therapeutic success in malaria patients. In Senegal (34) and Thailand (24), antibody responses to the repetitive circumsporozoite protein antigen and ring erythrocyte surface antigen, respectively, were shown to be more prevalent in patients who recovered than in patients with treatment failure. The third study, in Gabon (23), showed no association between outcome and antibody responses to several recombinant merozoite surface protein 1 (MSP-1) polypeptides, including the conserved 19-kDa C-terminal MSP-1₁₉, although there was an association with antibody to K1 and MAD 20 allelic families of the MSP-1 block 2 region. Although acquired immune responses may be more important in drug-resistant infections, parasite genotypes were not determined. Mutations in the *Pfcr*t and *Pfmdr*1 genes are associated with resistance to chloroquine (CQ), but the *Pfcr*t-76Thr variant appears most strongly associated with CQ resistance in all populations examined so far (9, 36, 38). In addition, in Malawi, the prevalence of the *Pfcr*t-76Thr variant decreased in the parasite population after CQ treatment was discontinued, while that of the *Pfmdr*1-86Tyr variant remained unchanged (28).

In much of West Africa, where CQ monotherapy has been the first-line therapy for uncomplicated malaria, treatment failure rates tend to be lower among older children (10), and a similar tendency is seen even when resistance to the treatment drug is very low (13); these observations may reflect a contribution from acquired immunity. Djimdé and colleagues (11) therefore proposed that treatment response is a phenotype for

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identifying host responses that provide protective immunity against malaria. Elimination of parasites by the immune system requires effectors that target blood-stage asexual parasites, and B cells and antibodies are known to play a key role in protective immunity against these stages, as shown by passive immunization studies with mice and humans (8, 21, 25).

Antibodies may act in a variety of ways, for example by preventing merozoite invasion of erythrocytes, by attacking infected erythrocytes, probably in conjunction with monocytes, or by preventing cytoadhesion of infected erythrocytes (19). Molecules important to all three of these processes have been identified as candidate vaccines, but those involved in merozoite invasion have been the most studied. Several merozoite surface molecules are well characterized (e.g., MSP-1 to -9, erythrocyte binding antigen-175, and apical merozoite antigen 1 [AMA-1]). There is evidence to implicate antibodies from malaria-immune donors in the inhibition of parasite invasion of erythrocytes, especially antibodies specific for MSP-1₁₉ (2, 20) and the ectodomain of AMA-1 (recently reviewed in reference 32). Some cohort studies have shown that the presence of antibody to MSP-1 is associated with lower rates of clinical malaria in the following high-transmission season (1, 15), although other studies (e.g., reference 12) found no such association. Similarly, evidence for a protective role for AMA-1 in vivo had been elusive until a recent cohort study showed an association of antibodies to the ectodomain, with protection from clinical malaria in Kenyans (32). This variation in results may reflect limitations in the cohort method as outlined above or the need for a more precise definition of the antibody. For example, the fine specificity of MSP-1₁₉ antibodies, together with their prevalence and titer level, may better predict protection (30, 31, 39). Some MSP-1₁₉-specific antibodies actually block the binding of protective antibodies, highlighting the need to measure functional antibody (20).

We hypothesized that following drug treatment, antibodies to these merozoite surface molecules play a role in the elimination of *Plasmodium falciparum* in areas where drug resistance is highly prevalent. In 2000 and 2001, pediatric treatment trials for uncomplicated malaria conducted in The Gambia included CQ comparison arms (14, 37). We have used plasma and data from these trials and examined whether antibodies to AMA-1 and MSP-1₁₉ are associated with clinical recovery and elimination of parasites. Three forms of MSP-1₁₉ were used as antigens: a naturally occurring sequence and two variants that have been modified to reduce the binding of blocking antibodies (20).

MATERIALS AND METHODS

Study population and samples. During the 2000 and 2001 malaria high-transmission seasons, children of 1 to 10 years of age attending Farafenni MCH clinic with symptoms of uncomplicated malaria were screened, and eligible children were invited to participate in a clinical trial of antimalarial drug treatment. Two hundred fifty-three children (130 in 2000 and 123 in 2001) were treated with CQ and followed for 28 days (details in references 14 and 37). Finger pick plasma samples donated at enrollment (day 0) were available from 58 patients in 2000, all of whom were enrolled in the first 7 weeks of the 9-week trial; there were no differences in screening characteristics and outcomes between those with and those without plasma samples (results not shown). In addition, children found to have gametocytes on day 7 during these trials were asked to give a venous blood sample for transmission experiments (14). Day 7 plasma samples were therefore available for a subset of 47 patients. Plasma samples were stored at -20°C , and whole blood samples, also collected on day 0 and day 7, were stored dried on

filter paper for DNA extraction and genotyping. Clinical and parasitological data were collected for 28 days after treatment. Parasites were quantified by microscopic examination of Giemsa-stained thick blood smears (16). Children lost to follow-up were excluded, since their response to treatment could not be classified, leaving 46 day 0 and 44 day 7 plasma samples, 12 of which were from the same children, for analysis.

To compare antibody responses in the malaria cases with those in healthy children, control samples were selected from those taken in a cross-sectional community-based survey of children in the study area shortly before the start of the 2000 high-transmission season and frequency matched by age to the malaria cases in 1-year age bands. These studies were approved by the Joint Gambian Government/MRC Ethics Committee.

Parasite genotyping. Extracted DNA was genotyped for *Pfcr1-76Thr/Lys* by PCR-restriction fragment length polymorphism using methods described previously (9, 36).

Enzyme-linked immunosorbent assay (ELISA). The ectodomain of AMA-1 FVO strain expressed in *Pichia pastoris* was donated by Alan Thomas, BPRC, The Netherlands (22). It was purified by affinity chromatography with nickel resin and ammonium sulfate precipitation and found to be pure by gel electrophoresis. Recombinant MSP-1₁₉ (wild-type [WT] Wellcome sequence, accession number P04933) was prepared by standard techniques as a glutathione *S*-transferase (GST) fusion protein. Two modified MSP-1₁₉ antigens were also prepared as GST fusion proteins: a triple variant that carries three amino acid substitutions (Glu27 to Tyr, Leu31 to Arg, and Glu43 to Leu) and a double variant with two substitutions (Cys12 to Ile and Cys28 to Trp, which thus lacks a disulfide bond). (Amino acid residues are numbered from Asn1 at the N terminus of MSP-1₁₉.) Both variants lack one or more epitopes for blocking antibodies (39). GST served as a control for the recombinant MSP-1₁₉ antigens.

Plasma samples were relabeled so that laboratory staff would be unaware of treatment outcome. ELISAs were conducted as described in references 6 and 32. Antigens were coated onto 96-well polystyrene plates at 1 $\mu\text{g}/\text{ml}$ (Immulon 4; Thermo Lab Systems, United Kingdom), and plasma samples were assayed in duplicate at a 1:1,000 dilution. Bound antibodies were detected with a horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (IgG) (Dako, Glostrup, Denmark) and developed with *o*-phenylenediamine dihydrochloride substrate (Sigma, United Kingdom).

A positive standard (PS) high-titer adult serum sample was included on every plate, and mean optical densities (ODs) were normalized between assays using the following formula: (OD of sample) \times (overall mean OD of PS)/(day mean OD of PS). ODs for the MSP-1₁₉ recombinants were corrected for the binding of IgG to GST alone. Negative responses for all ELISAs were defined as those below the mean OD + 3 SD of a single batch of 40 individual sera from adults who had never previously visited an area of malaria endemicity. Background mean and SD values for AMA-1 were 0.235 and 0.144, giving a cutoff value of 0.668. For MSP-1₁₉ antigens, the background mean and SD values for GST, WT, triple variant, and double variant were 0.126 and 0.060, 0.088 and 0.034, 0.073 and 0.029, and 0.090 and 0.033, respectively, giving cutoff values of 0.104, 0.107, and 0.085. Plasma samples positive for the total IgG conjugate were also analyzed with an IgG subclass ELISA by use of sheep anti-human horseradish peroxidase conjugates (The Binding Site, Birmingham, United Kingdom); cutoff values for AMA-1, MSP-1₁₉ WT, and the triple variant were 0.058, 0.021, and 0.014, respectively, for IgG1 and 0.107, 0.016, and 0.018, respectively, for IgG3. To quantify IgG to MSP-1₁₉, WT, and the triple variant more precisely, assays were repeated with serial dilutions and antibody concentrations were estimated by comparison to standard curves generated using purified polyclonal human IgG (The Binding Site, United Kingdom) (7).

Statistical methods. Statistical analysis was done using STATA version 8 (Stata Corporation, College Station, Texas) and logXact version 6 (Cytel Software Corporation). The primary analysis was a determination of the influence of specific antibody in plasma taken at enrollment (day 0) on the risk of clinical failure by day 28. Clinical failure was defined as presence of (i) severe malaria on days 1 to 28, (ii) parasitemia and fever ($>37.5^{\circ}\text{C}$) on days 3 to 28, and (iii) parasitemia on day 3 $>25\%$ of that on day 0 (40). In a secondary analysis, we investigated the association of antibody responses on day 7 with late parasitological failure, defined as parasitemia on day 14 or day 28, and with the density of parasitemia on these days. Children who had neither clinical nor parasitological failure as defined above were considered to have cleared their parasites. CQ-resistant infections were defined as those where the *Pfcr1-76Thr* allele was detected in the day 0 sample. Characteristics thought to be potential confounders of the relationship between immunological variables and treatment outcome were age and the presence of drug-resistant parasite genotypes. Odds ratios for treatment failure were calculated with mid-*P* values, which are less conservative than exact tests, and exact confidence intervals. The two-sample Wilcoxon rank-

TABLE 1. Study population

Characteristic	Value for indicated characteristic for group sampled on day:	
	0 (<i>n</i> = 46)	7 (<i>n</i> = 32)
Mean age in yr (SD)	4.54 (2.19)	4.74 (2.38)
Mean packed cell volume (%) (SD)	31.72 (5.75)	29.84 (4.59)
Ethnic type (no. [%] within sample group)		
Mandinka	17 (37)	17 (53)
Wolof	14 (30)	9 (28)
Fula	11 (24)	3 (9)
Other	4 (9)	3 (9)
Geometric mean no. of asexual <i>P. falciparum</i> parasites/ μ l on day 0 (range)	2,673 (1,500–270,000)	2,124 (500–278,000)
No. within sample group positive for presence of <i>Pfcr-t-76Thr</i> allele at day 0	31 (67%)	30 (94%)

sum (Mann-Whitney) test was used to compare antibody titers or concentrations between groups. Correlation between parasitemia level, including zero values, and IgG concentration was examined using Spearman's rank correlation test, and the magnitude of the effect on parasitemia was further assessed by calculating the arithmetic mean parasitemia level by category of IgG response.

RESULTS

Study group characteristics. Plasma samples and parasite genotyping data were available from 78 patients: 46 sampled on day 0, of whom 12 were sampled again on day 7, and an additional 32 patients sampled on day 7. These groups of patients were similar in age, anemia level (as estimated by packed cell volume), ethnicity, and asexual parasite density (Table 1), but carriage of parasites with the *Pfcr-t-76Thr* genotype was higher among patients sampled only on day 7: 30/32 individuals (95%) compared to 31/46 (67%) among those sampled on day 0. This increase was likely due to the higher proportion of gametocytemic donors in the day 7 group, as there is a correlation between gametocytemia and drug resis-

tance (17, 26). Clinical failures occurred soon after treatment, 13/16 (81%) before day 14, and all parasitological failures were detected on day 14 (29/45) or on day 28 (16/45), with a mean detection time point of 18.9 days.

Association of pretreatment IgG with clinical outcome. Prevalence of antibody for each of the antigens was higher in children who did not experience clinical failure than in those that did (Table 2); for example, 6 of the 37 children with detectable pretreatment antibody that recognized WT MSP-1₁₉ failed treatment, while 5 of the 9 children without such antibody failed treatment. Similar results were obtained for the other proteins: children with antibody to these merozoite antigens were between three and six times as likely to recover without relapse. Odds ratios adjusted for age were similar to unadjusted odds ratios, indicating that age was not a significant confounder in this analysis (Table 2). Antibody titers, estimated as median OD values in responders, were not significantly associated with clinical outcome (results not shown).

Two-thirds (31/46 [67%]) of patients were carrying the *Pfcr-t-76Thr* allele, which is strongly associated with failure of CQ treatment. Although the association between the presence of antibody and treatment outcome appeared to be stronger in the group that had drug-resistant parasite genotypes on day 0, this observation was difficult to test formally because of the small sample size.

There was considerable homogeneity in responder status for the different antigens; only 8 out of the 46 children (17%) were discrepant in their response to AMA-1 and WT MSP-1₁₉, and none of these experienced clinical failure. There were discrepancies in responses to the variant MSP-1₁₉ antigens; two samples were negative for the triple variant alone and one sample was negative for both the triple and double variants while containing antibodies reacting with the wild-type antigen.

Comparison of antibody response in malaria patients and in healthy children. To explore the dynamics of these responses in relation to malarial disease, we compared the prevalence and titers of IgG to AMA-1 and WT MSP-1₁₉ found in the malaria patients on day 0 to those in clinically normal children sampled before the start of the high-transmission season. As

TABLE 2. Treatment outcome and the presence of IgG to AMA-1 or MSP-1₁₉ antigens on day 0^a

Status for indicated antibody on day 0	No. of patients with clinical failure/total (%)	Odds ratio (95% CI; <i>P</i> value)	
		Unadjusted	Adjusted for age
AMA-1			
Negative	5/11 (45)	1	1
Positive	6/35 (17)	0.27 (0.05, 1.46; 0.076)	0.32 (0.05, 1.87; 0.168)
WT MSP-1 ₁₉			
Negative	5/9 (56)	1	1
Positive	6/37 (16)	0.18 (0.03, 1.06; 0.017)	0.19 (0.03, 1.11; 0.019)
Triple-variant MSP-1 ₁₉			
Negative	6/12 (50)	1	1
Positive	5/34 (15)	0.19 (0.03, 0.98; 0.013)	0.18 (0.03, 0.97; 0.013)
Double-variant MSP-1 ₁₉			
Negative	6/11 (55)	1	1
Positive	5/35 (14)	0.23 (0.04, 1.25; 0.028)	0.24 (0.04, 1.31; 0.032)

^a Specific IgG was detected by ELISA in plasma from children on day 0 and antibody and therapeutic responses classified as indicated in Materials and Methods. Exact 95% confidence intervals (95% CI) and mid-*P* values are given for the crude odds ratios and after adjustment for age.

TABLE 3. Parasitological outcome and day 7 IgG titers to AMA-1 and MSP-1₁₉ variants^a

Antigen	No. in group	Median outcome value (25th, 75th percentiles) in cases of parasitological outcome					
		OD			IgG concn (ng/ml)		
		Failure	Clearance	<i>P</i> value	Failure	Clearance	<i>P</i> value
AMA-1	27	3.215 (2.016, 3.843 [0.58])	3.048 (1.505, 3.341)	0.58	ND	ND	
WT MSP-1 ₁₉	30	1.980 (1.567, 3.019 [0.083])	2.848 (2.484, 3.941)	0.083	37.03 (21.89, 67.33 [0.057])	103.68 (52.22, 402.94)	0.057
Triple-variant MSP-1 ₁₉	29	1.627 (1.257, 2.730 [0.097])	2.536 (2.140, 3.745)	0.097	17.63 (12.00, 49.35 [0.057])	66.83 (29.95, 300.96)	0.057
Double-variant MSP-1 ₁₉	29	1.497 (1.086, 2.325 [0.223])	2.416 (1.513, 3.681)	0.223	ND	ND	

^a Specific IgG was detected by ELISA in plasma collected on day 7 from children infected with the *Pfcr-76Thr* genotype parasite, and results of positive responders are presented according to parasitological failure or clearance. For AMA-1, seven of the 27 positive responders cleared their parasites, and for the MSP-1₁₉ antigens eight of the 30 or 29 positive responders cleared theirs. Samples were tested at a 1:1,000 dilution for all antigens, and median OD values are presented. IgG concentrations for WT and triple-variant MSP-1₁₉ antigens were then obtained by titration (see Materials and Methods). *P* values were estimated using the two-sample Wilcoxon rank-sum test. ND, not done.

would be expected in this region of malaria endemicity, almost half (21/46) of the clinically normal children had detectable parasitemia, but the mean parasite density was over 100-fold lower than that in the malaria patients. Prevalence of IgG to these antigens was similar between the groups, but titers varied: anti-MSP-1₁₉ titers were significantly higher in the clinical cases (median OD of 0.487 and 25th and 75th quartiles [IQR] of 0.241 and 1.411 versus OD of 2.609 and IQR of 1.745 and 3.352; *P* < 0.0001), while AMA-1 titers appeared lower (median OD of 3.367 and IQR of 1.931 and 3.826 versus OD of 2.075 and IQR of 1.322 and 3.571), but not significantly so.

Association of posttreatment IgG with parasitological outcome. In contrast to the patients who provided plasma on day 0, most patients who donated plasma on day 7 were infected with *Pfcr-76Thr* parasites (Table 1). We selected day 7 donors carrying *Pfcr-76Thr* parasites to compare IgG responses and parasitological outcomes. There were 31 donors in total, of whom 8 cleared their parasites. The frequency of responders was high—over 87% for all antigens—and there was no significant association between IgG responder status and parasitological failure (results not shown). However, antibody titers, estimated as median OD values in positive samples, to the MSP-1₁₉ antigens were associated with parasitological clearance at marginal significance (*P* values were 0.083 for the wild type and 0.097 for the double variant [Table 3]). To examine this relationship more precisely, specific IgG concentrations in positive day 7 plasma samples were calculated. The data obtained supported the conclusion that IgG concentrations were higher in children with controlled parasitemia (Table 3).

These results prompted us to examine the relationship between level of parasitemia, defined as the highest value found on day 14 or 28, and specific IgG to the antigens. Categorizing responders for each antigen into approximately equal groups of low, medium, and high responders revealed a trend toward descending parasitemia: for WT MSP-1₁₉, the corresponding mean values were 21,419, 15,783, and 638 asexual *P. falciparum*/μl, and for the triple variant, mean parasitemia values were 27,625, 12,567, and 622 asexual *P. falciparum*/μl. Spearman's correlation coefficients were −0.550 and −0.590, with *P* values of 0.002 and 0.001, for WT MSP-1₁₉ and the triple variant, respectively. The concentration of these antibodies was not associated with age; *P* values were 0.497 and 0.548 for WT MSP-1₁₉ and the triple variant, respectively. (In contrast,

AMA-1-specific antibody levels, estimated from OD values of positive responders, showed a marginally significant association with age [*P* = 0.086].)

Further analysis of posttreatment IgG to wild-type and triple-variant MSP-1₁₉. The presence of IgG1 and IgG3 subclasses in the specific response was determined by ELISA for all positive plasma samples from day 7, including those from patients with clinical failure. IgG1 was detected in over 90% of IgG-positive samples, and IgG1 titers correlated well with those found using the non-class-specific conjugate. IgG3 specific for AMA-1 was present in only 1/33 samples, but IgG3 specific for MSP-1₁₉ was common. For the MSP-1₁₉ triple variant, 21/32 samples tested positive for specific IgG3, and titers were higher in patients with parasitological cure (median ODs, 0.533 versus 0.107; *P* = 0.048). Similar results were found with responses to the wild-type antigen.

The triple-variant protein used in these studies lacks blocking epitopes present in the wild-type protein, and the antibody responses found in the present study were consistent with this. Mean day 7 anti-MSP-1₁₉ IgG levels were higher to the WT than to the triple-variant protein (Table 3), and at an individual level, IgG binding to the triple variant was always similar to or lower than that binding to WT antigen (Fig. 1). Several individuals who cleared their infections show concordant responses (Fig. 1); however, the figure also shows that children are more likely to clear their infections if they have antibody levels above the median. Thus, although this method may be suitable to examine the importance of differential fine specificity in naturally occurring antibodies, larger group sizes will be necessary.

DISCUSSION

In this retrospective study, we have investigated whether antibodies to two key *P. falciparum* merozoite surface molecules, AMA-1 and MSP-1₁₉, are associated with clinical recovery and control of parasitemia in children undergoing treatment for uncomplicated malaria in an area of high prevalence of drug resistance. Anti-MSP-1₁₉ antibodies were associated with clinical recovery and control of parasitemia, while the presence of antibodies to AMA-1 was only weakly associated with clinical recovery and was not associated with control of parasitemia. If these associations are causal, the results suggest

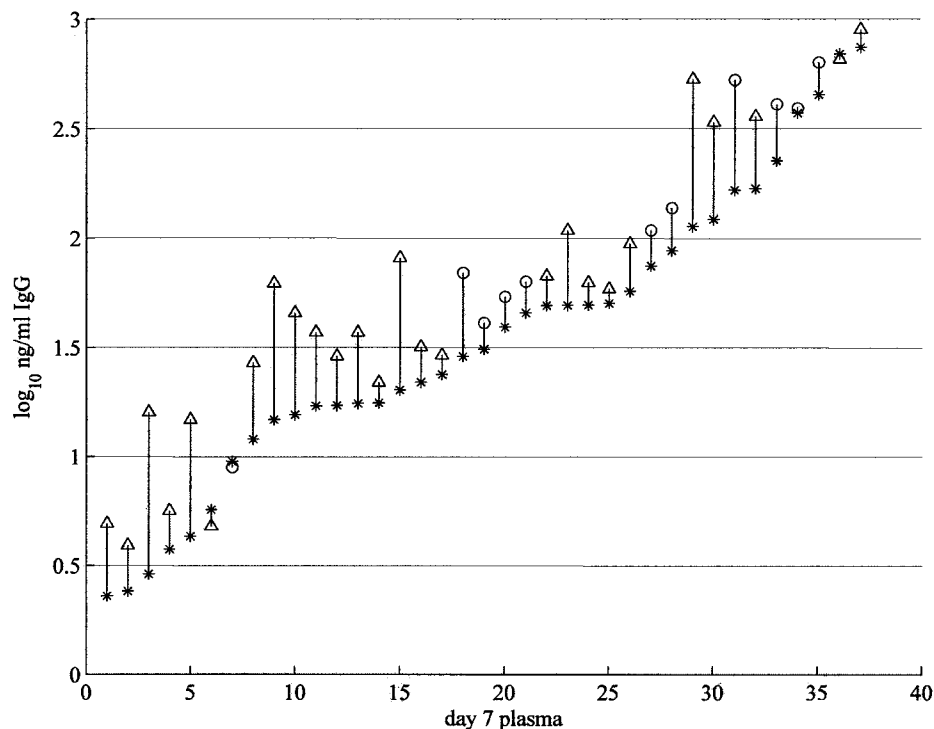


FIG. 1. Comparison of IgG concentrations to wild-type MSP-1₁₉ and to the triple variant in individual day 7 plasma samples. Variation between the IgG concentration (ng/ml) in day 7 plasma specific for WT and that for triple-variant MSP-1₁₉ is shown. Data from 37 subjects were ranked along the x axis according to their responses to the triple variant. ○ and △, WT MSP-1₁₉, with △ indicating cases with parasitological failure; *, triple variant.

that therapeutic responses to malaria treatment can be useful measures of effective clinical immunity.

As this was a retrospective study, selection of samples for analysis was constrained by their availability. This could have caused bias, but we found no difference in age, clinical outcome, or ethnicity between children who provided sera and those who did not. Two of the most likely confounders of the association of antibody and therapeutic response, age and drug-resistant genotype (10), were examined in detail. Age was not associated with anti-MSP-1₁₉ concentrations in the present study, but the association between treatment outcome and antibody levels on day 0 appeared to be stronger in children infected with parasites carrying the *Pfcr-76Thr* allele. The influence of this confounder was avoided when studying the day 7 responses by restricting the analysis to those carrying the *Pfcr-76Thr* allele. Genetic variations associated with the sickle cell trait (HbS) or ethnicity also influence the occurrence of malaria in African populations, and both appear to be associated with higher antibody response levels to malaria antigens (3, 5). In the present study, no pattern linking therapeutic response or antibody levels and ethnicity was discernible (results not shown), but this, along with the influence of HbS, needs to be studied in a larger group.

Whereas our findings are not incompatible with those of previous studies that have identified AMA-1 and MSP-1₁₉ as candidate target antigens for vaccines, we have no specific evidence that antibodies recognizing these proteins are protective. We have shown that children who clinically recover after

treatment are more likely to carry antibody recognizing these antigens. Extensive data from studies conducted in vitro, in animal models, and some malaria-exposed populations support AMA-1 as a vaccine candidate (e.g., reference 32); we found that IgG recognizing MSP-1₁₉ is more robustly associated with functional immunity than is that recognizing AMA-1. Direct comparisons between these antigens, however, should be made with caution, as they were not expressed in the same vector and the dynamics of the humoral response may vary between antigens. Our findings with MSP-1₁₉ agree with other studies linking anti-MSP-1₁₉ antibodies and protection from clinical malaria (e.g., references 1 and 15) and with a longitudinal study of Kenyan infants showing a clear relationship between the presence of anti-MSP-1₁₉ antibodies and afebrile, as opposed to febrile, infection (4). However, epidemiological evidence of an association of anti-MSP-1₁₉ responses with protection from clinical malaria has been conflicting, possibly reflecting the different methodologies that have been employed. The only previous study of anti-MSP-1 antibodies and therapeutic responses (23) found an association between therapeutic success and prevalence of antibody to MSP-1 block 2 antigens but not to MSP-1₁₉. This might reflect the lower prevalence of drug resistance in that study (33% compared to 79% for our patients), and stronger associations may be expected among patients infected with drug-resistant parasites. Also, some cohort studies (e.g., reference 12) found no correlation between anti-MSP-1₁₉ antibodies and malaria attacks, but our results show that anti-MSP-1₁₉ antibody measured during the low-

transmission season may not be a good predictor of levels or prevalence during clinical attacks in the high-transmission season. The dynamics of these responses may explain the variable association between anti-MSP-1₁₉ antibodies and protection from clinical attacks found in different studies and are worthy of further investigation.

Our results are, as far as we are aware, the first to show an association of parasite density with anti-MSP-1₁₉ IgG concentration during a malaria episode. The presence of a biological gradient increases the likelihood of causality and underlines an advantage in the use of therapeutic responses as an assay of functional immunity. It also supports the possibility that anti-MSP-1₁₉ IgG is a component of protection in these children. However, the associations we have observed also simply could reflect the higher level of exposure experienced by those children who are semi-immune, and the truly protective components of acquired immunity may be elicited by other antigens or factors we have not examined.

Cohort studies and therapeutic responses to drug treatment studies may measure different aspects of immunity. In cohort studies, functional immunity can be defined as a lower probability of developing clinical malaria, a higher parasitemia level, or infection (15, 32) and thus includes preerythrocytic immune responses against sporozoite and liver stages. The therapeutic approach, on the other hand, can define functional immunity as the ability to recover clinically or to control parasitemia, both of which may differ in mechanism from the ability to maintain parasitemia below clinically significant levels or to prevent infection. The therapeutic approach may thus identify protective mechanisms of greater relevance to reducing the clinical burden of disease. For example, in the current study, the presence of anti-MSP-1₁₉ IgG antibodies at consultation was associated with clinical recovery; if the relationship is causal, such antibodies may play a role in reducing child morbidity.

The IgG subclass analysis confirmed results from previous population studies with the IgG1 antibody subclass predominating for both AMA-1 and MSP-1₁₉, and with an additional IgG3 response to MSP-1₁₉ (7, 32). The relative balance of antibody binding to the wild-type and triple-variant MSP-1₁₉ supports the hypothesis that one or more epitopes on the wild-type antigen are recognized by some malaria-exposed individuals but not by others (30). Future studies could examine the association between differences in antibody fine specificity and clinical outcome. The similarities in IgG responses in patients with malaria and in clinically well, malaria-exposed populations, together with the associations between therapeutic and IgG responses, are consistent with results obtained by use of several other approaches. These similarities indicate that the current methods could be usefully applied to analyze other antigens.

Antimalarial drug treatment policy in sub-Saharan Africa requires accurate data on the degree and distribution of drug resistance in the parasite population, data that are typically generated from in vivo therapeutic efficacy studies. Collaboration on future therapeutic efficacy studies to allow collection of plasma from well-characterized patients may thus be possible, and this approach has been suggested as an ethical alternative to population-based studies (11). A recent review suggests that the number of available antigens is not a rate-limiting step for

vaccine development (29) and that a better definition of the antigens we already have, for example an analysis using the modified MSP-1₁₉ antigens as shown here, might be an efficient approach to define the antigenic components of effective malaria vaccines.

In conclusion, our results demonstrate that the presence of IgG to AMA-1 and MSP-1₁₉ on the day of consultation may protect against clinical failure, and that higher titers of IgG to MSP-1₁₉ enhance the likelihood of parasitological clearance in individuals treated with a suboptimal drug regime; they therefore support the use of therapeutic response studies to characterize candidate vaccine antigens.

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REFERENCES

- Al-Yaman, F., B. Genton, K. J. Krammer, S. P. Chang, G. S. Hui, M. Baisor, and M. P. Alpers. 1996. Assessment of the role of naturally acquired antibody levels to *Plasmodium falciparum* merozoite surface protein-1 in protecting Papua New Guinean children from malaria morbidity. *Am. J. Trop. Med. Hyg.* **54**:443–448.
- Blackman, M. J., H.-G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder. 1990. A single fragment of a malaria merozoite surface protein remains on the parasite surface during red cell invasion and is the target of invasion-inhibiting antibodies. *J. Exp. Med.* **172**:379–382.
- Bolad, A., S. E. Farouk, E. Israelsson, A. Dolo, O. K. Doumbo, I. Nebie, B. Maiga, B. Kouriba, G. Luoni, B. S. Sirima, D. Modiano, K. Berzins, and M. Troye-Blomberg. 2005. Distinct interethnic differences in immunoglobulin G class/subclass and immunoglobulin M antibody responses to malaria antigens but not in immunoglobulin G responses to nonmalarial antigens in sympatric tribes living in West Africa. *Scand. J. Immunol.* **61**:380–386.
- Branch, O. H., V. Udhayakumar, A. W. Hightower, A. J. Oloo, W. A. Hawley, B. L. Nahlen, P. B. Bloland, D. C. Kaslow, and A. A. Lal. 1998. A longitudinal investigation of IgG and IgM antibody responses to the merozoite surface protein-1 19-kilodalton domain of *Plasmodium falciparum* in pregnant women and infants: associations with febrile illness, parasitemia, and anemia. *Am. J. Trop. Med. Hyg.* **58**:211–219.
- Cabrera, G., M. Cot, F. Migot-Nabias, P. G. Kremsner, P. Deloron, and A. J. Luty. 2005. The sickle cell trait is associated with enhanced immunoglobulin G antibody responses to *Plasmodium falciparum* variant surface antigens. *J. Infect. Dis.* **191**:1631–1638.
- Cavanagh, D. R., I. M. Elhassan, C. J. Roper, and S. McBride. 1998. A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface antigen protein-1 in an area of unstable malaria in Sudan. *J. Immunol.* **161**:347–359.
- Cavanagh, D. R., C. Dobaño, I. M. Elhassan, K. Marsh, A. Elhassan, L. Hviid, E. A. T. G. Khalil, T. G. Theander, D. E. Arnot, and J. S. McBride. 2001. Differential patterns of human immunoglobulin G subclass responses to distinct regions of a single protein, the merozoite surface protein 1 of *Plasmodium falciparum*. *Infect. Immun.* **69**:1207–1211.
- Cohen, S., I. A. McGregor, and S. C. Carrington. 1961. Gamma-globulin and acquired immunity to human malaria. *Nature* **192**:733–737.
- Djimdé, A., O. K. Doumbo, J. F. Cortese, K. Kayentao, S. Duombo, Y. Diourte, A. Dicko, X.-Z. Su, T. Nomura, D. Fidock, T. E. Wellems, and C. V. Plowe. 2001. A molecular marker for chloroquine resistant falciparum malaria. *N. Engl. J. Med.* **344**:257–263.
- Djimdé, A., O. K. Doumbo, O. Traore, A. Guindo, K. Kayentao, Y. Diourte, S. N. Doumbo, D. Coulibaly, A. Koné, M. Tekete, B. Fofana, Y. Cissoko, and C. V. Plowe. 2001. Application of a molecular marker as an epidemiological surveillance tool for chloroquine resistant malaria. *Lancet* **358**:890–891.
- Djimdé, A. A., O. K. Doumbo, O. Traore, A. B. Guindo, K. Kayentao, Y. Diourte, S. Niare-Doumbo, D. Coubali, A. K. Kone, Y. Cissoko, M. Tekete,

- B. Fofana, A. Dicko, D. A. Diallo, T. E. Wellems, D. Kwiatkowski, and C. V. Plowe. 2003. Clearance of drug resistant parasites as a model for protective immunity in *P. falciparum* malaria. *Am. J. Trop. Med. Hyg.* **69**:558–563.
12. Dodo, D., T. G. Theander, J. A. Kurtzals, K. Kojo, E. Riley, B. D. Akanmori, F. K. Nkrumah, and L. Hviid. 1999. Levels of antibody to conserved parts of *Plasmodium falciparum* merozoite surface antigen 1 in Ghanaian children are not associated with protection from clinical malaria. *Infect. Immun.* **67**:2131–2137.
 13. Dorsey, G., A. Gasasira, R. Machezano, M. R. Kamya, S. G. Staedke, and A. Hubbard. 2004. The impact of age, temperature, and parasite density on treatment outcomes from antimalarial clinical trials in Kampala, Uganda. *Am. J. Trop. Med. Hyg.* **71**:531–536.
 14. Drakeley, C. J., M. Jawara, G. A. T. Targett, G. Walraven, U. Obisike, R. Coleman, M. Pinder, and C. J. Sutherland. 2004. Addition of artesunate to chloroquine for treatment of *Plasmodium falciparum* malaria in Gambian children causes a significant but short-lived reduction in infectiousness for mosquitoes. *Trop. Med. Int. Health* **9**:53–61.
 15. Egan, A., J. Morris, G. Barnish, S. Allen, B. M. Greenwood, D. C. Kaslow, A. A. Holder, and E. M. Riley. 1996. Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19 kDa C-terminal fragment of the merozoite surface antigen PfMSP1. *J. Infect. Dis.* **173**:765–769.
 16. Greenwood, B. M., and J. R. M. Armstrong. 1991. Comparison of two simple methods for determining malaria parasite density. *Trans. R. Soc. Trop. Med. Hyg.* **85**:186–188.
 17. Hallett, R., C. J. Sutherland, N. Alexander, R. Ord, M. Jawara, C. Drakeley, M. Pinder, G. Walraven, G. A. T. Targett, and A. Allouche. 2004. Combination therapy counteracts the enhanced transmission of drug-resistant malaria parasites to mosquitoes. *Antimicrob. Agents Chemother.* **48**:3940–3943.
 18. Hoffman, S. L., C. N. Oster, C. V. Plowe, G. R. Woollett, J. C. Beier, J. D. Chulay, R. A. Wirtz, M. R. Hollingdale, and M. Mugambi. 1987. Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. *Science* **237**:639–642.
 19. Hoffman, S. L., and L. H. Miller. 1996. Perspectives on malaria vaccine development, p. 1–14. In S. L. Hoffman (ed.), *Malaria vaccine development: a multi-immune response approach*. ASM Press, Washington, D.C.
 20. Holder, A. A., J. A. Guevara Patiño, C. Uthaipibull, S. E. H. Syed, I. T. Ling, T. Scott-Finnigan, and M. J. Blackman. 1999. Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria. *Parasitologia* **41**:409–414.
 21. Jarra, W., L. A. Hills, J. C. March, and K. N. Brown. 1986. Protective immunity to malaria. Studies with cloned lines of *Plasmodium chabaudi chabaudi* and *P. berghei* in CBA/Ca mice. II. The effectiveness and inter- or intra-species specificity of the passive transfer of immunity with serum. *Parasite Immunol.* **8**:239–254.
 22. Kocken, C. H., C. Withers-Martinez, M. A. Dubbeld, A. van der Wel, F. Hackett, A. Valderrama, M. J. Blackman, and A. W. Thomas. 2002. High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and induction of antibodies that inhibit erythrocyte invasion. *Infect. Immun.* **70**:4471–4476. (Erratum **70**:5901.)
 23. Mawili-Mboumba, D. P., S. Borrmann, D. R. Cavanagh, J. S. McBride, P. B. Matsigui, M. A. Missinou, P. G. Kremsner, and F. Ntoumi. 2003. Antibody responses to *Plasmodium falciparum* merozoite surface protein-1 and efficacy of amodiaquine in Gabonese children with *P. falciparum* malaria. *J. Infect. Dis.* **187**:1137–1141.
 24. Mayxay, M., K. Chotivanich, S. Pukrittayakamee, P. Newton, S. Looareesuwan, and N. J. White. 2001. Contribution of humoral immunity to the therapeutic response in falciparum malaria. *Am. J. Trop. Med. Hyg.* **65**:918–923.
 25. McGregor, I. A., S. C. Carrington, and S. Cohen. 1963. Treatment of East African *Plasmodium falciparum* malaria with West African human gamma-globulin. *Trans. R. Soc. Trop. Med. Hyg.* **57**:170–175.
 26. Meerman, L., R. Ord, J. Teun Bousema, M. van Niekerk, E. Osman, R. Hallett, M. Pinder, G. Walraven, and C. J. Sutherland. 2005. Carriage of chloroquine-resistant parasites and delay of effective treatment increase the risk of severe malaria in Gambian children. *J. Infect. Dis.* **192**:1651–1657.
 27. Migot-Nabias, F., A. J. F. Luty, P. Ringwald, M. Vaillant, B. Dubois, A. Renault, R. J. Mayombo, T. N. Minh, N. Fievet, J. R. Mbessi, P. Millet, and P. Deloron. 1999. Immune responses against *Plasmodium falciparum* asexual blood-stage antigens and disease susceptibility in Gabonese and Cameroonian children. *Am. J. Trop. Med. Hyg.* **61**:488–494.
 28. Mita, T., A. Kaneko, J. K. Lum, B. Bwijo, M. Takechi, I. L. Zungu, T. Tsukahara, K. Tanabe, T. Kobayakawa, and A. Bjorkman. 2003. Recovery of chloroquine sensitivity and low prevalence of the *Plasmodium falciparum* chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. *Am. J. Trop. Med. Hyg.* **68**:413–415.
 29. Moorthy, V. S., M. F. Good, and A. V. Hill. 2004. Malaria vaccine developments. *Lancet* **363**:150–156.
 30. Nwuba, R. I., O. Sodeinde, C. I. Anumudu, Y. O. Omosun, A. B. Odaibo, A. A. Holder, and M. Nwagwu. 2002. The human immune response to *Plasmodium falciparum* includes both antibodies that inhibit merozoite surface protein 1 secondary processing and blocking antibodies. *Infect. Immun.* **70**:5328–5331.
 31. Okech, B. A., P. H. Corran, J. Todd, A. Joynson-Hicks, C. Uthaipibull, T. G. Egwang, A. A. Holder, and E. M. Riley. 2004. Fine specificity of serum antibodies to *Plasmodium falciparum* merozoite surface protein, PfMSP-1₉, predicts protection from malaria infection and high-density parasitemia. *Infect. Immun.* **72**:1557–1567.
 32. Polley, S. D., T. Mwang, C. H. M. Kocken, A. W. Thomas, S. Duttad, D. E. Lanard, E. Remarque, A. Ross, T. N. Williams, G. Mwambingub, B. Loweb, D. J. Conway, and K. Marsh. 2004. Human antibodies to recombinant protein constructs of *Plasmodium falciparum* apical membrane antigen 1 (AMA1) and their associations with protection from malaria. *Vaccine* **23**:718–728.
 33. Riley, E. M., S. J. Allen, J. G. Wheeler, M. J. Blackman, S. Bennett, B. Takacs, H.-J. Schönfeld, A. A. Holder, and B. M. Greenwood. 1992. Naturally-acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity. *Parasite Immunol.* **14**:321–338.
 34. Robert, V., W. Roeffen, P. Brasseur, G. Aribot, J. P. Verhave, and C. Roussillon. 2000. Anti-NANP antibody and treatment efficacy in patients with acute uncomplicated falciparum malaria attacks. *Parasite Immunol.* **22**:589–593.
 35. Sachs, J., and P. Malaney. 2002. The economic and social burden of malaria. *Nature* **415**:680–685.
 36. Sutherland, C. J., A. Allouche, J. Curtis, C. J. Drakeley, R. Ord, M. Duraisingh, B. M. Greenwood, M. Pinder, D. Warhurst, and G. A. T. Targett. 2002. Gambian children successfully treated with chloroquine can harbour and transmit *Plasmodium falciparum* gametocytes carrying resistance genes. *Am. J. Trop. Med. Hyg.* **67**:578–585.
 37. Sutherland, C. J., C. J. Drakeley, U. Obisike, R. Coleman, M. Jawara, G. A. T. Targett, P. Milligan, M. Pinder, and G. Walraven. 2003. The addition of artesunate to chloroquine for treatment of *Plasmodium falciparum* malaria in Gambian children delays, but does not prevent, treatment failure. *Am. J. Trop. Med. Hyg.* **69**:19–25.
 38. Talisuna, A. O., J. Kyosiimire-Lugemwa, P. Langi, T. K. Mutabingwa, W. Watkins, E. van Marke, T. Egwang, and U. D'Alessandro. 2002. Role of the *pfprt* codon 76 mutation as a molecular marker for population based surveillance of chloroquine (CQ)-resistant *Plasmodium falciparum* in Uganda sentinel sites with high CQ resistance. *Trans. R. Soc. Trop. Med. Hyg.* **96**:551–556.
 39. Uthaipibull, C., B. Aufiero, S. E. H. Syed, B. Hansen, J. A. G. Patiño, E. Angov, I. T. Ling, K. Fegeding, W. D. Morgan, C. Okenhouse, B. Birdsall, J. Feeney, J. A. Lyon, and A. A. Holder. 2001. Inhibitory and blocking monoclonal antibody epitopes on merozoite surface protein 1 of the malaria parasite *Plasmodium falciparum*. *J. Mol. Biol.* **307**:1381–1394.
 40. World Health Organization. 2002. Monitoring antimalarial drug resistance. Document WHO/CDS/EPH/2002.17. World Health Organization, Geneva, Switzerland.