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Differential Recognition of *Plasmodium falciparum* Merozoite Surface Protein 2 Variants by Antibodies from Malaria Patients in Brazil

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Four variants of merozoite surface protein 2 (MSP-2) of *Plasmodium falciparum* were used in serology to examine whether changes in repeat units affect its recognition by antibodies during infection with parasites of known MSP-2 types. The results indicate that variation in MSP-2 repeats may represent a mechanism for parasite immune evasion.

Most major malaria antigens, such as the vaccine candidate merozoite surface protein-2 (MSP-2) of *Plasmodium falciparum*, display repetitive amino acid sequences (2). Insertions and deletions of repeat units, resulting from either mitotic or meiotic recombination, generate new antigenic variants that may be positively selected if mutant parasites evade hosts' immunity. Thus, high mutation rates coupled with natural selection may accelerate the evolution of repetitive antigens, with clear implications for malaria vaccine development (9).

Extensive sequence diversity in MSP-2 occurs in immunodominant repeat arrays that are flanked by dimorphic and conserved domains. Two allelic families may be defined, but the central region differs both between and within these dimorphic families (Fig. 1). Repeats in the FC27 family consist of one to four copies of a relatively conserved 32-mer motif followed by zero to five copies of a 12-mer motif, while IC1-type alleles differ in length (2 to 10 amino acids), sequence, and number of copies of repetitive motifs (6).

Human antibodies recognize predominantly variable domains on MSP-2 (1, 10) and are putatively associated with clinical immunity (11). Deletion of the 12-mer repeat impairs human antibody recognition of FC27-type antigens, indicating that B-cell epitopes occur in this region (8). Moreover, a murine monoclonal antibody discriminates between IC1-type antigens differing in the number of copies of the amino acid motif GGSA (4). Although cross-reactivity is a major factor driving the emergence and persistence of novel antigenic variants of *P. falciparum* in human populations (7), little is known about cross-reactivity patterns, both between and within allelic families, of naturally acquired antibodies to MSP-2. Here we used recombinant peptides to examine the hypothesis that the number and arrangement of repetitive units within allelic families affect MSP-2 recognition by human antibodies during infection with parasites of known MSP-2 type.

We studied 54 men between 18 and 58 years of age (mean, 28.4 years) presenting with uncomplicated *P. falciparum* infection (median parasite count, 9,246/mm^3; range, 1,519 to 53,819/mm^3). They participated in a clinical trial of mefloquine in the town of Peixoto de Azevedo, in the southwestern Amazon Basin of Brazil (3), an area with unstable transmission of both *P. falciparum* and *Plasmodium vivax* associated with gold mining activities. Subjects had been living in areas where malaria is endemic for 7.2 years on average (range, <1 to 38 years). Serum and blood clot samples obtained at enrollment were used for serology and extraction of parasite DNA (5), respectively.

PCR and hybridization with allele-specific probes were used to type the *Msp-2* gene of *P. falciparum* isolates (6). Two pairs of oligonucleotide primers corresponding to sequences in blocks 1 and 5 were used in nested PCRs. Following agarose gel electrophoresis, amplification products were transferred to

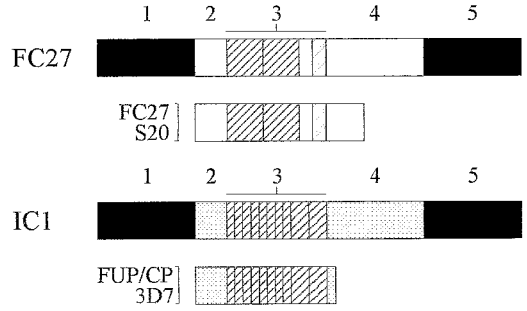

FIG. 1. Schematic representation of MSP-2 of *P. falciparum* and the recombinant peptides used in this study. This protein is divided into five domains: conserved blocks (1 and 5) are represented as black boxes, and dimorphic, nonrepetitive blocks (2 and 4) are represented as either white or gray boxes. The central repetitive region (block 3) is shown as it occurs in the representative isolates FC27 and IC1, which define the dimorphic allelic families. FC27 and S20 peptides belong to the FC27 family but mostly differ in the sequence of block 3: FC27 has two copies of the 32-mer motif ADTIASGSQRSTNSASTSTTNNG ESQTTTPTA followed by one copy of the 12-mer motif ESISPSPPIT TT, whereas S20 has one copy of the 32-mer motif ADTVASGSQSST NSASTSTTNNGESQTTTPTA followed by one copy of each of the 12-mer motifs ESNSPSPPITTT and KSNSPSPPITTT. The members of the IC1 family selected for this study, peptides FUP/CP and 3D7, share a common 4-mer motif, GGSA, which is repeated 12 times in FUP/CP and five times in 3D7.

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TABLE 1. Patterns of IgG antibody recognition of four MSP-2 peptides (FC27, S20, 3D7, and FUP/CP), as determined by enzyme-linked immunosorbent assay, in 54 adult malaria patients from Brazil[a]

Patient code	Length of exposure (years)	MSP-2 type	Age	MSP-2 peptide			
				FC27	S20	FUP/CP	3D7
5	0	IC1	26				
18	0	FC27	19				
37	0	IC1	24				
52	0	IC1/FC27	23				
9	1	FC27	22				
22	1	FC27	23				
27	1	FC27	29				
34	1	FC27	20				
3	2	FC27	22				
25	2	IC1	38				
49	2	FC27	24				
1	3	FC27	36				
19	3	IC1	36				
32	3	IC1/FC27	21				
38	3	FC27	36				
43	3	FC27	23				
47	3	FC27	18				
57	3	IC1/FC27	25				
4	4	FC27	23				
6	4	FC27	21				
7	4	FC27	24				
12	4	IC1/FC27	44				
28	4	FC27	35				
45	4	FC27	19				
16	5	FC27	22				
20	5	IC1	30				
36	5	IC1	30				
26	6	FC27	22				
26	6	FC27	22				
48	6	IC1/FC27	29				
2	7	FC27	27				
14	7	FC27	30				
58	7	IC1/FC27	37				
15	8	IC1/FC27	25				
30	8	IC1/FC27	27				
8	9	IC1	23				
35	9	IC1/FC27	29				
46	9	IC1	18				
33	10	IC1	26				
42	10	IC1/FC27	50				
44	10	IC1	37				
55	10	FC27	22				
13	11	IC1/FC27	35				
54	11	FC27	23				
17	12	FC27	31				
31	12	FC27	58				
40	12	FC27	23				
41	13	FC27	22				
11	15	FC27	45				
59	15	FC27	23				
23	18	IC1/FC27	27				
56	19	FC27	19				
10	22	IC1/FC27	40				
39	38	FC27	51				

[a] Thirteen patients were infected with genetically heterogeneous parasite populations expressing both MSP-2 types FC27 and IC1. Black boxes represent strong IgG antibody responses (reactivity indices ≥ 5), and open boxes indicate that no IgG antibodies were detected (reactivity indices < 1). Light- and dark-shaded boxes represent reactivity indices between 1 and 2.4 and between 2.5 and 4.9, respectively. Reactivity indices were calculated as the ratio of absorbance value obtained with test sera and negative control sera assayed on the same microplate (see the text for details). Ages are given in years.

Hybond-N membranes (Amersham Pharmacia Biotech, Piscataway, N.J.) and hybridized with the γ-^{32}P-labeled probes S1 (targeting block 2 of IC1-type alleles) and S2 (targeting the 12-mer repeats of FC27-type alleles). Allelic types were defined according to the sizes of PCR products and the hybridization patterns (6).

Four MSP-2 versions were expressed as recombinant peptides fused to the C terminus of glutathione *S*-transferase (GST) of *Schistosoma japonicum* (Fig. 1) and affinity purified (12). Naturally acquired immunoglobulin G (IgG) responses to these antigens were analyzed by enzyme immunoassay (12). Microplates (Nunc, Roskilde, Denmark) were coated with the peptides FC27, S20, FUP/CP, and 3D7 and GST alone (1 μg/well). Test sera and 28 negative controls (from malaria-free São Paulo, in southeastern Brazil) were tested at a 1:100 dilution. A peroxidase-conjugated goat immunoglobulin, anti-human IgG (Biolab Mérieux, Rio de Janeiro, Brazil) was used at a 1:10,000 dilution to detect IgG binding. After use of tetramethylbenzidine and hydrogen peroxide at an acid pH, absorbance values were measured at 450 nm. Reactivity indices were calculated as the ratio of the net absorbance value (after subtracting readings obtained with GST alone) of test sera to the average net absorbance value for four negative controls assayed on the same microplate. Positive samples had reactivity indices of >1.

Co-occurrences of FC27 and IC1 alleles were found in 13 (24%) subjects. Of the 67 alleles typed, 44 (66%) were FC27 and 23 (34%) were IC1 (Table 1). A single PCR fragment, from patient 39, failed to hybridize with both probes; standard DNA sequencing analysis (6) revealed a FC27-type allele with deletion of the 12-mer repeat motif (targeted by the S2 probe) (GenBank accession number AY102606), as previously shown in *Msp-2* alleles of various geographical origins but not in South America (6).

The prevalence of antibodies to FC27-type and IC1-type peptides mirrors the distribution of alleles found in infecting parasites (Table 1). At least one FC27-type antigen was recognized by antibodies from 39 (72%) patients, while only 19 (28%) of them had detectable IgG antibodies to at least one IC1-type antigen. These results contrast with those recently described for malaria-exposed Karitiana Indians in northwestern Brazil, who predominantly had antibodies that recognized IC1-type antigens. However, the distribution of MSP-2 alleles was not simultaneously investigated in the local parasite population (12).

Reactivity indices for FC27-type peptides were positively correlated with the length of malaria exposure (measured as the time, in years, the individual lived in an area where malaria is endemic); Pearson correlation coefficients (r) were 0.277 ($P = 0.04$) and 0.312 ($P = 0.02$) for FC27 and S20, respectively. Significant correlation was also found between reactivity indices for these antigens and the patient-reported number of microscopically confirmed *P. falciparum* infections in the past 2 years ($r = 0.327$ [$P = 0.02$] for FC27 and $r = 0.264$ [$P = 0.05$] for S20). No significant correlation was found between age and reactivity indices. The only variable correlated with reactivity indices for IC1-type peptides was the length of illness (in days) before admission ($r = 0.273$ [$P = 0.02$] for 3D7 and $r = 0.366$ [$P = 0.007$] for FUP/CP), suggesting that the duration of current exposure to the parasite, rather than cumulative malaria exposure, modulated the levels of IgG antibodies against the highly polymorphic IC1 family.

Antibody reactivity was more frequent in homologous (i.e., infecting parasites and peptides belonging to the same family) than heterologous antigen-parasite combinations (Table 1). For example, 79% of the patients harboring FC27-type parasites, but only 40% of those without FC27-type parasites, had IgG antibodies to peptide S20. However, the specificity of the predominant antibodies did not match the allelic type of MSP-2 in patients 1, 7, 40, and 46 (Table 1). This may result from a selective unresponsiveness to some antigenic variants or the co-occurrence of clones expressing the alternate MSP-2 version that were undetected by PCR (3a). Fifteen patients, seven of whom harbored only IC1-type parasites, failed to respond to all MSP-2 peptides. All of them reported at least one *P. falciparum* episode in the last 2 years.

Different proportions of patients (4 versus 35%, $P = 0.003$ by the χ^2 test) had antibodies that recognized the peptides 3D7 and FUP/CP, which differ in the number of copies of the GGSA motif (Table 1). Six patients harboring IC1-type parasites (patients 15, 32, 35, 36, 41, and 57) developed strong anti-FUP/CP responses (reactivity index ≥ 5) in the absence of detectable anti-3D7 antibodies, suggesting that the decreased number of copies of GGSA in peptide 3D7 may impair its recognition by naturally acquired antibodies in this population. Differences in the proportion of patients with antibodies recognizing the peptides FC27 and S20 were not statistically significant (59 versus 70%, $P = 0.315$ by the χ^2 test), but most (59%) patients with FC27-type parasites had higher reactivity indices for S20 than for FC27 peptide. Patient 52 developed a strong anti-S20 response in the absence of detectable anti-FC27 antibodies.

In conclusion, we detected both family-specific and variant-specific anti-MSP-2 IgG antibodies during acute malaria infections. Unresponsiveness to IC1-type peptides occurred in several subjects exposed to IC1-type parasites, underscoring the immunologic impact of the extensive antigenic diversity found in this family. Antibodies recognizing only or predominantly a single member of an allelic family were frequently found, especially for IC1-type peptides. Thus, variation in the number and arrange of repetitive arrays affects the recognition of MSP-2 peptides by naturally acquired human antibodies and may represent an efficient mechanism for parasites' immune evasion.

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REFERENCES

1. **Al-Yaman, F., B. Genton, R. F. Anders, M. Falk, T. Triglia, D. Lewis, J. Hii, H.-P. Beck, and M. P. Alpers.** 1994. Relationship between humoral response to *Plasmodium falciparum* merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. Am. J. Trop. Med. Hyg. **51:**593–602.
2. **Berzins, K., and R. F. Anders.** 1999. The malaria antigens, p. 181–216. *In* M. Wahlgren and P. Perlmann (ed.), Malaria—molecular and clinical aspects. Harwood Academic Publishers, Amsterdam, The Netherlands.
3. **Cerutti, C., Jr., R. R. Durlacher, F. E. C. de Alencar, A. A. C. Segurado, and**

L. W. Pang. 1999. In vivo efficacy of mefloquine for the treatment of falciparum malaria in Brazil. J. Infect. Dis. **180:**2077–2080.

3a. **da Silveira, L. A., M. L. Dorta, E. A. S. Kimura, A. M. Katzin, F. Kawamoto, K. Tanabe, and M. U. Ferreira.** 1999. Allelic diversity and antibody recognition of *Plasmodium falciparum* merozoite surface protein 1 during hypoendemic malaria transmission in the Brazilian Amazon region. Infect. Immun. **67:**5906–5916.

4. **Fenton, B., J. T. Clark, A. Khan, V. J. Robinson, D. Walliker, R. Ridley, J. G. Scaife, and J. S. McBride.** 1991. Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*. Mol. Cell. Biol. **11:**963–971.

5. **Ferreira, M. U., Q. Liu, O. Kaneko, M. Kimura, K. Tanabe, E. A. S. Kimura, A. M. Katzin, S. Isomura, and F. Kawamoto.** 1998. Allelic diversity at the merozoite surface protein-1 locus of *Plasmodium falciparum* in clinical isolates from the southwestern Brazilian Amazon. Am. J. Trop. Med. Hyg. **59:**474–480.

6. **Hoffmann, E. H. E., L. A. Silveira, R. Tonhosolo, F. J. T. Pereira, W. L. Ribeiro, A. P. Tonon, F. Kawamoto, and M. U. Ferreira.** 2001. Geographical patterns of allelic diversity in the *Plasmodium falciparum* malaria-vaccine candidate, merozoite surface protein-2. Ann. Trop. Med. Parasitol. **95:**117–132.

7. **McKenzie, F. E., M. U. Ferreira, J. K. Baird, G. Snounou, and W. H. Bossert.** 2001. Meiotic recombination, cross-reactivity, and persistence in *Plasmodium falciparum*. Evol. Int. J. Org. Evol. **55:**1299–1307.

8. **Ranford-Cartwright, L. C., R. R. Taylor, N. Asfari-Jirhandeh, D. B. Smith, P. E. Roberts, V. J. Robinson, H. A. Babiker, E. M. Riley, D. Walliker, and J. S. McBride.** 1996. Differential antibody recognition of FC27-like *Plasmodium falciparum* merozoite surface protein MSP2 antigens which lack 12 amino acid repeats. Parasite Immunol. **18:**411–420.

9. **Rich, S. M., M. U. Ferreira, and F. J. Ayala.** 2000. The origin of antigenic diversity in *Plasmodium falciparum*. Parasitol. Today **16:**390–396.

10. **Taylor, R. R., D. B. Smith, V. J. Robinson, J. S. McBride, and E. M. Riley.** 1995. Human antibody response to *Plasmodium falciparum* merozoite surface protein 2 is subgroup specific and predominantly of the immunoglobulin G3 subclass. Infect. Immun. **63:**4382–4388.

11. **Taylor, R. R., S. J. Allen, B. M. Greenwood, and E. M. Riley.** 1998. IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP-2): increasing prevalence with age and association with clinical immunity to malaria. Am. J. Trop. Med. Hyg. **58:**406–413.

12. **Tonhosolo, R., G. Wunderlich, and M. U. Ferreira.** 2001. Differential antibody recognition of four allelic variants of the merozoite surface protein-2 (MSP-2) of *Plasmodium falciparum*. J. Eukaryot. Microbiol. **48:**556–564.