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Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria

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SUMMARY

The merozoite surface protein 2 (MSP2) of Plasmodium falciparum *is recognized by human antibodies elicited during natural infections, and may be a target of protective immunity. In this prospective study, serum IgG antibodies to MSP2 were determined in a cohort of 329 Gambian children immediately before the annual malaria transmission season, and the incidence of clinical malaria in the following 5 months was monitored. Three recombinant MSP2 antigens were used, representing each of the two major allelic serogroups and a conserved region. The prevalence of serum IgG to each antigen correlated positively with age and with the presence of parasitaemia at the time of sampling. These antibodies were associated with a reduced subsequent incidence of clinical malaria during the follow-up. This trend was seen for both IgG1 and IgG3, although the statistical significance was greater for IgG3, the most common subclass against MSP2. After adjusting for potentially confounding effects of age and pre-season parasitaemia, IgG3 reactivities against each of the major serogroups of MSP2 remained significantly associated with a lower prospective risk of clinical malaria. Individuals who had IgG3 reactivity to both of the MSP2 serogroup antigens had an even more significantly reduced risk. Importantly, this effect remained significant after adjusting for a simultaneous strong protective association of antibodies to another antigen (MSP1 block 2) which itself remained highly significant.*

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INTRODUCTION

Plasmodium falciparum malaria is a major cause of human morbidity and mortality, and there is a need for an effective vaccine. Identification of the optimal parasite antigens on which to base a vaccine is a challenging task, because there are many antigenic proteins in the parasite, but the role of most of these in protective immunity is unknown. The merozoite surface protein 2 (MSP2) is located on the parasite stage that invades red blood cells (1,2). Monoclonal antibodies to native MSP2 and rabbit antibodies raised against a recombinant MSP2 protein can inhibit *P. falciparum* merozoite invasion *in vitro* (1), indicating that this antigen could be a potential target of immunity. A recombinant vaccine containing MSP2 sequences has shown immunogenicity in human volunteers, although the search for an optimal formulation continues (3,4).

The single *msp2* locus of *P. falciparum* is highly polymorphic (5–8), and antigenic polymorphism has been documented in particular regions of the protein (6). However, different *msp2* alleles can all be grouped into one or the other of only two major allelic types encoding antigenically distinct major forms of MSP2, serogroup A (3D7-like) and serogroup B (FC27-like), originally defined by monoclonal antibodies (6) and also recognized by human polyclonal sera (9). Both of the *msp2* major allelic types are common in most populations, and their frequencies are unusually even throughout the world (8,10), compared with alleles of other *P. falciparum* loci (11,12). This suggests that the allelic types may be selectively maintained within populations, because of acquired human immunity to serogroup-specific epitopes, as demonstrated for another *P. falciparum* merozoite antigen (13). Interestingly, a recent field trial of a vaccine containing an MSP2 recombinant protein shows an allele-specific effect on the *P. falciparum* parasites subsequently detected in the natural infections of vaccinees (4).

Consistent with this are some characteristics of anti-MSP2 antibodies produced by people exposed to natural *P. falciparum* infections. Human antibodies to MSP2 that are prevalent in endemic areas recognize predominantly serogroup-specific epitopes (9,14), and epitopes that are present on particular variants or subtypes within each serogroup (15,16). Moreover, there is some evidence of association between anti-MSP2 antibodies and protection from malaria. In Papua New Guinea, a longitudinal cohort study has shown that antibodies to serogroup A (3D7-like) are correlated with a reduced prospective risk of clinical malaria in children, but no correlation was found between antibodies to serogroup B (FC27-like) and protection (17). Most antibodies to MSP2 are of the IgG3 subclass (9,18,19), and to a lesser extent IgG1. A cohort study in The Gambia indicated that the IgG3 subclass might be a particularly important component of protective anti-MSP2 antibodies (19).

The present study involves analysis of another prospective longitudinal cohort in The Gambia, to address whether serum IgG reactivity to MSP2 is associated with a reduced risk of clinical malaria, and whether there is a particularly effective contribution from the cytophilic subclasses (IgG1 or IgG3) against either of the two MSP2 serogroups. Results show that serum IgG3 against each of the MSP2 serogroups is strongly associated with a reduced risk of malaria, even after adjustment for potentially confounding variables.

MATERIALS AND METHODS

Study population

329 children (aged 3–7 years) living in villages near Basse (Upper River Division, The Gambia) were included in a cross-sectional survey at the beginning of the malaria transmission season in June 1996, and then followed up clinically and parasitologically for a period of 5 months (July to November). At the cross-sectional survey, slides were prepared for determination of malaria parasitaemia and serum samples were collected for measurement of specific antibodies against MSP2. During the 5 months follow-up, blood slides were prepared from children with suspected malaria during passive and active surveillance, and confirmed clinical cases of malaria were treated and documented. The outcome variable of clinical malaria is defined as a fever (> 37·5°C) plus coincident parasitaemia of > 5000 per microlitre at any point during the follow-up, either by passive or active case detection. This cohort was studied previously for antibodies to MSP1 (a previous cohort size of 337 included eight individuals from whom sera were no longer available for the present study) (13). The study was approved by the Medical Research Council/Gambia Government Joint Ethical Committee, and informed consent was obtained from all study participants or their guardians.

MSP2 antigens and antibody assays

Three recombinant MSP2 antigens were produced in *E. coli* as fusion proteins, with MSP2 sequences at the C-terminus of glutathione S-transferase (GST) (9). One of the proteins contains a highly conserved sequence of 57 amino acids from the C-terminal region of MSP2 (protein K1 17/14, aa 207–263 as encoded by the K1 allele) (2). The same conserved sequence is also included in the two larger antigens that represent mature near full-length serogroup A and serogroup B MSP2 proteins, respectively. The serogroup A product is based on the MSP2 allele from the *P. falciparum* clone T9/96 (protein T9/96 13/14, aa 22–286 of T9/96) (6), which is of the 3D7-like type. The serogroup B product is based on the Dd2 allele (protein Dd2 13/14, aa 22–247 of Dd2) which is of the FC27-like type.

ELISA assays to measure MSP2 specific IgG and IgG subclasses were carried out as previously described (9,20). Briefly, wells of microtitre plates (Immulon-4; Dynatech, Billingshurst, UK) were coated overnight with 100 µL of antigen at 0·5 µg/mL in 0·1 M carbonate buffer (Na_2CO_3/$NaHCO_3$) and blocked for 5 h at room temperature with 200 µL of blocking buffer (1% milk powder in PBS, 0·05% Tween 20). Plates were washed three times and 100 µL of serum (diluted 1/1000) was added to each of duplicate wells and incubated overnight at 4°C. Plates were washed and incubated with a 1/6000 dilution of horseradish peroxidase conjugated rabbit anti-human IgG antibody (Dako Ltd) for 3 h at room temperature. All plates were developed with H_2O_2 and o-phenylenediamine at 4°C for 10 min, the reaction was stopped with 20 µL of 2 M H_2SO_4 per well, and the optical density (OD) was measured at 492 nm. For each tested serum, the OD value against GST alone was subtracted from the value obtained for each MSP2 fusion protein in order to obtain specific OD values for each antigen. Tested sera were defined as positive if they gave a specific OD above the normal cut-off (mean + 2 SD of 15 negative control sera from individuals living in the United Kingdom who had not been exposed to malaria).

Human IgG1 and IgG3 subclass reactivity to the antigens was tested on sera that were positive for total IgG. Previous studies have shown that individuals who are ELISA-negative for total IgG to these MSP2 proteins are invariably negative for IgG1 or IgG3 tested separately (9,19). Sera were tested at 1/1000 dilution, and HRP-conjugated affinity-purified anti-IgG1 and IgG3 second antibodies (AP006 and AP008, Binding Site, UK) were used at 1/1000 dilution. ELISA was performed as for the total IgG assay, and cut-off OD values were determined for each antigen as the mean

+ 2 SD of 20 control sera from adults living in the United Kingdom.

Statistical methods

Correlations between values of continuous variables (such as ELISA absorbance (OD) levels to different antigens) were calculated using the Spearman rank correlation coefficient. Associations between continuous variables (such as OD levels in a particular ELISA assay) and binary categorical variables (such as the occurrence or non-occurrence of clinical malaria) were studied using the Mann–Whitney non-parametric *U*-test, or by multiple logistic regression analysis. Associations between categorical variables were tested by chi-squared and multiple logistic regression analyses, and relative risks (RR) with 95% confidence intervals (CI) were calculated where appropriate. To adjust for confounding variables (such as age) in the examination of associations between the presence of antibodies in June and subsequent clinical malaria, binomial regression with a log-link was used to estimate crude and adjusted relative risks, with 95% confidence intervals (CI).

RESULTS

Anti-MSP2 antibodies before the malaria transmission season

At the time of serum sampling in June 1996, prior to the transmission season, 132 (40%) of the 329 children were parasitaemic (geometric mean parasitaemia of 69 parasites per microlitre; range 5–7500). These infections were asymptomatic and are presumed to be retained from the previous transmission season (> 6 months before). Table 1 shows the prevalence of anti-MSP2 antibodies, and its relationship with age and parasitaemia at the time of sampling. Older children were significantly more likely than younger children to have antibodies to either of the major MSP2 serogroups A or B ($P < 0{\cdot}001$). There was also a significant positive association between parasitaemia and the presence of antibodies to either MSP2 serogroup (almost all individuals with > 500 parasites per microlitre had antibodies to one or both of these antigens) ($P < 0{\cdot}001$).

Only 17 (5%) of 329 individuals had detectable antibodies to the conserved C-terminal region of MSP2. As expected, all these individuals had antibodies reactive with the full-length serogroup A and B proteins in which this conserved sequence is included. Apart from the C-terminal sequence, and a short conserved sequence at the N-terminus, the serogroup A and B proteins are very distinct structurally. Over all individuals, antibody levels (OD values) against the serogroup A and serogroup B proteins were significantly correlated with each other (Spearman's $r = 0{\cdot}59$, $P < 0{\cdot}001$), consistent with the usual inter-individual variation in levels of antibodies to malaria antigens generally. One hundred and forty (42·6%) individuals had positive OD values (above the cut-off) to both serogroups A and B, 42 (12·8%) to serogroup A only, and 31 (9·4%) to serogroup B only.

Table 1 Antibodies to MSP2 prior to the malaria transmission season and their relationship with *P. falciparum* parasitaemia and age of individuals at time of sampling

	Proportion (% of individuals) with IgG to MSP2 antigens		
Number of individuals (*n*)	Serogroup A (3D7-like)	Serogroup B (FC27-like)	Conserved C-terminal
Total ($n = 329$)	55·3	52·0	5·2
By age			
3 year ($n = 129$)	38·8	29·5	5·4
4 year ($n = 64$)	54·7	54·7	4·7
5 year ($n = 49$)	65·3	67·3	2·0
6 year ($n = 45$)	75·6	75·6	6·7
7 year ($n = 42$)	73·8	73·8	7·1
By parasitaemia			
Negative ($n = 197$)	47·2	41·1	3·0
1–499 μL^{-1} ($n = 87$)	52·9	52·9	5·7
≥ 500 μL^{-1} ($n = 45$)	95·6	97·8	13·3

Anti-MSP2 antibodies and their relationship to the subsequent incidence of malaria

Clinical malaria was detected in 179/329 (54%) of children during follow-up (geometric mean parasitaemia 43 833 parasites per microlitre; range 5000–400 000). Risk of clinical malaria was inversely associated with age, with incidence ranging from 65% of children aged 3–4 years to 40% of children aged 7 years (P for trend < 0·001). The presence of parasitaemia in June (prior to the transmission season) was not significantly associated with the risk of clinical malaria during follow-up ($P = 0{\cdot}30$). It was considered that age and parasitaemia could both be potential confounders in an analysis of the relationship between antibodies and clinical malaria, and thus it was decided to incorporate these into multivariate analyses.

In a univariate analysis, the presence of IgG in June against each of the MSP2 serogroup A and B antigens was associated with a significantly reduced risk (where relative risk, RR < 1·0) of clinical malaria during follow-up (serogroup A, RR = 0·80, 95% CI = 0·66–0·97; serogroup B, RR = 0·80, 95% CI = 0·65–0·97), but the association did not persist after adjustment for age and parasitaemia in June (Table 2). The presence of antibodies to the C-terminal conserved region, though uncommon, was associated with a

Table 2 Proportions of children acquiring clinical malaria (July to November) according the presence of serum antibodies to MSP2 in June

Recombinant MSP2 antigen	Proportions acquiring clinical malaria according to antibody (Ab) reactivities			
	Ab positives	Ab negatives	Crude RR[a] (95% CI)	Adjusted RR[b] (95% CI)
Total IgG				
Serogroup A	48·9% (89/182)	61·2% (90/147)	0·80 (0·66–0·97)*	0·94 (0·77–1·16)
Serogroup B	48·5% (83/171)	60·8% (96/158)	0·80 (0·65–0·97)*	0·95 (0·77–1·17)
Serogroups A and B	44·3% (62/140)	59·5% (69/116)	0·54 (0·33–0·89)*	0·81 (0·50–1·33)
IgG1				
Serogroup A	0% (0/3)	54·9% (179/326)	–	–
Serogroup B	35·3% (12/34)	56·6% (167/295)	0·62 (0·39–0·99)*	0·68 (0·43–1·08)
Serogroups A and B	0% (0/2)	56·8% (167/294)	–	–
IgG3				
Serogroup A	37·6% (32/85)	60·2% (147/244)	0·62 (0·47–0·84)**	0·70 (0·52–0·96)*
Serogroup B	37·0% (30/81)	60·1% (149/248)	0·62 (0·46–0·83)**	0·68 (0·49–0·94)*
Serogroups A and B	30% (15/50)	62·0% (132/213)	0·48 (0·31–0·75)**	0·54 (0·34–0·84)**

[a]Relative risks by univariate analysis; [b]Relative risks after adjustment for age and pre-season parasitaemia by multivariate analysis; *$P < 0·05$, **$P < 0·01$.

lower risk of clinical malaria (adjusted RR = 0·55, 95% CI 0·27–1·14). Examination of ELISA OD values as a crude measure of antibody levels (rather than simple positivity) showed that children who did not get malaria had higher levels to the serogroup A and B antigens than those who did get malaria (Mann–Whitney *U*-test: serogroup A, $P = 0·005$; serogroup B, $P = 0·002$), but there was no difference in the levels to the conserved C-terminal region ($P = 0·71$).

To test whether protective associations may be particularly attributable to IgG1 or IgG3, the reactivities of these subclasses were assayed against both the full-length serogroup A and B antigens. The proportion of individuals with detectable IgG3 to these antigens was much higher than the proportion with detectable IgG1. Moreover, the presence of detectable IgG3 to either serogroup was very strongly associated with a reduced risk of clinical malaria in univariate analyses (serogroup A, RR = 0·62, 95% CI = 0·47–0·84; serogroup B, RR = 0·62, 95% CI = 0·46–0·83; $P < 0·01$ for each, Table 2), and this remained significant after adjustment for age and parasitaemia at the time of serum collection in June (serogroup A, RR = 0·70, 95% CI = 0·52–0·96; serogroup B, RR = 0·68, 95% CI = 0·49–0·94; $P < 0·05$ for each, Table 2). The presence of IgG1 to serogroup B was also associated with a reduced risk of clinical malaria, but less significantly (Table 2); only three individuals had detectable IgG1 to the serogroup A antigen.

Combinations of different antibody reactivities

As shown in Table 2, the children who had detectable serum IgG3 in June against both the MSP2 serogroup A and serogroup B antigens had a very significantly reduced risk of acquiring clinical malaria during the follow-up, compared to those without IgG3 to either, even after adjustment for age and parasitaemia in June (adjusted RR = 0·54, 95% CI = 0·34–0·84, $P < 0·01$). This stronger association suggests an additive protective effect of antibodies to each serogroup, consistent with a mechanism of serogroup-specific immunity.

A previous study has shown that serum IgG to recombinant antigens representing the block 2 region of MSP1 (the major merozoite surface protein) were also strongly associated with protection from clinical malaria in this cohort of children (13). In particular, children with antibodies to both the K1-like type and the MAD20-like type of MSP1 block 2 (represented by the 3D7 and Wellcome sequence recombinant antigens, respectively) had a very significantly reduced risk of clinical malaria ($P < 0·001$), even after adjustment for age and presence of parasitaemia at the time of serum collection in June. An important critical question is whether the antibody specificities measured are causally important, or whether they are merely markers that correlate with other protective responses. To address this, a multiple logistic regression analysis was performed incorporating together the antibody reactivities to MSP2 measured here, and MSP1 block 2 measured previously. Remarkably, this highly stringent combined analysis (which also adjusted for age and presence of parasitaemia at the time of serum collection) showed that the protective associations were independently statistically significant for antibodies to MSP2 ($P = 0·037$ for the presence of IgG3 to both MSP2 serogroups A and B) and MSP1 block 2 ($P = 0·005$ for the presence of IgG to both the K1-like and MAD20-like types represented by the 3D7 and Wellcome recombinant antigens).

DISCUSSION

The finding that naturally acquired serum IgG3 antibodies to both of the serogroups of *P. falciparum* MSP2 are associated with a significantly reduced risk of clinical malaria supports the vaccine candidacy of this antigen. The results confirm and extend the evidence from previous studies which indicated that this antigen may be an important target of immunity. It had been predicted that IgG3 antibodies to MSP2 may give stronger protection than IgG1 (19). Here, serum IgG1 against MSP2 was much less commonly detected than IgG3, as in other studies (9,18,19). Protective associations are seen here for IgG3 antibodies against each of the major allelic serogroups A and B, and are shown to be significant even after accounting for the potential confounding variables of age (an important confounding variable here) and the presence of pre-transmission season parasitaemia (a variable that had virtually no confounding effect here).

Moreover, the significant protective association of IgG3 reactivity to both MSP2 serogroups is independent of the protective association seen with antibodies to MSP1 block 2 in the same cohort of children (13). The significant protective associations of antibodies to these two different *P. falciparum* antigens, MSP2 and MSP1 block 2, after adjustment for confounding variables within the same multivariate analysis, is an exceptional finding that strongly implicates both antigens as independent targets of protective immune responses. Such combined analyses of antibodies to different *P. falciparum* antigens have been rarely performed, although it has been shown that inclusion of different antibody reactivities in the same analysis can alter protective associations markedly (21,22). It is therefore worthwhile to study antibodies to other *P. falciparum* antigens, and to analyse these together in studies of cohorts such as this.

Both IgG1 and IgG3 are cytophilic and may be effective in antibody-dependent cellular mechanisms against *P. falciparum* (23). However, recent evidence indicates that IgG3 plays the major role in parasite inhibition by monocytes *in vitro* (24). It is usually expected that after several exposures to a protein antigen, IgG1 will become the major subclass with IgG2, IgG3 and IgG4 present in only small amounts. The abundance of IgG3 is unusual, and it is notable that IgG3 dominates not only the response to MSP2 but also responses to some parts of other *P. falciparum* merozoite proteins (20,25,26). Interestingly, IgG against the block 2 region of the MSP1 antigen is strongly associated with protection (13), and is predominantly of the IgG3 subclass in surveys of separate populations (20). The mechanisms of induction of human IgG3 responses to merozoite proteins, and the means by which this subclass may influence the protective efficacy of the response (positively or negatively) in each case, are important matters for investigation.

Human IgG3 has a short half-life, and thus continuous production is required to sustain its levels. This production could be stimulated either by persistent infection or by new infections. The fact that 40% of children in this study were harbouring *P. falciparum* before the annual rains (which mark the beginning of the transmission season) indicates that parasites survived throughout the dry season in these individuals. There was an association between parasitaemia and the presence of anti-MSP2 antibodies in the individuals prior to the transmission season, but the association of IgG3 antibodies with a lower subsequent risk of malaria remained statistically significant after adjusting for the presence of parasitaemia prior to the transmission season (as well as adjustment for the age of each child). It will be relevant to investigate the factors that determine the persistence of detectable antibody responses to MSP2 as well as to other malaria antigens. However, detectable antibodies at the beginning of the malaria transmission season may not be the protective parameters *per se*, but they may rather be correlated with the presence of memory B cells of the corresponding specificities. It is difficult to study memory B cells directly, due to the low frequency of cells of any given specificity, but studies of antibody production by cultured *ex vivo* B cell populations may be useful in characterizing natural IgG subclass responses further (27,28).

MSP2 is being evaluated as a promising candidate for a malaria vaccine (3,4). The present and previous studies provide strong evidence that naturally acquired antibodies to both serogroups of MSP2 are associated with a lower risk of malaria, and that these antibodies are predominantly of the IgG3 subclass. It will be important to determine whether protective antibodies are broadly specific (either to serogroup A or to serogroup B) or whether they are narrowly subtype-specific (against epitopes which differ among allelic forms within each serogroup). A recent study has shown extensive antibody cross-reactivity among different sequence variants within the MSP2 serogroups (14), encouraging the idea that a vaccine based on MSP2 may only need to include a small repertoire of allelic sequences, and that a minimal set of such sequences may be definable. It would be desirable to know whether particular IgG subclasses of antibodies induced by a vaccine can determine its efficacy, and also to know how the precise antigenic components of a vaccine could influence this.

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