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Naturally Acquired Antibody Responses to *Plasmodium falciparum* Merozoite Surface Protein 4 in a Population Living in an Area of Endemicity in Vietnam

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Merozoite surface protein 4 (MSP4) of *Plasmodium falciparum* is a glycosylphosphatidylinositol-anchored integral membrane protein that is being developed as a component of a subunit vaccine against malaria. We report here the measurement of naturally acquired antibodies to MSP4 in a population of individuals living in the Khanh-Hoa region of Vietnam, an area where malaria is highly endemic. Antibodies to MSP4 were detected in 94% of the study population at titers of 1:5,000 or greater. Two forms of recombinant MSP4 produced in either *Escherichia coli* or *Saccharomyces cerevisiae* were compared as substrates in the enzyme-linked immunosorbent assay. There was an excellent correlation between reactivity measured to either, although the yeast substrate was recognized by a higher percentage of sera. Four different regions of MSP4 were recognized by human antibodies, demonstrating that there are at least four distinct epitopes in this protein. In the carboxyl terminus, where the single epidermal growth factor-like domain is located, the reactive epitope(s) was shown to be conformation dependent, as disruption of the disulfide bonds almost completely abolished reactivity with human antibodies. The anti-MSP4 antibodies were mainly of the immunoglobulin G1 (IgG1) and IgG3 subclasses, suggesting that such antibodies may play a role in opsonization and complement-mediated lysis of free merozoites. Individuals in the study population were drug-cured and followed up for 6 months; no significant correlation was observed between the anti-MSP4 antibodies and the absence of parasitemia during the surveillance period. As a comparison, antibodies to $MSP1_{19}$, a leading vaccine candidate, were measured, and no correlation with protection was observed in these individuals. The anti-$MSP1_{19}$ antibodies were predominantly of the IgG1 isotype, in contrast to the IgG3 predominance noted for MSP4.

Immunity to *Plasmodium falciparum* blood stage infection can be passively transferred by immune sera, suggesting that antibodies against asexual blood stage parasites play an important role in protective immunity (16, 34). Identification of the antigenic targets of such potentially protective antibody responses following natural infection can aid understanding of the host-parasite relationship and provide information beneficial to the selection of candidate antigens for malaria vaccines. Several *P. falciparum* asexual blood stage antigens have been examined in immunoepidemiological surveys conducted in malaria-endemic areas and are recognized by the immune responses of individuals exposed to natural infection. These antigens include MSP1 (3, 9, 20, 21, 31, 32, 35), MSP2 (1, 2, 33, 38, 39), MSP3 (30), AMA1 (40), RESA (1, 7), and rhoptry-associated proteins 1 and 2 (37). In some cases, positive associations are observed between the antibody responses and clinical protection against malaria infection (1–3, 7, 21, 31, 35).

Merozoite surface protein 4 (MSP4) is a newly identified glycosylphosphatidylinositol (GPI)-anchored integral membrane protein that possesses an epidermal growth factor (EGF)-like domain at the carboxyl terminus of the protein (29). MSP4 is immunogenic in laboratory animals (41), and antibodies raised to it can inhibit parasite growth in vitro (T. Wu, unpublished data). Studies with the murine homologue of MSP4 indicate that this protein is capable of inducing protective immunity in mice against lethal challenge with *Plasmodium yoelii* (26). This in turn suggests that MSP4 has potential as a component of a subunit vaccine for human malaria. Evaluation of MSP4 as a potential vaccine candidate requires an understanding of the antibody responses induced by natural infection. In this paper, we report a study examining the naturally acquired antibodies to MSP4 in a population living in the Khanh-Hoa region of southern-central Vietnam, where malaria is highly endemic. The correlation between MSP4-specific antibodies and protective immunity to *P. falciparum* infection was also investigated. As a comparison, antibodies to $MSP1_{19}$, a leading vaccine candidate, were measured in the same study population, and their correlation with protective immunity was determined.

MATERIALS AND METHODS

Study subjects and serum samples. The serum samples examined in this study were collected from residents living in the Khanh-Nam Commune, located 60 km inland from the coastal city of Nha Trang in the Khanh Vinh District of Khanh-Hoa Province in southern central Vietnam. We classified older children and adults living in this area as semi-immune based on the observation that only about half of those with parasitemia described symptoms consistent with malaria infection and that these symptoms were often mild (e.g., headache). Three species of human malaria-causing organisms are endemic to this area. Surveys taken at the time of this study (1994) showed blood smear positivity rates of

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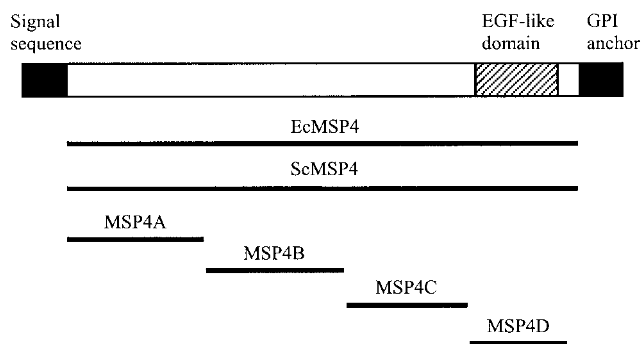

FIG. 1. Schematic (to scale) showing the positions of the various recombinant MSP4 proteins. EcMSP4-His and ScMSP4-His contain the same predicted mature MSP4 coding sequence but are produced in different expression systems. Both proteins contain a hexahistidine tag at the C terminus. MSP4A, MSP4B, MSP4C, and MSP4D are four recombinant MSP4 fragments, each spanning approximately one-quarter of the mature MSP4. All four MSP4 fragments are produced as GST fusion proteins. GPI, glycosylphosphatidylinositol.

between 25 and 30%, with approximately 60% of these infections due to *P. falciparum*, 30% to *Plasmodium vivax*, and 10% to *Plasmodium malariae*. At the commencement of the study in June 1994 (T0), blood samples were obtained with informed consent from 134 volunteers aged from 9 to 55 years (mean age, 27.5 years). These volunteers were radically treated with quinine sulfate (10 mg/kg three times a day, days 0 to 3), doxycycline hyclate (100 mg twice a day, days 0 to 10), and primaquine phosphate (30 mg base once a day, days 0 to 14), a regimen that in our experience consistently eliminates all preerythrocytic and erythrocytic stage parasites, including hypnozoites. These volunteers were followed up daily by questioning for symptoms and weekly by obtaining a peripheral blood smear by finger prick, for a period of 6 months. Additional blood smears were collected from individuals complaining of symptoms consistent with malaria infection. All smears were read on site, and all volunteers with positive blood smears were treated with mefloquine (15 mg/kg) and followed up for 28 days to ensure clearance of parasitemia. A second blood sample (T1) was collected at the time of treatment, and a third (T28) was collected 28 days later. No volunteers developing parasitemia during weekly surveillance had recurrent parasitemia during the 28 days of follow-up after mefloquine treatment. All blood smears were later reread by an expert microscopist in order to confirm the accuracy of field readings.

Of the 112 individuals who completed surveillance, 47 became parasitemic with *P. falciparum*, 32 became parasitemic with another species of *Plasmodium* but not *P. falciparum*, and 33 did not develop a positive blood smear during the 6-month study period. Overall, infections were detected in about 70% of the individuals over the 6-month study period; their parasitemia-free intervals (time to infection) were between 36 and 156 days.

Recombinant MSP4 proteins. Two recombinant full-length MSP4 proteins were used as target antigens to measure total anti-MSP4 antibodies, each of which contained the same sequence encoding the mature MSP4 protein but was produced in a different expression system (Fig. 1). EcMSP4-His was a product expressed in *Escherichia coli* (41), and ScMSP4-His was a product derived from the yeast *Saccharomyces cerevisiae* (42). Both proteins contained a hexahistidine tag at the C terminus. For measurement of the epitope specificity of the anti-MSP4 antibodies, four glutathione S-transferase (GST) fusion proteins (GST-MSP4A, GST-MSP4B, GST-MSP4C, and GST-MSP4D) were used, each of which contained a sequence spanning approximately one quarter of the mature MSP4 (41) (Fig. 1). To determine conformation-dependent epitopes, the recombinant MSP4 proteins were reduced and alkylated as described previously (41).

Recombinant $MSP1_{19}$. $MSP1_{19}$, the carboxyl-terminal fragment of MSP1 that contains two EGF-like domains, was expressed as a GST fusion protein in *E. coli*. The correct conformation of this recombinant protein has been demonstrated by its reactivity with several biologically important monoclonal antibodies (11).

Antibody assays. The reactivity of human sera with recombinant proteins was examined by enzyme-linked immunosorbent assay (ELISA) as follows. Flat-bottomed microtiter plates (Immulon 2; Dynatech Laboratories, Chantilly, Va.) were coated with 50 μl of recombinant proteins at 1 μg/ml (for the hexahistidine-tagged proteins) or 2 μg/ml (for the GST fusion proteins) per well diluted in phosphate-buffered saline (PBS). After incubation overnight at 4°C, the plates were washed five times with PBS–0.05% Tween 20 (vol/vol) (PBST) using a Bio-Rad model 1575 Immuno Wash (Bio-Rad Laboratories, Hercules, Calif.) and blocked with 400 μl of 5% (wt/vol) skim milk in PBST per well for 1 h at room temperature (RT). Serum samples were diluted 1:500 or 1:5,000 in blocking buffer and added at 50 μl/well to duplicate wells. After incubation at RT for 2 h, the plates were washed and incubated for 2 h at RT with 50 μl of alkaline phosphatase-conjugated sheep anti-human immunoglobulins (Igs; Silenus Laboratories, Melbourne, Victoria, Australia) per well diluted 1:2,000 in blocking buffer. The plates were then washed, 75 μl of ρ-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, Mo.) (1 mg/ml dissolved in 0.1 M carbonate buffer [pH 9.6]) with 1 mM $MgCl_2$ was added to each well, and the plates were incubated for another 2 h at RT. The optical density (OD) was determined at 405 nm using a Bio-Rad model 405 microplate reader (Bio-Rad Laboratories). Each serum sample was tested against either GST or PBS as a negative control for the GST fusion proteins and the hexahistidine-tagged proteins, respectively. The specific OD values were calculated by subtracting the control OD value from the value obtained from the fusion protein. Positive sera were defined as those that give an OD value greater than the mean plus 3 standard deviations of OD values obtained with sera taken from 30 Australian blood donors with no history of exposure to malaria.

For detection of the Ig isotypes of the anti-MSP4 and anti-$MSP1_{19}$ antibodies, an isotype-specific ELISA was performed. Briefly, microtiter plates were coated with a recombinant protein, followed by incubation with the human sera as described above. A panel of monoclonal antibodies specific to human Ig subclasses (Sigma Chemical Co.) were diluted 1:1,000 in blocking buffer and applied to separate wells at 50 μl/well. These monoclonal antibodies were anti-human IgG1 (clone SG-16), anti-human IgG2 (clone HP-6014), anti-human IgG3 (clone HP-6050), anti-human IgG4 (clone HP-6025), and anti-human IgM (clone MB-11). The plates were incubated at RT for 1 h and washed with PBST, followed by addition of 50 μl of alkaline phosphatase-conjugated sheep anti-mouse Igs (Silenus Laboratories, Melbourne, Victoria, Australia) per well. The plates were then developed with ρ-nitrophenyl phosphate as detailed above. In light of the affinity difference between the isotype-specific monoclonal antibodies, the isotype-specific OD values were adjusted by calibrating the assay using a reference serum (human standard serum NOR-01; Nordic Immunology) in which the content of each Ig subclass had been precisely determined. By coating microtiter plates with the reference serum and incubating with the human isotype-specific monoclonal antibodies, the OD values obtained were compared with the actual values for the reference serum and used to calculate compensation factors for the different isotypes, which are the ratios of OD for the given isotype to that of IgG1. The derived compensation factors for IgG1, IgG2, IgG3, and IgG4 were 1, 0.37, 1.07, and 1.71, respectively, and they were used to adjust the ELISA values.

Data analysis. Statistical analysis was performed using Gradhpad Prism software (Gradhpad Software Incorporated). The chi-square test was used to compare proportions of antibody responders in different groups, whereas the Wilcoxon and Mann-Whitney tests were used to compare the antibody levels between groups for paired and unpaired data, respectively. Spearman's rank correlation test was used to correlate antibody reactivity with pairs of individual antigens and to assess associations between antibody levels of different isotypes.

RESULTS

Prevalence and magnitude of total anti-MSP4 antibodies. A total of 342 serum samples taken from 134 subjects were examined for antibody responses to MSP4, including samples collected at the beginning of the survey (prior to radical cure, T0), samples taken at the time of treatment from individuals acquiring *Plasmodium* parasitemia (T1), and samples from the same treated individuals collected 28 days after treatment (T28). All sera were tested at a 1:5,000 dilution against the two full-length recombinant MSP4 proteins EcMSP4-His and ScMSP4-His. As summarized in Table 1, a high prevalence of anti-MSP4 antibodies was observed in these serum samples, and the level of the anti-MSP4 antibodies was also high. Although the endpoint titers were not determined, the fact that 82 and 94% of sera were positive at a 1:5,000 dilution against EcMSP4 and ScMSP4, respectively, suggested that most of the sera had a specific antibody titer greater than 1:5,000.

TABLE 1. Prevalence and magnitude of anti-MSP4 antibodies[a]

Target antigen	Cut-off OD[b]	Positive proportion[c] (%)	OD values[d]				
			Min	Max	Median	LQ	UQ
EcMSP4-His	0.072	82.2	−0.012	2.325	0.316	0.107	0.715
ScMSP4-His	0.014	93.9	−0.004	2.397	0.294	0.091	0.745

[a] A total of 342 serum samples collected from the Vietnam residents were tested by ELISA;
[b] The cut-off OD values are calculated as the mean + 3 standard deviations for the ODs of 30 control sera from nonimmune individuals;
[c] The positive sera are defined as those that give an OD value greater than the cut-off OD.
[d] OD values are shown as minimum (Min), maximum (Max), median, and lower (LQ) and upper (UQ) quartiles.

A comparison of the OD values measured against EcMSP4-His and ScMSP4-His showed a very high correlation (r_s = 0.986, $P < 0.001$); however, the percentage of positive sera measured against ScMSP4-His was higher (Table 1). The level of anti-MSP4 antibodies measured against either EcMSP4-His or ScMSP4-His had no correlation with the age of the residents, with Spearman's correlation coefficient being −0.047 (P = 0.442) and −0.064 (P = 0.293), respectively.

Epitope specificity of anti-MSP4 antibodies. A subset of the serum samples (n = 174) were examined for epitope specificity of the anti-MSP4 antibodies. The sera were tested at a 1:500 dilution against the four recombinant MSP4 fragments MSP4A, MSP4B, MSP4C, and MSP4D. All regions of MSP4 were recognized by these human sera. The percentage of positive responses to MSP4A, MSP4B, MSP4C, and MSP4D was 78.7, 92.5, 75.3, and 71.3%, respectively. There was a range of reactivity detected with OD values; the medians (lower quartile, upper quartile) were 0.376 (0.116, 1.359) for MSP4A, 0.789 (0.308, 1.602) for MSP4B, 0.346 (0.127, 0.775) for MSP4C, and 0.206 (0.060, 0.498) for MSP4D.

To determine whether recognition of the EGF-like domain by human antibodies was conformation dependent, the recombinant MSP4D was reduced and alkylated and its reactivity with the serum samples was compared to that with the nonreduced form of MSP4D. The epitope(s) recognized by human antibodies in MSP4D was shown to be reduction sensitive, and reactivity was almost completely abolished after reduction and alkylation of the recombinant protein. Only 1.7% of the sera were positive for the reduced and alkylated MSP4D, versus 71.3% for the nonreduced MSP4D. The median (lower quartile, upper quartile) of the OD values was −0.001 (−0.016, 0.008) for the reduced and alkylated MSP4D and 0.206 (0.060, 0.498) for the nonreduced MSP4D (Wilcoxon test, $P < 0.001$). In contrast, the reactivity of human sera with the other three fragments (MSP4A, MSP4B, and MSP4C) was not affected by reduction and alkylation (data not shown).

Individuals tended to respond to multiple epitopes on the protein, and significant correlations were observed between antibody responses to different regions of MSP4 except that between MSP4B and MSP4D. The Spearman's correlation coefficients (P values) between MSP4A and MS4B, MSP4A and MSP4C, MSP4A and MSP4D, MSP4B and MSP4C, MSP4B and MSP4D, and MSP4C and MSP4D were 0.286 (0.001), 0.484 (0.001), 0.268 (0.001), 0.377 (0.001), 0.122 (0.110), and 0.360 (0.001), respectively. However, a number of sera had high levels of antibodies to one region but low or no response to the others (data not shown).

Isotype distribution of anti-MSP4 antibodies. It has previously been suggested that the isotypes or isotype balance of antibodies rather than the levels of antibodies per se are important in antibody-mediated protection against malaria (10). Therefore, isotype distribution of the anti-MSP4 antibodies was examined using the same set of serum samples that were used for the determination of epitope specificity. The percentages of sera positive for IgG1, IgG2, IgG3, IgG4, and IgM were 85.6, 36.8, 77.0, 5.2, and 25.3%, respectively. OD levels were higher for the IgG3 isotype, followed by IgG1 (Fig. 2). In contrast, IgG2 was present at a low level, and IgG4 was hardly detectable. IgM was present at a level higher than IgG2 but lower than IgG1. This pattern was not dependent on whether the ELISA plates were coated with EcMSP4-His or ScMSP4-His (data not shown).

Correlations between the ODs measured for the different antibody isotypes were analyzed except for IgG4, for which there was an insufficient number of positive responses to allow testing. Significant correlations were observed between all of the analyzed antibody isotypes; the highest correlation was found between the two cytophilic isotypes IgG1 and IgG3 (r_s = 0.448, $P < 0.001$).

Comparison of anti-MSP4 antibodies at different time points. The serum samples taken at T0, T1, and T28 from the 47 individuals who acquired *P. falciparum* parasitemia were analyzed to compare the change in anti-MSP4 antibodies at different time points. As shown in Fig. 3A and B, the total anti-MSP4 antibodies and antibodies directed to MSP4A, MSP4B, and MSP4C remained at similar levels at T1 compared to T0, but increased significantly at T28. IgG1 increased

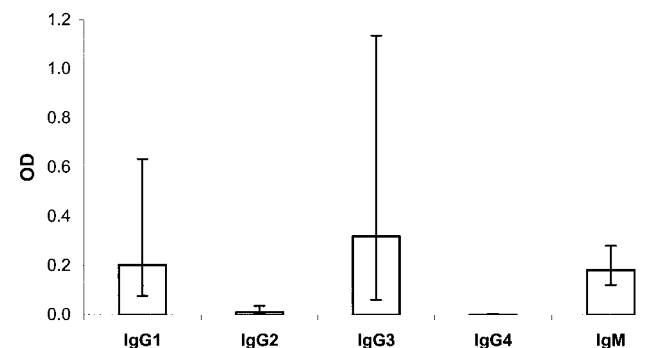

FIG. 2. Isotype-specific antibody responses to MSP4. Bars indicate medians of the OD values, and error bars indicate the upper and lower quartile values.

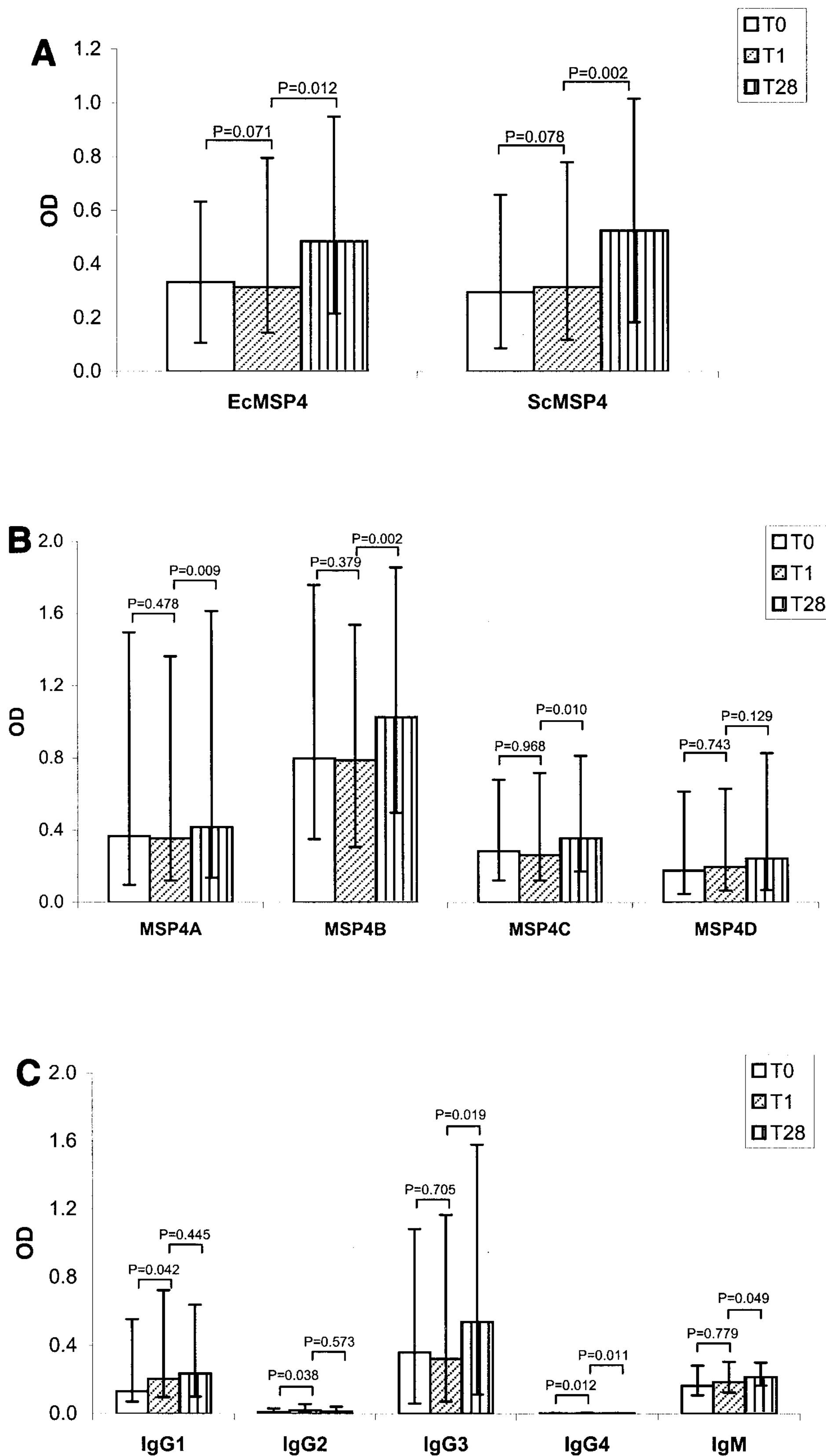

FIG. 3. Comparison of antibody responses to MSP4 in serum samples collected from Vietnam residents at different time points. (A) Antibody responses to full-length MSP4 proteins. (B) Antibody responses to four different regions of MSP4. (C) Anti-MSP4 antibodies of different isotypes. T0 samples were collected from individuals at the beginning of the survey, and T1 and T28 samples were collected from individuals who acquired and were treated for *P. falciparum* parasitemia (T1) and 28 days after treatment (T28) respectively. Bars indicate medians of the OD values, and error bars indicate the upper and lower quartile values. *P* values (Wilcoxon test) between antibody levels in the matched pairs of serum samples are shown on the graphs.

TABLE 2. Antibody responses to MSP4 proteins in Vietnam residents susceptible to and potentially protected against *P. falciparum* infection based on the presence or absence of *P. falciparum* parasitemia during the surveillance period

Target antigen	Antibody prevalence (%)[a]			Median antibody level (OD)[b]		
	Susceptible (n = 47)	Protected (n = 33)	P (χ^2)[c]	Susceptible (n = 47)	Protected (n = 33)	P (U)[c]
EcMSP4-His	80.9	84.8	0.643	0.333 (0.107, 0.633)	0.246 (0.112, 0.404)	0.390
ScMSP4-His	93.6	90.9	0.651	0.296 (0.087, 0.659)	0.210 (0.089, 0.404)	0.280
MSP4A	76.6	78.8	0.817	0.368 (0.098, 1.496)	0.384 (0.133, 0.900)	0.864
MSP4B	91.5	84.8	0.355	0.799 (0.352, 1.759)	0.541 (0.162, 1.179)	0.186
MSP4C	70.2	81.8	0.238	0.285 (0.122, 0.681)	0.461 (0.168, 1.119)	0.113
MSP4D	63.8	75.8	0.257	0.177 (0.048, 0.615)	0.173 (0.077, 0.383)	0.657

[a] Antibody prevalence is represented as the proportion of positive sera, which are defined as those giving an OD value greater than the mean + 3 standard deviations for the ODs of 30 control sera from nonimmune individuals.
[b] Antibody level is represented as the median (lower quartile, upper quartile) of the OD values.
[c] Antibody prevalence in susceptible and protected individuals is compared using the χ^2 test, whereas antibody level in the two different groups is compared using the Mann-Whitney test. P (χ^2) and P (U) are the associated levels of significance, respectively. The threshold for significance is 0.0085 after an adjustment is made for the number of comparisons.

significantly between time points T0 and T1, whereas IgG3 and IgM showed significantly increased levels during the convalescent period (Fig. 3C). The matched pairs of serum samples collected at T0, T1, and T28 were all closely correlated in the levels of anti-MSP4 antibodies, either to specific regions or of specific isotypes (data not shown).

Association of anti-MSP4 antibodies with protective immunity. Forty-seven of the 112 individuals (42%) completing radical cure acquired *P. falciparum* parasitemia during the 6-month surveillance period, while 65 remained smear negative or acquired parasitemia with *P. vivax* or *P. malariae*. It is possible that these different outcomes reflect the level of protective antibodies against *P. falciparum* present at the beginning of surveillance. Based on this assumption, we have classified those acquiring *P. falciparum* parasitemia as susceptible and those not acquiring parasitemia with any species of malaria as potentially protected. The individuals who developed parasitemia with *P. vivax* or *P. malariae* were excluded from this analysis due to the possibility that they were indeed susceptible to *P. falciparum* but that this susceptibility was not revealed because of an intervening infection with another species. The MSP4 homologues in *P. vivax* and *P. malariae* have different sequences from that in *P. falciparum* (C. Black, unpublished data), making cross-protection unlikely.

The antibody responses to MSP4 in the serum samples taken at T0 from the different groups are summarized in Table 2 and Table 3. Statistical analysis revealed no significant difference between the proportion of positive sera from individuals susceptible to and potentially protected against *P. falciparum* infection. The levels of the antibodies were not significantly higher in the potentially protected group than in the susceptible group. In the group of susceptible individuals, no correlation was observed between the antibody responses to MSP4, either to specific regions or of specific isotypes, and the time to reinfection with *P. falciparum* ($P > 0.05$ in all cases). Excluding the individuals who were parasitemic at T0 from the analysis did not change the result (data not shown).

Analysis of anti-MSP1$_{19}$ antibodies in the study population. Several immunoepidemiological studies have demonstrated positive associations between protective immunity against malaria infection and the antibody responses to MSP1, a well-studied protein that is a leading vaccine candidate (3, 21, 31, 35). To compare the antibody responses to MSP4 in our study population with those to MSP1, we have analyzed the anti-MSP1$_{19}$ antibodies in the same cohort of individuals. The 174 serum samples that were used to determine the epitope specificity and isotype distribution of anti-MSP4 antibodies were tested against a conformationally correct form of MSP1$_{19}$, the carboxyl-terminal region of MSP1 containing two EGF-like domains (11). The anti-MSP1$_{19}$ antibodies were observed at a high prevalence and level: 96.5% of the tested sera were positive at a 1:5,000 dilution, the range of the OD values being 0.612, 0.171, and 1.823 for the median and the lower and upper quartiles, respectively. The percentage of sera positive for IgG1, IgG2, IgG3, IgG4, and IgM was 75.4, 8.2, 40.9, 0.0, and 37.4%, respectively. The medians (lower quartile, upper quartile) of the OD values were 0.344 (0.077, 1.128) for IgG1, 0.014 (0.006, 0.025) for IgG2, 0.014 (0.004, 0.082) for IgG3, 0.000

TABLE 3. Ig-specific anti-MSP4 antibodies in Vietnam residents susceptible to and potentially protected against *P. falciparum* infection based on the presence or absence of *P. falciparum* parasitemia during the surveillance period[a]

Isotype	Antibody prevalence (%)			Median antibody level (OD)		
	Susceptible (n = 47)	Protected (n = 33)	P (χ^2)	Susceptible (n = 47)	Protected (n = 33)	P (U)
IgG1	87.2	66.7	0.027	0.132 (0.071, 0.555)	0.204 (0.031, 0.674)	0.584
IgG2	27.7	36.4	0.408	0.010 (0.005, 0.031)	0.010 (0.004, 0.053)	0.914
IgG3	76.6	72.7	0.694	0.360 (0.060, 1.082)	0.241 (0.048, 0.726)	0.476
IgG4	0	3.0	0.230	0.002 (−0.001, 0.005)	0.002 (0.000, 0.003)	0.445
IgM	23.4	21.2	0.817	0.164 (0.109, 0.283)	0.132 (0.089, 0.232)	0.485

[a] See Table 2, footnotes *a, b*, and *c*. The threshold for significance is 0.0102 after an adjustment is made for the number of comparisons.

TABLE 4. Anti-$MSP1_{19}$ antibodies in Vietnam residents susceptible to and potentially protected against *P. falciparum* infection based on the presence or absence of *P. falciparum* parasitemia during the surveillance period[a]

Isotype	Antibody prevalence (%)			Median antibody level (OD)		
	Susceptible (n = 47)	Protected (n = 33)	P (χ^2)	Susceptible (n = 47)	Protected (n = 33)	P (U)
Total Ig	95.7	97.0	1.000	0.667 (0.134, 1.775)	0.418 (0.118, 1.084)	0.636
IgG1	66.0	72.7	0.627	0.362 (0.037, 1.206)	0.315 (0.061, 0.673)	0.973
IgG2	6.4	9.1	0.687	0.014 (0.006, 0.024)	0.012 (0.007, 0.029)	0.476
IgG3	40.4	45.5	0.819	0.017 (0.004, 0.082)	0.014 (0.008, 0.114)	0.338
IgG4	0.0	0.0	—[b]	−0.001 (−0.005, 0.008)	0.000 (−0.003, 0.004)	0.696
IgM	34.0	42.4	0.488	0.312 (0.252, 0.536)	0.389 (0.233, 0.580)	0.513

[a] See Table 2, footnotes *a, b*, and *c*. The threshold for significance is 0.0085 after an adjustment is made for the number of comparisons.
[b] —, not compared due to negative value in both groups.

(−0.003, 0.007) for IgG4, and 0.330 (0.225, 0.539) for IgM. In contrast to MSP4, the anti-$MSP1_{19}$ antibodies have the highest level of IgG1 isotype.

Comparison of the anti-$MSP1_{19}$ antibodies, either the total Ig or the individual Ig isotypes, in the potentially protected and susceptible groups revealed no significant difference (Table 4). Analysis of the individuals in the susceptible group also did not reveal any significant correlation between anti-$MSP1_{19}$ antibodies and time to reinfection with *P. falciparum* ($P > 0.05$ in all cases). Comparison of the total anti-$MSP1_{19}$ antibodies at T0, T1, and T28 showed no significant change during the sampling times (data not shown).

DISCUSSION

This is the first study to examine the antibody responses to MSP4 induced by natural infection. It has been widely believed that a large proportion of the naturally acquired immunity to the asexual blood stage parasites is antibody based (4). Therefore, in this study work has focused on the antibody responses to MSP4, although cell-mediated immune responses may also exist and play a role in the immune state. It was observed that anti-MSP4 antibodies are highly prevalent and present at a high level in the individuals living in this malaria-endemic area, suggesting that MSP4 is a well-recognized asexual-stage parasite antigen. The lack of antibody response to MSP4 in some of the individuals is unlikely to be due to lack of exposure, since all residents have been exposed to parasites repeatedly. Although there is abundant evidence for genetic restriction of immune responses to discrete epitopes (12, 23), it is unlikely that the nonresponders are genetically unable to mount significant antibody responses to MSP4, as there are at least four distinct, non-cross-reactive epitopes in this protein (41). As some of these individuals were resistant to malaria infection, presumably they possessed antibodies and/or specific cellular responses to other erythrocytic stage antigens. Further studies are needed to examine whether these individuals have antibody responses to other merozoite surface proteins or to proteins targeted by other types of defense mechanism against erythrocytic or preerythrocytic stages.

It is interesting that the human sera reacted similarly with the two recombinant MSP4 proteins expressed in either *E. coli* or *S. cerevisiae*. It has been generally considered that proteins with disulfide bonds may not be properly folded in *E. coli* due to the reducing intracellular environment, and recombinant proteins secreted from *S. cerevisiae* are considered more likely to have a conformation that mimics their native counterparts (6, 13). The similarity of the two recombinant MSP4 proteins with respect to their reactivity with immune human sera could indicate that they have a similar secondary and tertiary conformation as well as the same sequence. ScMSP4-His appears to be a superior substrate, as reactivity was slightly higher. This may be due to expression of a more correctly conformed MSP4D region, which is a weak epitope relative to the others but heavily conformation dependent. Alternatively, the fact that ScMSP4-His is purer than EcMSP4-His may lead to a higher number of MSP4 molecules used in the ELISA, with consequently higher reactivity.

We found that MSP4D was more weakly recognized by immune sera than the other three regions of MSP4. This finding supports the proposition that EGF-like domains are relatively poorly immunogenic, although strongly conformationally dependent. Egan et al. have shown that $MSP1_{19}$, the C-terminal 19-kDa fragment of MSP1 which contains two EGF-like domains, is less well recognized than the 33-kDa processing fragment $MSP1_{33}$ (19), although the recombinant $MSP1_{19}$ proteins used are believed to conform closely to the secondary and tertiary structures of the native protein (11, 15, 25). It has been hypothesized that the complex disulfide-bonded structure of native $MSP1_{19}$ may inhibit antigen processing or presentation, and the lack of T-cell help may contribute to the lower prevalence of anti-$MSP1_{19}$ antibodies (19). The reduction sensitivity of MSP4D is also in agreement with the findings of Egan et al. (20), who demonstrated that antibodies to $MSP1_{19}$ in immune human sera tend to recognize disulfide bond-dependent epitopes, although minor linear epitopes are also present.

Overall, there was a moderately good correlation between an individual's response to the different regions of MSP4; however, a number of individuals exhibited remarkably different antibody responses to the different regions. These data demonstrated that the different regions of MSP4 have differential immunogenicity in the human host and the immunogenicity varies between individuals. The data also suggest that an antibody response to one region of a protein should not be taken as indicative of the overall response to the protein as a whole, even for a relatively small protein such as MSP4. A similar phenomenon has been described for MSP1 (9). This point may be of particular importance when antibodies to certain regions of the protein, but not to other regions, are involved in protective immunity, as in the case of MSP1 (8, 14, 27, 28, 31).

The anti-MSP4 antibodies were found to be mainly IgG1 and IgG3. Both subclasses have been reported to have opsonizing and complex-fixing properties (24). The predominance of IgG3 in the antibody responses to MSP4 is unusual, although it has been seen in another genetically distinct human population (unpublished data). This pattern has been described for MSP2 (22, 33, 38, 39) but not for other malaria antigens. For example, in MSP2-seropositive individuals in the Gambia, IgG1 antibodies are prevalent in children less than 10 years of age, whereas in adolescents and adults MSP2-specific antibodies are predominantly of IgG3 (38). In contrast, antibodies to MSP1 were found to be predominantly of IgG1 in all age groups (20). Our study in this Vietnamese population also revealed the predominance of IgG1 in the antibody responses to $MSP1_{19}$. This isotype difference is intriguing, given that MSP4 and $MSP1_{19}$ have sequence features in common, such as the presence of EGF-like domains and the lack of repeat regions. Several studies have reported that the prevalence of IgG3 responses to various malaria antigens increases with age and/or exposure, but for antigens other than MSP2, this does not lead to IgG3 predominance (5, 7, 17, 18, 36). Since all of the individuals investigated in this study were semi-immune adults or adolescents, it is unknown whether the subclass distribution of anti-MSP4 antibodies is the same in children. Further studies are required to determine whether an age-related switch from IgG1 to IgG3 also exists for the MSP4-specific antibodies.

The levels of the anti-MSP4 antibodies in the Vietnam residents did not decrease during the convalescent period, indicating that these antibodies are not short-lived. This stability in antibody levels is shared by the responses to $MSP1_{19}$. This may be a feature of this population of adults and adolescents with a history of many years of exposure to repeated infections. Alternatively, it is possible that the time scale (28 days), originally selected to monitor possible relapsed recrudescent infections, is not appropriate to define the duration of antibody responses. IgG3 has a serum half-life of only 8 days, but our data obtained from this Vietnamese population indicate that its preponderance in MSP4 recognition does not necessarily result in short-lived responses, as suggested for responses to other antigens in other areas where malaria is endemic (22).

No correlation has been observed in the study population between the presence of MSP4-specific antibodies at T0 and the absence of parasitemia during surveillance. Similarly, no such association has observed for antibodies to $MSP1_{19}$, a well-studied protein that is frequently reported to be positively correlated with protection from high parasitemias and reduced morbidity (3, 21, 31, 35). This suggests that the state of sterile immunity may be due to a different set of host factors than those responsible for controlling parasitemia or limiting morbidity. Accordingly, it would be worthwhile examining antibody responses to MSP4 in a different population for which these clinical and parasitological data are available. Such studies are now under way in a population of transmigrants who have experienced sequential malaria infections.

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