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Associations between Responses to the Rhoptry-Associated Membrane Antigen of *Plasmodium falciparum* and Immunity to Malaria Infection

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Rhoptry proteins participate in the invasion of red blood cells by merozoites during the malaria parasite's asexual-stage cycle. Interference with the rhoptry protein function has been shown to prevent invasion, and three rhoptry proteins have been suggested as potential components of a vaccine against malaria. Rhoptry-associated membrane antigen (RAMA) is a 170-kDa protein of *Plasmodium falciparum* which is processed to a 60-kDa mature form in the rhoptries. p60/RAMA is discharged from rhoptries of free merozoites and binds to the red-cell membrane before being internalized to form part of the parasitophorous vacuole of the newly developing ring. We examined the range of anti-RAMA responses in individuals living in an area of endemicity for malaria and determined its association with clinical immunity. RAMA is immunogenic during infections, and at least three epitopes within RAMA are recognized by hyperimmune sera in immunoblots. Sera from individuals living in a region of Vietnam where malaria is endemic possessed strong antibody responses toward two C-terminal regions of RAMA. Cytophilic antibody isotypes (immunoglobulin G1 [IgG1] and IgG3) predominated in humoral responses to both C-terminal epitopes. Acute episodes of *P. falciparum* infection result in significant boosting of levels of antibody to an epitope at the extreme C terminus of RAMA that harbors the red-cell-binding domain. Immunity to *P. falciparum* infection was linked to elevated levels of IgG3 responses to this functional domain of RAMA, suggesting that the region may contain a protective epitope useful for inclusion in a multiepitope vaccine against malaria.

Plasmodium falciparum malaria is responsible for >2 million deaths each year. With the increasing resistance of *Plasmodium* parasites to antimalarial drugs and of the *Anopheles* mosquito vector to commonly available insecticides, an effective vaccine that would protect nonimmune individuals from death would be valuable. The life cycle of malaria parasites is quite complex and provides a number of potential vaccine targets. Several proteins expressed by *P. falciparum* during the erythrocytic stages are being investigated as candidates for a subunit vaccine, among them rhoptry-associated proteins. The most important immune response for immunity to asexual-stage infection is believed to be humoral, although cell-mediated responses may also contribute to immunity.

Rhoptries are intracellular organelles of malaria parasites involved in the invasion of red blood cells (RBCs) by *Plasmodium* merozoites. Although their contents are only transiently accessible to antibodies, seroepidemiological studies have demonstrated the development of antibody responses to the rhoptry proteins RhopH3, RAP1, and RAP2 following infection (8, 12, 20, 23). In vitro growth inhibition assays have indicated that antibodies directed against the RAP1 and RAP2 proteins have inhibitory effects on *P. falciparum* growth in in vitro culture (6, 9, 14, 18). Moreover, immunization of *Saimiri* monkeys with RAP1 protected the animals from a lethal malaria parasite infection (17), suggesting that interference with the rhoptry protein function by immune responses can prevent RBC invasion.

Recently, the rhoptry-associated membrane antigen (RAMA) of *P. falciparum* was characterized (21). RAMA is expressed as a 170-kDa protein (p170) that contains three distinct repeat regions (R1, R2, and R3) within the N-terminal half. p170/RAMA is proteolytically processed in rhoptries to a 60-kDa mature form (p60), comprising the C-terminal part of the full-length precursor. The p60 form of RAMA is present in rhoptries of free merozoites and is discharged when the merozoites attach to RBCs, binding to the RBC membrane. The binding domain is located at the extreme C terminus of RAMA within an 82-residue region we call RAMA-E. In early ring stage parasites, p60/RAMA is found associated with the parasitophorous vacuole. Here, we show that there are at least three epitopes within RAMA recognized by sera from individuals chronically exposed to malaria and that the immunodominant epitopes are located within the middle and C-terminal parts of the protein. We also examine antibody responses to RAMA in a population of individuals living in a region of Vietnam where malaria is endemic and show a correlation between immunoglobulin G3 (IgG3) responses to RAMA-E, the region of the protein that contains the RBC-binding domain, and a state of clinical immunity to infection.

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MATERIALS AND METHODS

Ethical committees. This study has the approval of the Ethical Review Committee at Naval Medical Research Unit 2, Jakarta, Indonesia; the Ethical Review Committee at the Institute of Malariology, Parasitology and Entomology, Hanoi,

Vietnam; and the Monash University Standing Committee on Ethics in Research Involving Humans, Melbourne, Australia.

Serum samples and recombinant proteins. The serum samples examined in this study were collected from residents living in the Khanh-Nam Commune of Khanh-Hoa Province in south-central Vietnam. Three species of *Plasmodium*, *P. falciparum*, *P. vivax*, and *P. malariae*, are endemic to this area. Surveys taken at the time of this study (1994) showed blood smear positivity rates of 14.4% for *P. falciparum*, 11.3% for *P. vivax*, and 1.0% for *P. malariae*. We classified older children and adults living in this area as semi-immune based on the observation that only half of parasitemic individuals described symptoms consistent with malaria and that these symptoms were often mild (e.g., headache). The study subjects and collection of serum samples have been described previously (22). Briefly, at the commencement of the study in June 1994 (T_0), blood samples were obtained with informed consent from 134 volunteers aged between 9 and 55 years, followed by radical treatment with quinine sulfate, doxycycline hyclate, and primaquine phosphate. These volunteers were then monitored daily by questioning them for symptoms and weekly by obtaining peripheral blood smears by finger prick for a period of 6 months. Individuals who developed patent parasitemia over the course of the study were treated, and blood samples were collected at the time of treatment (T_1) and 28 days later (T_{28}). Parasitemia-free intervals (times to infection) were between 36 and 156 days. Of the 110 individuals who completed surveillance, 48 became parasitemic with *P. falciparum*, 31 became parasitemic with another species of *Plasmodium* but not *P. falciparum*, and 31 did not develop a positive blood smear. Overall, infections were detected in ~72% of the individuals over the 6-month surveillance period. No volunteers had recurrent parasitemia during the 28 days of follow-up after mefloquine treatment.

Samples taken from 30 Australian individuals with no exposure to malaria parasites and hyperimmune sera obtained from 26 Papua New Guinean (PNG) individuals chronically exposed to malaria parasites were used as negative and positive controls, respectively.

The target antigens for measuring antibody responses were five recombinant RAMA fragments and the C-terminal region of RhopH3 (Ag44) (5), expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* as described previously (21). An extreme N-terminal fragment of RAMA (RAMA-A) was expressed as a GST fusion protein as before (21), using primers p869 (5′-gctaggatccACATATTTAGAACAAATAAAAAATGGT-3′) and p1005 (5′-tagcgtcgacGTCATTTTCCAACTTATTTATTTC-3′) for PCR amplification from a cDNA template (added extensions are shown in lowercase letters and introduced restriction sites are underlined). GST was used as a negative control to determine antibody responses specific to the RAMA or Ag44 portions of fusion proteins.

ELISAs. Anti-RAMA and anti-RhopH3 reactivities were examined by enzyme-linked immunosorbent assay (ELISA) as described previously (22) with the following modifications. Fifty microliters of a 1-μg/ml dilution of target proteins was used to coat the microtiter plates. Serum samples were tested at dilutions of 1:500. For the determination of antibody isotypes, the optical density (OD) was measured after 24 h of incubation with *p*-nitrophenyl sulfate. The cutoff for defining sera as positive was set as the mean plus 2 standard deviations of OD values for sera from Australian donors with no exposure to malaria parasites.

Data analysis. Statistical analysis of data was performed using Prism version 3.0 (Graphpad Software Inc.). Fisher's exact test was used to compare the prevalences of antibody responses. To compare antibody responses between groups, the Mann-Whitney test and the Wilcoxon test were used for the unpaired and paired data, respectively. Spearman's rank correlation test was used to determine the association of antibody responses with patients' ages and time to reinfection, as well as to correlate the responses to different target antigens. The significance thresholds for analyzing total and isotype-specific antibody response data were adjusted for multiple comparisons to maintain the chance of randomly obtaining at least one statistically significant result at 5%. Based on the formula $P_t = 1.00 - 0.95^{1/n}$, where P_t is the significance threshold and n is the number of comparisons, the significance thresholds for analyzing total antibody responses and isotype-specific antibody responses were calculated and set as for six (0.0085) or five (0.0102) comparisons, respectively.

SDS-PAGE and immunoblotting. Recombinant proteins (100 ng) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and blotted as described previously (2). Recombinant fragments A and B of merozoite surface protein 4 (MSP4) were kindly provided by Lina Wang. Pooled hyperimmune human sera were used as primary antibodies and detected with anti-human immunoglobin conjugated with horseradish peroxidase and developed as described previously (2).

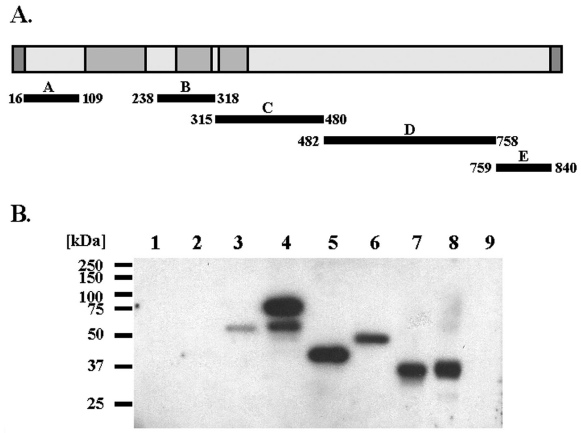

FIG. 1. (A) Schematic representation of RAMA protein and expressed fragments. Boxed and shaded are the signal peptide (residues 1-15); the repeat regions R1 (residues 96 to 225), R2 (residues 260 to 314), and R3 (residues 319 to 373); and the C-terminal peptide for glycosylphosphatidylinositol anchoring (residues 841 to 861). The positions of expression constructs (in residues) are indicated. (B) Epitope mapping. Shown is an immunoblot with immune human sera on 100 ng of recombinant proteins. Lane 1, RAMA-A; lane 2, RAMA-B; lane 3, RAMA-C; lane 4, RAMA-D; lane 5, RAMA-E; lane 6, Ag44; lane 7, MSP4/A; lane 8, MSP4/B; lane 9, GST. The positions of molecular mass markers are shown on the left.

RESULTS

RAMA epitope mapping. In order to detect and map epitopes within RAMA that are commonly recognized by humans exposed to repeated *P. falciparum* infections, we probed a panel of malaria parasite proteins with pooled sera from 26 PNG individuals chronically exposed to malaria. The immunoblots were loaded with 100 ng of each RAMA fragment, Ag44, and fragments MSP4/A and MSP4/B (all proteins were expressed as recombinant GST fusions) and GST alone as a negative control. As shown in Fig. 1, immune human sera recognized three regions of RAMA, fragments C, D, and E (lanes 3, 4, and 5), indicating that there are at least three epitopes within the RAMA protein that are recognized by humoral immune responses induced by *P. falciparum* infections in a field population. The reactivities with fragments RAMA-D and -E were much stronger than with fragment RAMA-C and comparable to reactivities with the known malaria antigens Ag44 (lane 6) and MSP4 (lanes 7 and 8). Two bands were observed in the RAMA-D lane corresponding to prominent breakdown products of the full-length fusion protein. No GST band was detected in lane 9, indicating that the observed reactivity with recombinant proteins was indeed directed against the *P. falciparum* protein portions of fusions. All recombinant proteins were recognized by anti-GST sera (not shown).

Anti-RAMA serum reactivities. The optimal dilution for testing anti-RAMA antibody responses was determined by performing preliminary ELISAs with pooled sera from PNG individuals and with sera from 10 randomly chosen individuals from the Vietnam field study. RAMA fragments B, C, D, and E were used in this initial testing, and the optimal serum dilution was determined to be 1:500, giving specific reactivity OD values of 0.15 (RAMA-B), 0.24 (RAMA-C), 1.48 (RAMA-D), and 1.59 (RAMA-E) for the PNG sera and mean

TABLE 1. Prevalences and magnitudes of anti-RAMA antibody responses in study population[a]

Antigen	Cutoff OD[b]	Prevalence[c]	OD values[d]		
			Median	LQ	UQ
RAMA-A	0.75	21.33	0.440	0.250	0.700
RAMA-B	0.04	60.84	0.090	0.000	0.495
RAMA-C	0.20	72.03	0.510	0.155	1.125
RAMA-D	0.09	86.71	0.755	0.290	1.430
RAMA-E	0.05	83.22	0.525	0.155	1.260

[a] Results from testing of 286 serum samples against five RAMA fragments and Ag44 by ELISA.
[b] Cutoff OD values were set as the mean plus 2 standard deviations of OD values for sera from Australian donors not exposed to malaria.
[c] Percentage of positive sera defined as those that gave OD values greater than the cutoff OD.
[d] OD values are presented as the median and lower (LQ) and upper (UQ) quartiles.

OD values of 0.43 (RAMA-B), 0.71 (RAMA-C), 0.79 (RAMA-D), and 0.78 (RAMA-E) for the Vietnamese sera. However, even at 1:5,000 dilution, the PNG and Vietnamese sera still showed specific reactivity, with OD values for the PNG sera of 0.01 (RAMA-C), 0.25 (RAMA-D), and 0.25 (RAMA-E) and for the Vietnamese sera of 0.03 (RAMA-B), 0.10 (RAMA-C), 0.15 (RAMA-D), and 0.10 (RAMA-E).

Prevalences and magnitudes of antibody responses. A total of 286 serum samples obtained from 119 individuals during the course of the field study were tested for reactivity against recombinant fragments of RAMA. As summarized in Table 1, a high prevalence of positive sera reacting at high magnitude with the C-terminal part of RAMA, fragments D and E, was found. Reactivity was also observed to a lesser extent with fragment C, which contains the R3 repeat region. The level of antibody responses to fragment B of RAMA, comprising the R2 repeat region (21), was very low, although the percentage of positive sera was quite high. Interestingly, a high cutoff value, based on the antibody responses of individuals with no history of exposure to malaria, was observed for fragment A of RAMA.

Individuals tended to respond with similar strengths to different epitopes within RAMA, with the exception of fragment A. A high correlation was observed between responses to fragments B, C, D, and E, with Spearman's correlation coefficients (P values) of <0.0001 for all combinations of fragments. Although there was no correlation between antibody responses and an individual's age or time to reinfection, a trend could be observed such that individuals with higher antibody responses to RAMA fragments tended to be older and to have longer time intervals before reinfection.

Association of antibody responses with antimalarial protection. Not all individuals participating in the study became parasitemic with *P. falciparum* during the surveillance period, and the observed lack of infection may reflect higher levels of protective antibodies in these subjects. To determine whether anti-RAMA antibodies are associated with the clinically immune state, we compared the antibody responses of the susceptible group (48 individuals who acquired *P. falciparum* parasitemia) and the protected group (31 individuals who did not develop a *Plasmodium*-positive blood smear) to RAMA fragments. As summarized in Table 2, there were no statistically significant differences in either the prevalence or magnitude of the total antibody response to any RAMA fragment in the two groups. However, as a general trend, antibody responses in the protected group tended to be higher than in the susceptible individuals, particularly against fragment RAMA-E.

Association of antibody isotype distribution with protective immunity. Fragments D and E comprise the C-terminal region of RAMA present in the mature 60-kDa form of the protein, with RAMA-E containing the RBC-binding domain and harboring the immunodominant epitopes of RAMA. Since it has been suggested that not only the magnitude of antibody responses but also the balance of antibody isotypes may be important in antimalarial immunity (3), we examined the distribution of antibody isotypes to RAMA-D and RAMA-E. We analyzed T_0 serum samples of positive responders (with an OD value greater than the cutoff OD) and found that, overall, the cytophilic antibody isotypes IgG1 and IgG3 were predominant in antibody responses to both RAMA-D and -E. Next, we compared the isotype distribution of responses at T_0 in the protected and susceptible groups to determine whether a predominance of specific anti-RAMA isotypes might contribute to clinical immunity. As summarized in Table 3, anti-RAMA-D antibody responses had similar isotype distributions in both groups. For RAMA-E, however, significant differences were observed between the protected and susceptible individuals (Table 4). IgG3 responses to RAMA-E were more prevalent and of greater magnitude in the protected group. The difference in magnitude is particularly significant, even after correcting for multiple comparisons. There was a clear difference observed between the ratios of IgG3 to IgG1, with a median

TABLE 2. Comparison of antibody responses of individuals susceptible to and protected against *P. falciparum* infection during the course of the study

Antigen	Prevalence[a]			Antibody level[b]		
	Protected (n = 31)	Susceptible (n = 48)	P[c]	Protected (n = 31)	Susceptible (n = 48)	P[d]
RAMA-A	16.13	12.50	0.744	0.490 (0.340, 0.690)	0.350 (0.200, 0.460)	0.016
RAMA-B	67.74	58.33	0.480	0.120 (0.025, 0.630)	0.095 (0.000, 0.520)	0.691
RAMA-C	70.97	79.17	0.430	0.570 (0.165, 1.480)	0.575 (0.260, 1.200)	0.916
RAMA-D	90.32	87.50	1.000	0.890 (0.300, 1.505)	0.875 (0.415, 1.505)	0.511
RAMA-E	80.65	85.42	0.757	1.070 (0.345, 1.735)	0.365 (0.145, 1.020)	0.062

[a] Percentage of positive sera defined as those that gave OD values greater than the cutoff OD.
[b] Antibody levels are presented as median OD values (lower quartile, upper quartile).
[c] Antibody prevalences of protected and susceptible groups were compared using Fisher's exact test.
[d] Antibody levels of protected and susceptible groups were compared using the Mann-Whitney test.

TABLE 3. Comparison of antibody isotypes in responders to RAMA-D among individuals susceptible to and protected against *P. falciparum* infection during the course of the study

Antibody isotype	Prevalence[a]			Antibody level[b]		
	Protected (n = 28)	Susceptible (n = 42)	P^c	Protected (n = 28)	Susceptible (n = 42)	P^d
IgG1	67.86	78.57	0.4047	0.155 (0.050, 0.605)	0.295 (0.105, 0.580)	0.544
IgG2	39.29	30.95	0.6080	0.020 (0.005, 0.035)	0.020 (0.010, 0.045)	0.866
IgG3	85.71	83.33	1.0000	0.060 (0.025, 0.130)	0.085 (0.025, 0.205)	0.560
IgG4	14.29	38.10	0.0349	0.000 (0.000, 0.000)	0.010 (0.000, 0.020)	0.013
IgM	78.57	69.05	0.4241	0.100 (0.055, 0.480)	0.165 (0.025, 0.330)	0.871

[a] Percentage of positive sera defined as those that gave OD values greater than the cutoff OD.
[b] Antibody levels are presented as median OD values (lower quartile, upper quartile).
[c] Antibody prevalences of protected and susceptible groups were compared using Fisher's exact test.
[d] Antibody levels of protected and susceptible groups were compared using the Mann-Whitney test.

value of 1.13 in protected individuals and 0.33 in the susceptible group. In addition, the balance of cytophilic and noncytophilic antibody classes also differed between two groups, with median ratio values of 5.550 and 1.525 in the protected and susceptible groups, respectively. As IgG3-type antibodies have been implicated in anti-malarial immunity (3), the elevated levels of anti-RAMA-E IgG3 antibodies in the group of protected individuals might be linked to protection from *P. falciparum* infection.

Magnitude of antibody responses during convalescence. Serum samples taken from 48 individuals reinfected with *P. falciparum* during the course of the study were analyzed to compare the antibody responses at the time points T_0 (the beginning of the study), T_1 (reinfection first detected; drug administered), and T_{28} (28 days post-T_1). As shown in Fig. 2, there was no one trend observed in changes of antibody response levels for all of the antigens tested. While a gradual increase from T_0 to T_1 to T_{28} was observed for antibody responses to RAMA fragments A and E, a slight drop between T_0 and T_1 and a rise between T_1 and T_{28} were observed for RAMA-B. A gradual decrease was observed in antibody responses to fragment D of RAMA, whereas responses to RAMA-C remained at relatively constant levels at all time points. The rise of antibody responses to RAMA-E following reinfection with *P. falciparum* (T_{28} compared to T_1) proved to be statistically significant, with a P value of 0.004.

Antibody responses to Ag44 in the study population. Ag44 comprises the 134-residue C-terminal fragment of the well-characterized rhoptry protein RhopH3. Antibody responses to the C-terminal region of RhopH3 are present in individuals living in areas where malaria is endemic and are associated with *P. falciparum* infections (23). To test for antibody responses in the study population and to compare them with responses to RAMA, we analyzed anti-Ag44 antibodies in the same set of 286 serum samples from 119 individuals. The prevalence of anti-Ag44 responses, 30.25%, was lower than that observed for all RAMA fragments except RAMA-A. This may be due to a slightly higher cutoff OD of 0.39. The magnitudes of anti-Ag44 responses were relatively low but comparable to those for anti-RAMA fragments A and B, with a median OD value (lower quartile, upper quartile) of 0.230 (0.095, 0.460). Antibody responses to Ag44 were highly correlated with responses to RAMA fragments (except for RAMA-E), with Spearman's correlation coefficients (P values) of <0.0001 for Ag44/RAMA-A, 0.0003 for Ag44/RAMA-B, 0.0001 for Ag44/RAMA-C, and 0.0013 for Ag44/RAMA-D. No correlation between anti-Ag44 responses and the individual's age, time to reinfection, or gender was observed. When responses to Ag44 were analyzed at three different time points, a slight drop was observed between T_0 and T_1. However, a statistically significant increase in anti-Ag44 responses was observed at T_{28} compared to T_1 (P = 0.007), with median OD values (lower quartile, upper quartile) of 0.200 (0.075, 0.395) for T_0, 0.175 (0.085, 0.430) for T_1, and 0.205 (0.065, 0.480) for T_{28}. To determine whether antibody responses to Ag44 might be associated with the immune state, the anti-Ag44 responses of protected and susceptible patients were compared. Both the prevalence (35.48% for protected individuals versus 25% for susceptible individuals) and the magnitude [median OD values (lower quartile, upper quartile) of 0.270 (0.130, 0.475) versus 0.200 (0.075, 0.395)] of the responses of the protected individ-

TABLE 4. Comparison of antibody isotypes in responders to RAMA-E among individuals susceptible to and protected against *P. falciparum* infection during the course of the study

Antibody isotype	Prevalence[a]			Antibody level[b]		
	Protected (n = 25)	Susceptible (n = 41)	P^c	Protected (n = 25)	Susceptible (n = 41)	P^d
IgG1	92	80.49	0.296	0.140 (0.130, 0.465)	0.110 (0.030, 0.170)	0.0161
IgG2	32	21.95	0.396	0.020 (0.005, 0.045)	0.020 (0.000, 0.030)	0.4034
IgG3	88	68.29	0.083	0.160 (0.130, 0.295)	0.030 (0.010, 0.095)	0.0003
IgG4	12	17.07	0.730	0.000 (0.000, 0.010)	0.000 (0.000, 0.010)	0.7959
IgM	60	43.90	0.310	0.050 (0.005, 0.240)	0.010 (0.000, 0.140)	0.1502

[a] Percentage of positive sera defined as those that gave OD values greater than the cutoff OD.
[b] Antibody levels are presented as median OD values (lower quartile, upper quartile).
[c] Antibody prevalences of protected and susceptible groups were compared using Fisher's exact test.
[d] Antibody levels of protected and susceptible groups were compared using the Mann-Whitney test.

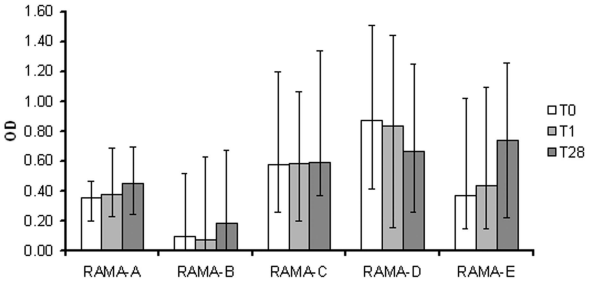

FIG. 2. Comparison of antibody responses of susceptible patients to RAMA fragments at three time points. The bars represent median OD values, with the error bars indicating the lower and upper quartiles.

uals tended to be higher, but the differences were not statistically significant.

DISCUSSION

Rhoptry proteins play a crucial role in the life cycle of *Plasmodium* parasites via their involvement in the invasion of RBCs by *Plasmodium* merozoites. Several studies have shown the importance of humoral responses to rhoptry proteins in antimalarial protection. In this study, we investigated the immunogenicity of a recently identified and characterized rhoptry protein named RAMA (21) in human infections and tried to determine whether anti-RAMA antibodies might be associated with the immune state. RAMA was first identified by screening expression libraries with immune human sera (19), and the clone identified encoded part of the mature form of the protein (p60). We had shown that p60/RAMA is involved in the invasion process and binds the RBC surface via the extreme C-terminal region, RAMA-E. Using nonoverlapping recombinant RAMA fragments, we showed that at least two distinct epitopes are present in p60 and that additional epitopes are found in the full-length protein, which is only transiently present in the intraerythrocytic parasite and is not found in the merozoite. Presumably, these are exposed to the immune system when parasitized RBCs are phagocytosed or otherwise destroyed prior to merozoite release.

We tested 286 serum samples from 119 individuals living in an area of Vietnam where malaria is endemic and constantly exposed to *Plasmodium* parasites. The analysis of total antibody responses agrees with our initial observations that the epitopes within the C-terminal part of RAMA are strongly immunogenic. The highest prevalence and magnitude were observed for antibody responses to fragments D and E of RAMA, present in the p60 form of the native protein, and in both cases the cytophilic IgG1 and IgG3 isotypes were the predominant antibody subclasses. High antibody responses to fragment C of RAMA, which contains the R3 repeat region, were also observed. Repeats have been implicated in humoral responses to malaria parasite antigens (15, 16), and the presence of a repetitive epitope within RAMA-C might explain the high level of antibody response to this fragment. Interestingly, we observed much lower serum reactivities to fragment RAMA-B, containing the R2 repeat region, indicating that the mere presence of repeats is not sufficient for significant immunogenicity. It was interesting to observe that relatively high antibody responses to fragment RAMA-A were detected in sera from individuals with no history of exposure to malaria parasites, resulting in a high cutoff value and thus a low prevalence of malaria-associated anti-RAMA-A antibodies. This could suggest the presence of an epitope that is highly cross-reactive with commonly encountered antigens.

To determine whether levels of antibodies to RAMA or Ag44 epitopes are associated with a state of resistance to infection, we compared the antibody responses of two groups of individuals in the study population. The protected group contained individuals who had no detectable parasites in regular blood smears over the course of the field study and who therefore appeared resistant to infection (parasitemia). The lack of a positive blood smear is unlikely to be due to a lack of exposure, since all participating individuals lived within a small community with a high incidence of infections (determined by a field survey to be 156 infections per 100 persons per year) and had been exposed to malaria parasites repeatedly. The susceptible group included individuals who became parasitemic with *P. falciparum* during the course of the field study and were therefore clearly not immune. Individuals who became parasitemic with other species of malaria parasites were excluded from the study, since this is a confounding factor that may limit the capacity of *P. falciparum* to coinfect either by raising cross-reactive immune responses or by nonspecific mechanisms that add up in a density-dependent manner (4). Overall, antibody responses in protected individuals tended to be slightly higher, particularly responses to fragment E, which is the functional region of the molecule involved in RBC binding. More importantly, we observed significantly higher levels of cytophilic IgG3-type antibodies in the protected group, as well as higher ratios of cytophilic to noncytophilic and of IgG3 to IgG1 isotypes. Previously, a number of studies had indicated an association between IgG-type antibody responses to the rhoptry protein RAP1 and lower parasitemia rates or protection from *P. falciparum* infection (1, 11, 13). The predominance of cytophilic isotypes, and more specifically the IgG3 class, rather than a total antibody level have been shown to be associated with antimalarial protection (3). Our results indicate that in the study population there was a clear link between the isotype distribution of cytophilic classes, a higher level of anti-RAMA-E IgG3-type antibodies, and the state of immunity to *P. falciparum* infection. The most likely mechanism would be that the anti-RAMA-E antibodies interfere with binding to RBCs and hinder the merozoites' entry into the host cell.

When we analyzed changes in serum reactivity associated with *P. falciparum* infection, we observed significantly increased antibody responses to fragment RAMA-E and to Ag44 28 days postinfection compared to T_1. Given the likelihood that individuals in the study population had been exposed to various malaria parasites' antigens on numerous occasions prior to the study, this result indicates that epitopes within fragment E of RAMA and within Ag44 actively and strongly boost humoral responses with each consecutive exposure and that the induced antibodies last for at least 28 days. A similar observation was made for RAP1, where increased antibody levels have been associated with exposure to *P. falciparum* and persisted for up to 30 days postinfection (10). Our results suggest that RAMA-E epitopes may be important for inducing more efficient antimalarial responses which develop over a

period of time with consecutive malaria parasite infections as opposed to epitopes within other regions of RAMA. Ag44 represents the C-terminal portion of RhopH3, and a previous seroepidemiological study indicated that this region is strongly immunogenic and that ongoing *P. falciparum* infection results in significant increases in antibody responses to the antigen (23). Monoclonal antibodies directed against epitopes within Ag44 showed growth-inhibitory properties in in vitro assays (6, 7). As in vitro RBC-binding assays indicated that Ag44 harbors an RBC-binding domain (21), there appears to be a link between protection and responses to RBC-binding domains for both RAMA and RhopH3.

In summary, our study shows that RAMA induces specific immune responses in individuals repetitively exposed to *P. falciparum* infection. At least three epitopes are present, with immunodominant epitopes located within the p60 mature form of RAMA, which is released into the bloodstream and exposed to antibody binding. Antibodies of the IgG3 isotype directed to fragment E are strongly associated with resistance to infection in our study population. Fragment E contains the RBC-binding domain and may be involved in the process of merozoite entry and the formation of the parasitophorous vacuole (21). As such, RAMA-E might have potential as a candidate for inclusion in an antimalarial vaccine. As it is only 82 residues, further mapping studies may identify a shorter region suitable for addition to a multiepitopic vaccine antigen. To further evaluate its efficacy, studies assessing the humoral responses to RAMA-E in individuals from other areas where malaria is endemic, in vitro growth inhibition assays with specific anti-RAMA-E antibodies, and challenge experiments in laboratory animals using recently identified rodent RAMA homologues (A. E. Topolska and R. L. Coppel, submitted for publication) are under way.

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