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Allelic Diversity and Antibody Recognition of *Plasmodium falciparum* Merozoite Surface Protein 1 during Hypoendemic Malaria Transmission in the Brazilian Amazon Region

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The polymorphic merozoite surface protein (MSP-1) of *Plasmodium falciparum* is a major asexual blood-stage malaria vaccine candidate. The impact of allelic diversity on recognition of MSP-1 during the immune response remains to be investigated in areas of hypoendemicity such as the Brazilian Amazon region. In this study, PCR was used to type variable regions, blocks 2, 4, and 10, of the *msp-1* gene and to characterize major gene types (unique combinations of allelic types in variable blocks) in *P. falciparum* isolates collected across the Amazon basin over a period of 12 years. Twelve of the 24 possible gene types were found among 181 isolates, and 68 (38%) of them had more than one gene type. Temporal, but not spatial, variation was found in the distribution of MSP-1 gene types in the Amazon. Interestingly, some gene types occurred more frequently than expected from random assortment of allelic types in different blocks, as previously found in other areas of endemicity. We also compared the antibody recognition of polymorphic (block 2), dimorphic (block 6), and conserved (block 3) regions of MSP-1 in Amazonian malaria patients and clinically immune Africans, using a panel of recombinant peptides. Results were summarized as follows. (i) All blocks were targeted by naturally acquired cytophilic antibodies of the subclasses IgG1 and IgG3, but the balance between IgG1 and IgG3 depended on the subjects' cumulative exposure to malaria. (ii) The balance between IgG1 and IgG3 subclasses and the duration of antibody responses differed in relation to distinct MSP-1 peptides. (iii) Antibody responses to variable blocks 2 and 6 were predominantly type specific, but variant-specific antibodies that target isolate-specific repetitive motifs within block 2 were more frequent in Amazonian patients than in previously studied African populations.

The hypothesis of strain dependence of malaria immunity has been revived by mathematical models that define clinical protection as the ability of generating effective responses against the antigenic variants to which subjects are locally exposed (34). *Plasmodium falciparum* malaria has been modeled as a heterogeneous disease caused by several independently transmitted and antigenically distinct parasite subpopulations, or strains. The strain theory postulates that a limited set of immunodominant polymorphic antigenic determinants elicits life-long responses associated with the early acquisition of immunity to disease, while weaker responses to conserved antigens are probably involved in the later development of antiparasite immunity (33). Multivalent vaccines based on polymorphic antigens, the composition of which is changed regularly to match locally prevalent antigenic variants, might therefore represent an alternative approach to antimalarial immunization, instead of relying on highly conserved but poorly immunogenic antigens (2).

Merozoite surface protein 1 (MSP-1) of *P. falciparum* provides a model to examine the role of variable and conserved epitopes in antimalarial immunity. MSP-1 emerged as a major asexual blood-stage malaria vaccine candidate because (i) immunization with both native and recombinant MSP-1 fragments partially or completely protects *Aotus* and *Saimiri* monkeys against experimental challenge with *P. falciparum* (31), (ii) polyclonal and monoclonal antibodies to MSP-1 are able to inhibit parasite growth in vitro (31), and (iii) MSP-1 is targeted by antibodies that inhibit merozoite dispersal in vitro (48). MSP-1 is a glycoprotein with a size of approximately 190 kDa. After proteolytic processing, only a 19-kDa C-terminal fragment remains anchored on the merozoite surface during erythrocyte invasion (37).

Sequence comparisons led Tanabe and colleagues to describe seven variable blocks in the *msp-1* gene that are interspersed with conserved or semiconserved regions (60). The 19-kDa C terminus corresponds approximately to conserved block 17 (Fig. 1). There are two basic versions of each block, named after the representative isolates K1 and MAD20. The only known exception to allelic dimorphism occurs in block 2, which has a third version originally found in isolate RO33. Most allelic diversity is generated by recombination near the 5′ end of the gene and variations in the tripeptide repeats found in the MAD20 and K1 versions of block 2 (51, 60).

Conserved and variable regions of MSP-1 are recognized by antibodies and reactive T cells from people naturally exposed to malaria (37). Several longitudinal studies (1, 21, 55, 61),

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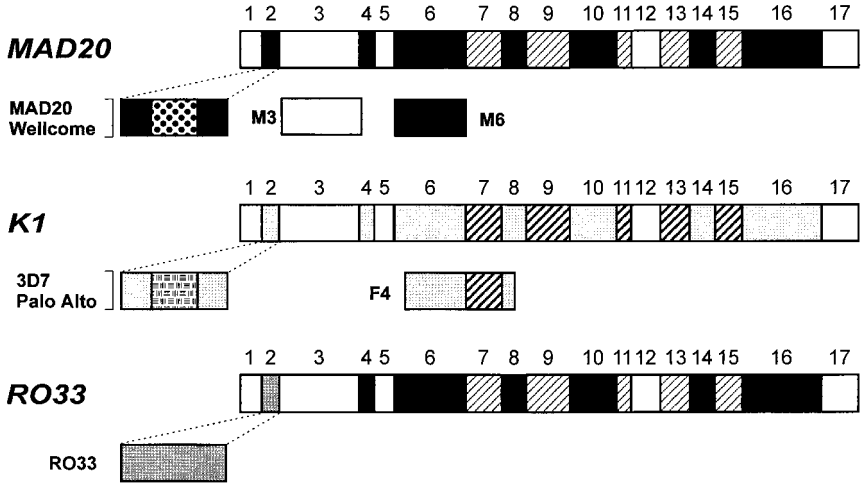

FIG. 1. Schematic representation of the *msp-1* gene of *P. falciparum* and of the recombinant peptides used in this study. This gene was divided into 17 blocks (60): conserved blocks are represented as open boxes, semiconserved blocks are represented as hatched boxes, and variable blocks are represented as closed boxes. The block 2 versions MAD20 and Wellcome belong to the MAD20 allelic family and differ in the central repetitive region but share a common sequence flanking the tripeptide repeats. The same patterns are observed in relation to the 3D7 and Palo Alto versions (K1 allelic family).

albeit not all (56), have detected positive associations between antibody responses to MSP-1 and protection from *P. falciparum* malaria. However, the relative role of different MSP-1 regions in protective immunity remains to be determined. Partial protection may be induced, for instance, in monkeys immunized with peptides derived from both the N terminus (14, 23, 35, 36) and the C terminus (13, 38, 45) of MSP-1. Similarly, monoclonal antibodies that inhibit parasite growth in vitro recognize epitopes on either the variable block 2 (47) or conserved block 17 (5).

Naturally acquired antibodies react more frequently against variable, rather than conserved, MSP-1 blocks (30, 52, 61) and are specific for one of the major versions of each variable block (12, 30). Further analyses are hampered, however, by the lack of data about the MSP-1 variants or types to which subjects are actually exposed in most areas of malaria endemicity. In the present study, we analyzed patterns of allelic diversity at the *msp-1* locus in *P. falciparum* isolates from an area of low malaria endemicity, the Brazilian Amazon region. We also examined antibody responses developed by local malaria patients against a panel of recombinant peptides derived from polymorphic block 2, conserved block 3, and dimorphic blocks 6 to 8 of MSP-1. Our focus was the IgG subclass distribution of these antibodies, because of the potential role of cytophilic antibodies of immunoglobulin G1 (IgG1) and IgG3 subclasses in immune protection against blood-stage infection (6, 32). Finally, we addressed two questions: (i) are anti-MSP-1 antibody responses short-lived and (ii) are there substantial differences, in terms of specificity and IgG subclass distribution, in anti-MSP-1 antibodies found in semi-immune Amazonian patients and clinically immune Africans?

MATERIALS AND METHODS

Study area. Hypoendemic malaria transmission by both *P. falciparum* and *P. vivax* occurs in the Brazilian Amazon region, where the main malaria vector is *Anopheles darlingi*. About 390,000 new cases were diagnosed in this region in 1997, and *P. vivax* accounted for two thirds of them (52a). Malaria transmission is heterogeneously distributed throughout the Amazon area (Fig. 2) and associated with agricultural settlements and professional activities such as construction of roads, wood extraction, and mining in the rain forest borders (50). Exposed people are mainly nonimmune migrants from malaria-free areas, and the status of clinical immunity commonly seen in African adults is rarely observed. Almost all malaria infections are symptomatic (9, 10).

***P. falciparum* isolates and DNA isolation.** A *P. falciparum* isolate was defined as a sample of parasites derived from a single patient at a single occasion. Venous blood was collected from 224 symptomatic malaria patients (76% males) aged between 9 months and 67 years (mean, 30.2 years) and stored in liquid nitrogen or at −20°C. All patients participated in studies of phenotypic and genetic diversity of malaria parasites and gave informed consent. Isolates collected between 1985 and 1989 were kindly provided by Judith K. Kloetzel (Institute of Tropical Medicine of São Paulo, São Paulo, Brazil). Collection dates and sites for the 181 isolates (81%) whose *msp-1* gene was fully typed are given in Table 1 and Fig. 2. *msp-1* diversity in most isolates collected in Rondônia in 1995 (see footnote *b* of Table 1) had been described in a previous publication (29), but data are included here to make temporal comparisons possible. Parasite DNA templates were prepared as described (29).

Serum and plasma samples. (i) Acute malaria patients from Rondônia. Blood samples were obtained, after informed consent, from 96 *P. falciparum*-infected symptomatic patients (69% males) aged between 1 and 65 years (mean, 29.6 years) who presented at the Center for Tropical Medicine of Rondônia between June and July 1995. Serum aliquots were kept at −20°C until tested. All patients lived in Porto Velho and surrounding areas in the northern part of Rondônia (Fig. 2), where parasite rates are typically below 2% (9, 10). Blood samples from most patients were also available for parasite DNA extraction.

(ii) Acute and convalescent malaria patients from Pará. Paired serum samples from 25 adult (ages, >18 years) male patients with a history of several past malaria infections were kindly provided by José Maria de Souza (Evandro Chagas Institute, Belém, Brazil). The first sample (acute phase) was collected during mildly symptomatic *P. falciparum* infection, while the second sample (convalescence) was obtained 63 days after the beginning of effective antimalarial chemotherapy. Only patients without detected parasite recrudescences were included in this sample. All patients had contracted their malaria infection in the region of Paragominas, eastern Pará (Fig. 2), and remained hospitalized throughout the 63-day observation period, without risk of reinfection (26). No parasite DNA from these subjects was available for parasite typing.

(iii) Clinically immune African subjects. Plasma samples from 30 inhabitants in an area of holoendemicity, the village of Dielmo (Senegal, West Africa), were kindly provided by Philippe Dubois (Pasteur Institute, Paris, France). Samples were obtained in June 1990, just before the beginning of the rainy season, from adults aged 18 to 71 years (mean, 40.0 years) without any symptoms of malaria or other infectious disease. One third of them had detectable *P. falciparum* parasitaemias at the time of bleeding. The donors' status of clinical immunity was defined according to the following criteria: (i) continuous, life-long exposure to intense malaria transmission in the absence of chemoprofilaxis and (ii) infrequency of clinical malaria attacks (62). All *msp-1* block 2 allelic types were found in local *P. falciparum* isolates, but K1 and RO33 predominate over MAD20 (44).

***msp-1* gene typing strategy.** Sequences of oligonucleotide primers and PCR protocols used to type the variable blocks 2, 4, and 10 are given elsewhere (29, 42). The typing procedure was designed to identify the 24 major *msp-1* gene types shown in Table 2 (42), which are defined as unique combinations of (i) one of three versions (K1, MAD20, or RO33) of block 2, (ii) one of four versions of

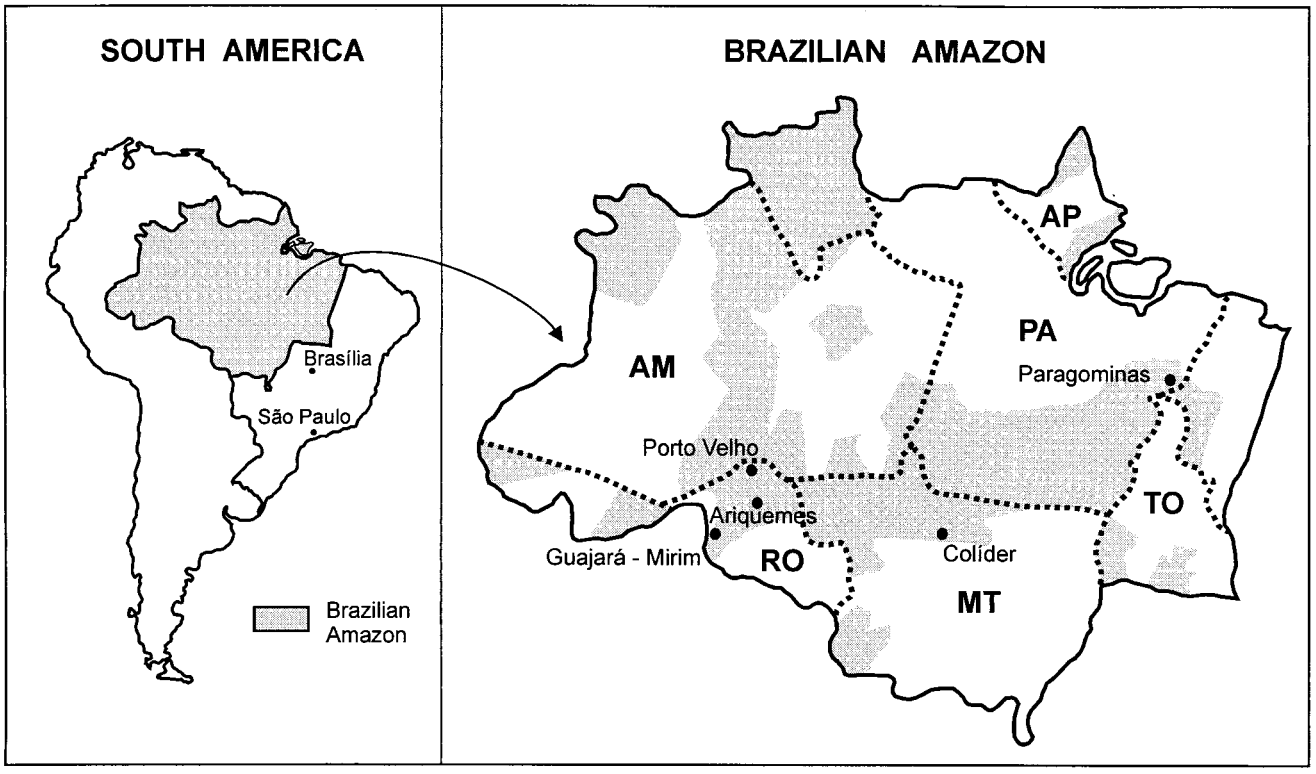

FIG. 2. (Left panel) Map of South America showing the Brazilian Amazon region. (Right panel) Collection sites of the Amazonian *P. falciparum* isolates analyzed in this study (Table 1). The shaded portions represent the areas with highest malaria transmission in the early 1990s (50). States are abbreviated as follows: AM, Amazonas; RO, Rondônia; MT, Mato Grosso; PA, Pará; TO, Tocantins; and AP, Amapá.

block 4 because recombination within this region generates K1/MAD20 and MAD20/K1 hybrids in addition to pure allelic types K1 and MAD20, and (iii) one of two versions (K1 or MAD20) in the segment between blocks 6 and 16 where intragenic recombination does not occur (42). The typing procedure may be summarized as follows. (i) Fragments between semiconserved block 9 and variable block 10 were amplified in two separate reactions with the common forward primer C9F and the type-specific reverse primer K10R or M10R. (ii) Segments between variable blocks 2 and 6 were amplified in three separate reactions with the type-specific forward primer K2F, M2F, or R2F and the type-specific reverse primers K6R and M6R. The allelic type determined for block 10 (either K1 or MAD20) was assumed to be the same for block 6, and this information was used to select the type-specific reverse primer, either K6R or M6R, for the second amplification step. (iii) Finally, block 4 was typed by nested PCR in four separate reactions with the type-specific forward primers K4F or M4F and the type-specific reverse primers K4R or M4R, by using as template the product amplified by the second step. Both the 5′ (block 4a) and the 3′ (block 4b) segments of block 4 were typed (16, 29, 43). The major advantage of this multistep strategy is the possibility of typing each parasite subpopulation present in genetically mixed infections (43).

Recombinant MSP-1 peptides. (i) Block 2. Glutathione *S*-transferase (GST) fusion proteins derived from block 2 of the isolates MAD20 and Wellcome (MAD20 allelic family), 3D7 and Palo Alto (K1 allelic family), and RO33 (RO33 allelic family) (Fig. 1) were kindly provided by David R. Cavanagh (University of Edinburgh, Edinburgh, Scotland). These proteins were previously shown to induce specific antibodies, upon immunization in mice, that recognize the native MSP-1 from members of the same allelic family (11). Antibodies to block 2 peptides may be either isolate or variant specific (antibodies that react against isolate-specific tripeptide repeats) and allelic family or type specific (antibodies that react against determinants flanking the repetitive sequence) (11, 12).

(ii) Blocks 3 and 6 to 8. Blocks 3 and 6 derived from isolate MAD20 and a fragment between blocks 6 and 8 of isolate K1 were expressed as histidine-tagged fusion proteins and purified as previously described (61) (Fig. 1). The original clones were kindly provided by Hermann Bujard (University of Heidelberg, Heidelberg, Germany), and fusion proteins were named, respectively, M3, M6, and F4 (Fig. 1). Block 3 is highly conserved, especially in the segment where the B-cell epitope described by Crisanti and colleagues (18) is probably situated (41). In contrast, the segment between blocks 6 and 16 is clearly dimorphic (51). The MAD20 version of block 6, which predominates in several areas of endemicity, including Brazil and West Africa (15), has previously been shown to be highly immunogenic in natural infections (30, 52, 61). Furthermore, naturally acquired antibodies that recognize this fragment (peptide M6) are type specific (30) and putatively associated with clinical protection against malaria (61). Therefore, analyses of antibody responses against M3, M6, and F4 may provide insights into the recognition of conserved and dimorphic (type-specific) regions of MSP-1 during the immune response.

ELISA. IgG subclass antibodies to recombinant peptides were measured by enzyme-linked immunoassay (ELISA) essentially as described previously (26). High-binding 96-well microplates (Costar, Cambridge, Mass.) were coated with, per well, either 50 ng of protein (block 2 peptides and F4) or 20 ng of protein (M3 and M6) dissolved in 50 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) as determined by checkerboard titration of known positive and negative controls. When block 2 peptides were tested, alternate rows were coated with either the recombinant peptide or GST control (50 ng/well). Serum or plasma samples (including positive and negative controls) were tested at a 1:100 dilution. The binding of IgG subclass antibodies was detected with mouse monoclonal antibodies to human IgG1 (clone HP-6012; Oxoid, Unipath, Bedford, United Kingdom), IgG2 (clone HP-6014; Sigma, St. Louis, Mo.), IgG3 (clone HP-6050; Sigma), or IgG4 (clone HP-6025; Sigma). Monoclonal-antibody binding was detected with peroxidade-conjugated, rabbit anti-mouse immunoglobulin (Sigma). After use of *o*-phenylenediamine and hydrogen peroxide at acid pH as substrate, absorbance values were measured at 492 nm. Quantitative comparisons of antibody concentrations of each IgG subclass were based on an indirect standardization procedure. Standard curves of 10 serial dilutions of purified myeloma proteins of each subclass (Sigma) were included in all microplates. Concentrations of each subclass of IgG against the recombinant peptides were interpolated from absorbance values by second-degree polynomial regression. Results were expressed as micrograms of antipeptide antibody per milliliter of serum or plasma. The sensitivity threshold of the assay is approximately 1 to 5 μg of peptide-specific antibody/ml. Concentrations of IgG antibodies were calculated by summing the concentrations of each IgG subclass.

Data analyses. The frequency of each *msp-1* gene type was computed as its proportion of the total of typed parasite populations among the isolates tested, including more than one subpopulation per isolate in cases of genetically mixed infections. The proportions expected from the null hypothesis of random association of variable block allelic types were derived from a simple probability model analogous to those used in population genetics to estimate the expected frequency of multilocus genotypes. For instance, the expected proportion of gene type 1, which has K1-type sequences in all variable blocks, is given by multiplying

TABLE 1. Collection dates and sites of the 181 *P. falciparum* isolates included in this study

Collection date (yr)	State[a]	Site	No. of isolates typed
1985–1986	Rondônia	Porto Velho	14
		Ariquemes	23
		Guajará-Mirim	7
		Other sites in Rondônia	5
	Pará	Paragominas	7
		Other sites in Pará	7
	Mato Grosso	Colíder	6
		Other sites in Mato Grosso	5
	Amapá	Unknown	2
	Tocantins	Unknown	1
	Unknown	Unknown	2
Total			79
1987–1989	Rondônia	Porto Velho	1
	Unknown	Unknown	2
Total			3
1995	Rondônia	Porto Velho	79[b]
1997	Rondônia	Porto Velho	14
		Ariquemes	5
	Amazonas	Unknown	1
Total			20

[a] The locations of mentioned states are shown in Fig. 2.
[b] The *msp-1* gene typing results for 54 of these isolates have been published previously (29).

the observed proportions of parasites with K1-type sequences in blocks 2, 4a, 4b, and 6 to 16. To test the null hypothesis of random assortment of allelic types in this parasite population, expected and observed frequencies were compared by using χ^2 statistics for goodness of fit. For this analysis, cells with expected frequencies of <5 were pooled. We have used χ^2 tests for independent samples to analyze temporal and spatial variations in *msp-1* gene type distributions. The nonparametric Spearman's correlation coefficient r_s was calculated to test correlation between age and the number of *msp-1* gene types harbored by each patient, as well as between concentrations of antibodies to different peptides. Antibody concentrations are presented as means ± standard errors of the means. The nonparametric tests of Kruskal-Wallis and Mann-Whitney (for independent samples) and Wilcoxon (for paired samples) were used to compare antibody concentrations. Significance was set at the 5% level.

RESULTS

***msp-1* gene type frequencies.** *msp-1* typing was completed for 181 isolates (81%), and partial typing (i.e., only one or two variable blocks were successfully amplified during the multistep typing strategy) was obtained for 12 isolates (5%). No PCR product was obtained from 31 (14%) isolates. Further analyses were restricted to fully typed isolates (Table 1). Twelve of the 24 possible *msp-1* gene types were identified, and 265 parasite populations were typed among 181 isolates (Table 2). Most (89%) parasite populations expressed one of the seven most common gene types (15–18, 22–24), and K1-type sequences in blocks 6 to 16 were rarely observed (6%). More than one gene type was found in 68 (38%) isolates (mean ± standard deviation, 1.46 ± 0.72 gene types per isolate), and one patient harbored as many as six different gene types. No significant correlation was detected between the number of different gene types harbored per host and the age of the patient ($r_s = 0.187, P > 0.05, n = 177$ patients whose ages were known).

TABLE 2. Observed frequencies of the 24 *msp-1* gene types in 181 *P. falciparum* isolates from the Brazilian Amazon region[a]

Gene type	Allelic type in variable *msp-1* block:				No. (%) of typed parasite populations[b]
	2	4a	4b	10	
1	K1	K1	K1	K1	6 (2.26)
2	MAD20	K1	K1	K1	10 (3.76)
3	RO33	K1	K1	K1	1 (0.38)
4	K1	MAD20	K1	K1	0 (0)
5	MAD20	MAD20	K1	K1	0 (0)
6	RO33	MAD20	K1	K1	0 (0)
7	K1	K1	MAD20	K1	0 (0)
8	MAD20	K1	MAD20	K1	0 (0)
9	RO33	K1	MAD20	K1	0 (0)
10	K1	MAD20	MAD20	K1	0 (0)
11	MAD20	MAD20	MAD20	K1	0 (0)
12	RO33	MAD20	MAD20	K1	0 (0)
13	K1	K1	K1	MAD20	9 (3.38)
14	MAD20	K1	K1	MAD20	0 (0)
15	RO33	K1	K1	MAD20	22 (8.27)
16	K1	MAD20	K1	MAD20	48 (18.11)
17	MAD20	MAD20	K1	MAD20	12 (4.51)
18	RO33	MAD20	K1	MAD20	72 (27.07)
19	K1	K1	MAD20	MAD20	0 (0)
20	MAD20	K1	MAD20	MAD20	0 (0)
21	RO33	K1	MAD20	MAD20	2 (0.75)
22	K1	MAD20	MAD20	MAD20	25 (9.40)
23	MAD20	MAD20	MAD20	MAD20	36 (13.53)
24	RO33	MAD20	MAD20	MAD20	22 (8.27)

[a] Gene types are defined as a unique combination of allelic types detected in the variable blocks 2, 4a (5′ segment of block 4), 4b (3′ segment of block 4), and 6 to 16 of the *msp-1* gene. Because there is no recombination in the gene segment comprised between blocks 6 and 16, the allelic type detected in block 10 is considered to be the same for variable blocks 6, 8, 14, and 16. Allelic types are named after the reference isolates K1, MAD20, and RO33.
[b] Sixty-eight isolates had more than one fully typed parasite population, and gene type frequencies (%) were computed by using the total number of gene types detected in this parasite population ($n = 265$). Data for 54 of these isolates (corresponding to 77 typed parasite populations) have been previously published (29).

Temporal but no spatial variation in the distribution of *msp-1* gene types. We compared *msp-1* gene type frequencies in isolates collected in Rondônia between 1985 and 1986 with those found in isolates collected in the same area in 1995 and

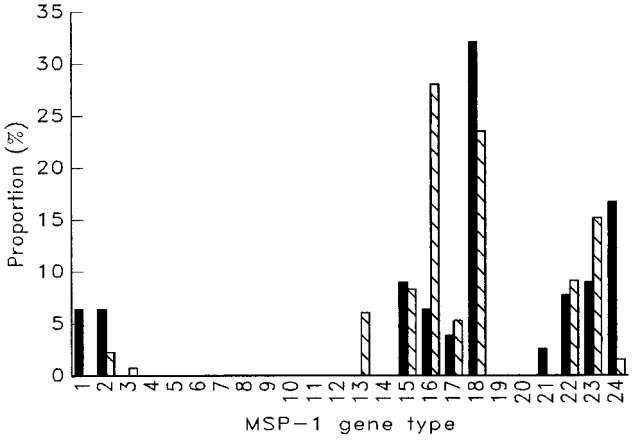

FIG. 3. Frequency distribution of *msp-1* gene types in *P. falciparum* isolates collected in Rondônia between 1985 and 1986 (78 typed populations in 49 isolates) (closed bars) and between 1995 and 1997 (132 typed populations in 98 isolates) (hatched bars).

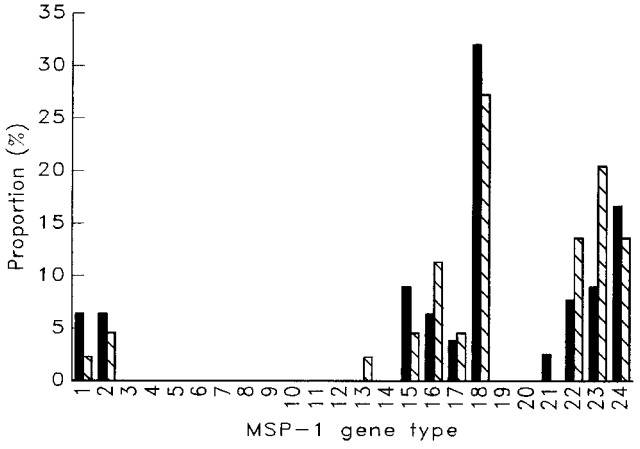

FIG. 4. Frequency distribution of *msp-1* gene types in *P. falciparum* isolates collected in Rondônia between 1985 and 1986 (78 typed populations in 49 isolates) (closed bars) and in isolates collected throughout the same period in other Amazonian states (44 typed populations in 28 isolates) (hatched bars).

1997 (Fig. 3). A statistically significant difference was detected by comparison of the overall distribution of gene types at both occasions ($\chi^2 = 36.95$, 6 degrees of freedom [df], $P < 0.0001$). A block-by-block comparison showed statistically significant differences in the frequencies of allelic types, at both occasions, in blocks 2 ($\chi^2 = 74.76$, 2 df, $P < 0.0001$), 4a ($\chi^2 = 29.55$, 1 df, $P < 0.00001$), and 6-16 ($\chi^2 = 7.55$, 1 df, $P = 0.006$). Interestingly, the largest variations were found in the frequencies of gene types 16, 18, 23, and 24 (Fig. 3). Types 16 and 18 differ only by the block 2 allelic type (either K1 or RO33), the same occurring in relation to types 23 and 24 (either MAD20 or RO33). The seven most frequent gene types (15–18, 22–24) accounted for 85% of the typed parasite populations in 1985 to 1986 and 91% in 1995 to 1997. Next we compared *msp-1* gene type frequencies in isolates collected in Rondônia between 1985 and 1986 with those found in isolates collected throughout the same period in other Amazonian states (Fig. 4). We were unable to detect statistically significant spatial variation ($\chi^2 = 6.08$, 5 df, $P > 0.05$). Negative results were also obtained in block-by-block comparisons.

Nonrandom associations of allelic types in variable blocks. We next compared the observed distribution of *msp-1* gene types with that expected from the hypothesis of random assortment allelic types in variable blocks. Because of temporal variations, two separate analyses were performed. The first set of data included isolates collected between 1985 and 1986, regardless of the collection site, whereas the second set of data included isolates collected in 1995 and 1997. Figure 5 shows expected and observed gene type distributions in these population samples. Significant departures from the expected distributions were detected in both samples ($\chi^2 = 24.09$, 7 df, $P = 0.015$ [1985 to 1986], and $\chi^2 = 56.27$, 8 df, $P < 0.0001$ [1995 and 1997]). Gene types 2, 18, and 23 were more prevalent than expected in both samples, the opposite being found in relation to gene types 14 and 17. Block-by-block analyses revealed some instances of apparent linkage between allelic types in variable blocks that are common to both population samples. For instance, all parasites with K1-type sequences in blocks 6 to 16 ($n = 13$) have concordant (K1-type) sequences in blocks 4a and 4b. The cooccurrence of K1-type sequences in block 4a with MAD20-type sequences in block 4b was much less frequent than expected from random assortment of allelic types.

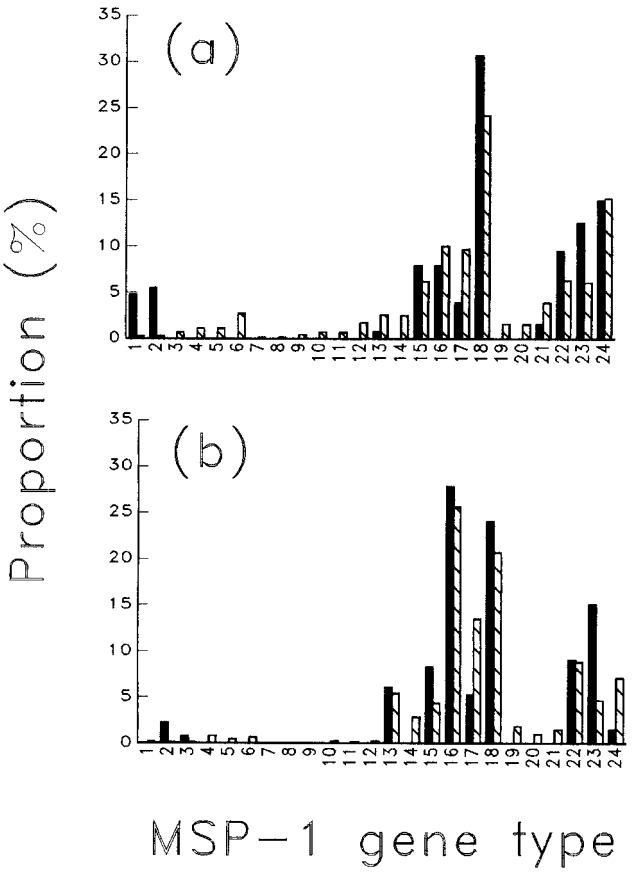

FIG. 5. Observed (closed bars) and expected (hatched bars) frequencies of *msp-1* gene types in *P. falciparum* isolates from the Brazilian Amazon region collected between 1985 and 1986 (127 typed populations in 79 isolates) (a) and between 1995 and 1997 (133 typed populations in 99 isolates) (b). Frequencies expected by the hypothesis of random association of allelic types were generated as described in Materials and Methods.

IgG subclass antibodies to MSP-1 in patients from Pará and clinically immune Africans. In Fig. 6 IgG subclass concentrations of antibodies to MSP-1 in malaria patients from Pará and clinically immune African adults are compared. Results may be summarized as follows. (i) Cytophilic antibodies of either the IgG1 or IgG3 subclass predominated against all MSP-1 peptides in both Africans and Amazonians. IgG1 predominated against blocks 3 and 6, while IgG3 frequently dominated responses to block 2. (ii) Higher proportions of IgG3 antibodies were usually found in Africans than in acutely ill Amazonians. For instance, among Africans, IgG3 accounted for 70 to 95% of all antibodies to block 2 peptides, with 31% of those being antibodies to M3 and 39% being those to M6. Among acute patients from Pará, the figures were, respectively, 28 to 67%, 7% and 8%. The opposite trend was found in relation to IgG1. (iii) Cytophilic antibodies to conserved (block 3) and dimorphic (block 6), but not polymorphic (block 2), regions of MSP-1 were short-lived in the absence of reexposure to the parasite, as suggested by antibody concentrations measured in paired serum samples from acute and convalescent Amazonian patients. (iv) Mean antibody concentrations tended to be higher against the predominant version of variable blocks in local parasites compared with those against conserved block 3. Accordingly, antibody concentrations against M6 were higher than those against F4 (the MAD20 version of block 6 predom-

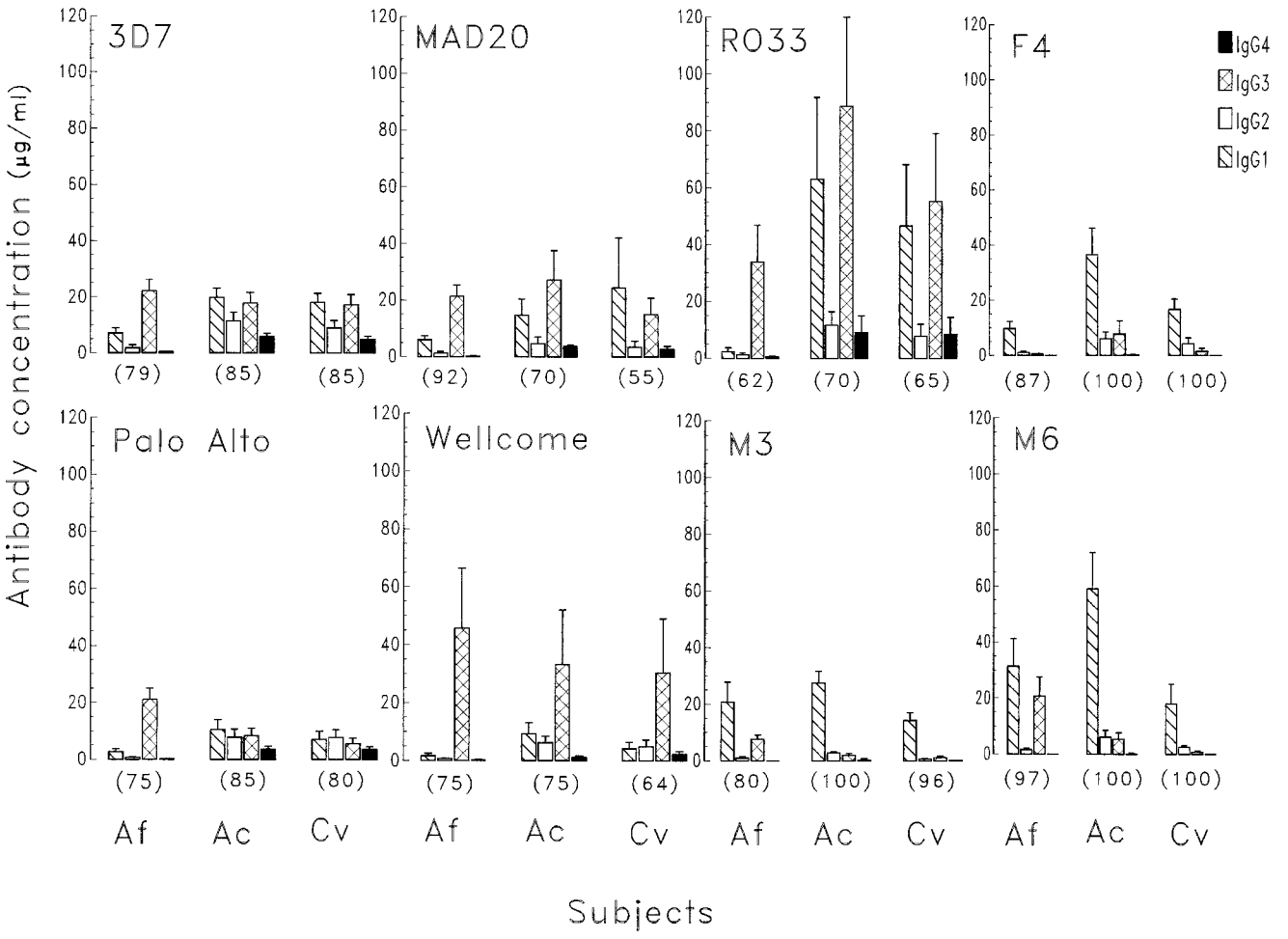

FIG. 6. Concentrations of IgG subclass antibodies to MSP-1 recombinant peptides in clinically immune Africans and Amazonian patients from Pará. Bars indicate means, and error bars indicate standard errors of the means. Numbers in parentheses indicate the percentage of patients with detectable IgG antibodies to each peptide, regardless of the IgG subclass. Af, clinically immune Africans ($n = 24$ for block 2 peptides and $n = 30$ for all other peptides); Ac, Amazonian patients with acute *P. falciparum* infection ($n = 20$ for block 2 peptides and $n = 25$ for all other peptides); Cv, the same Amazonian patients during convalescence ($n = 20$ for block 2 peptides and $n = 25$ for all other peptides). Statistical analysis compared concentrations of each IgG subclass to each peptide in different groups of subjects by either the Wilcoxon or Mann-Whitney test. The following statistically significant differences ($P < 0.05$) were found: (i) 3D7, IgG1, IgG2, and IgG4: Ac and Cv > Af; IgG3: Af > Ac and Cv; (ii) Palo Alto, IgG2, and IgG4: Ac and Cv > Af; IgG3: Af > Ac and Cv; (iii) MAD20, IgG4: Ac and Cv > Af; (iv) Wellcome, IgG2: Ac > Af; (v) RO33, IgG1: Ac and Cv > Af; IgG2: Ac > Af; (vi) M3, IgG1: Ac > Af and Cv; IgG3: Af > Ac and Cv; (vii) F4, IgG1: Ac and Cv > Af and Ac > Cv; IgG3: Ac > Af and Cv; and (viii) M6, IgG1: Ac > Af and Cv; IgG3: Af > Ac and Cv and Ac > Cv.

inates in Brazilian and West African isolates). No clear pattern emerged from type-specific block 2 recognition in Africans (all allelic types are present at similar proportions in local parasites), but high levels of anti-RO33 antibodies were detected in Amazonian patients (RO33 allelic type predominates in Brazil).

IgG subclass antibodies to MSP-1 in patients from Rondônia in relation to their cumulative exposure to malaria. We next measured anti-MSP-1 antibodies in acute *P. falciparum* malaria patients from Rondônia. Since almost all malaria infections are symptomatic in this region, the self-reported number of previous clinical episodes (including both *P. falciparum* and *P. vivax* infections) were used to classify these patients into three categories of cumulative exposure to malaria: (i) no past malaria attack; (ii) 1 to 10 past malaria attacks, including at least one *P. falciparum* episode confirmed by thick smear microscopy; and (iii) >10 malaria episodes. Figure 7 shows the concentrations of IgG subclass anti-MSP-1 antibodies in these patients. Results may be summarized as follows. (i) IgG1 against blocks 3 and 6 predominated, while high levels of both IgG1 and IgG3 against block 2 were found. (ii) The relative participation of cytophilic antibodies tended to increase in patients with more-frequent past exposure to the parasite. This trend is statistically significant for peptides Palo Alto, M3, F4, and M6. (iii) Most patients developed antibody responses against individual peptides, regardless of the subclass, during their primary malaria infections. All of them recognized at least one block 2 peptide, indicating that this region elicits antibodies even after a single contact with the parasite. (iv) Despite large individual variations, the highest mean antibody concentrations were found against the peptides RO33 and M6, which represent the predominant versions of blocks 2 and 6 in local isolates. The conserved block 3 was recognized by a high proportion of patients, but mean antibody concentrations were low.

Association between block 2 types in infecting parasites and antibody recognition of block 2 peptides. Block 2 typing data and block 2-specific antibody concentrations were available for 72 patients from Rondônia. Concentrations of block 2-specific

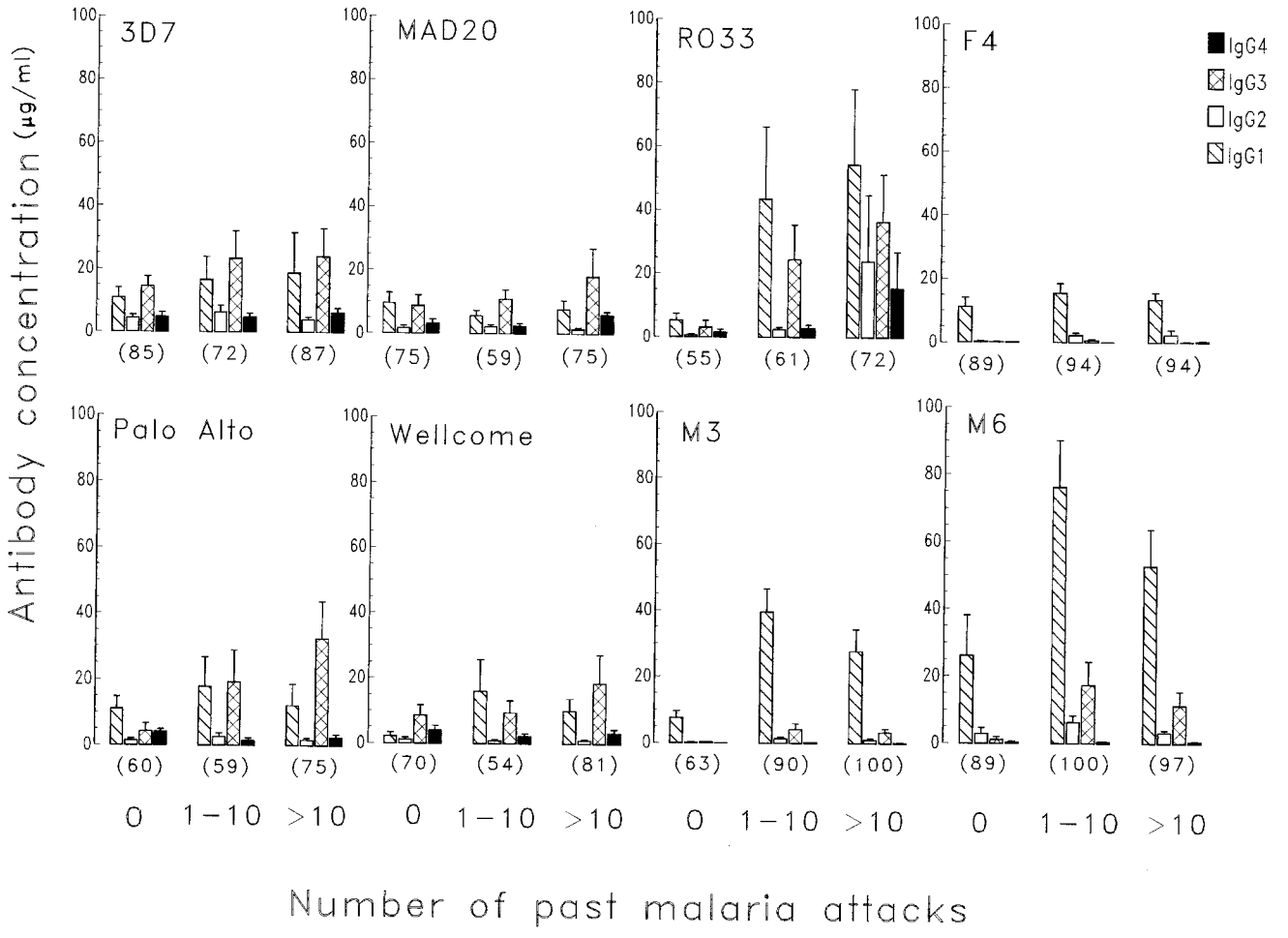

FIG. 7. Concentrations of IgG subclass antibodies to MSP-1 recombinant peptides in Amazonian patients from Rondônia in relation to cumulative exposure to malaria. Bars indicate means, and error bars indicate standard errors of the means. Numbers in parentheses indicate the percentage of patients with detectable IgG antibodies to a given peptide, regardless of the IgG subclass. Patients are grouped according to the self-reported number of past malaria infections. The number of tested samples in each group are as follows: (i) no past malaria infection $n = 20$ for block 2 antigens and $n = 19$ for all other antigens; (ii) 1 to 10 past malaria infections $n = 39$ for block 2 antigens and $n = 32$ for all other antigens; and (iii) >10 past malaria attacks $n = 32$ for block 2 antigens and $n = 33$ for all other antigens. Statistical analysis compared concentrations of each IgG subclass to each peptide in different groups of subjects by the Kruskal-Wallis test. The following statistically significant differences ($P < 0.05$) were found: (i) Palo Alto:IgG3, (ii) M3:IgG1, IgG2, and IgG3, (iii) F4:IgG1, and (iv) M6:IgG1 and IgG3.

IgG subclass antibodies were compared in the presence and absence of parasites that express a given version of block 2 (Fig. 8). Note that higher levels of cytophilic antibodies were usually found in homologous combinations compared with those in heterologous ones. Significant differences were detected for cytophilic, but not IgG2 and IgG4, antibodies to peptides 3D7, Palo Alto, and RO33. Data regarding block 6 typing and block 6-specific antibodies were available for 62 patients, but only 3 of these patients harbored parasites expressing the K1 version of block 6, precluding comparisons between homologous and heterologous responses. An analysis of 16 patients experiencing their primary malaria attack due to parasites expressing known block 2 types revealed a heterogeneous pattern of antibody recognition (Table 3). Three patients (R18, R42, and R143) had type-specific antibodies, while others (R28, R78, R83, R108, and R129) seemed to discriminate between different variants within the same allelic family. There are also instances where the specificity of the predominant antibodies did not match the block 2 allelic type(s) detected by PCR (patients R22, R45, and R117). To further investigate the relative participation of type-specific and variant-specific antibodies to block 2, correlation coefficients between IgG antibody concentrations were calculated to determine whether antibody responses to peptides derived from the same allelic family were associated (Table 4). The strongest correlations were usually found between antibody responses to peptides belonging to the same allelic family (within-type correlations), but most coefficients were rather low. The poor correlation between concentrations of antibodies that are specific for members of the same allelic family suggests that antibody recognition of block 2 peptides is largely variant-specific in these subjects.

DISCUSSION

The presence of several gene types in parasites from a single host makes possible the occurrence of crossfertilization and meiotic recombination at this locus within the mosquito vector. Diversity is expected to depend on the proportion of genetically mixed infections and the number of infectious clones per

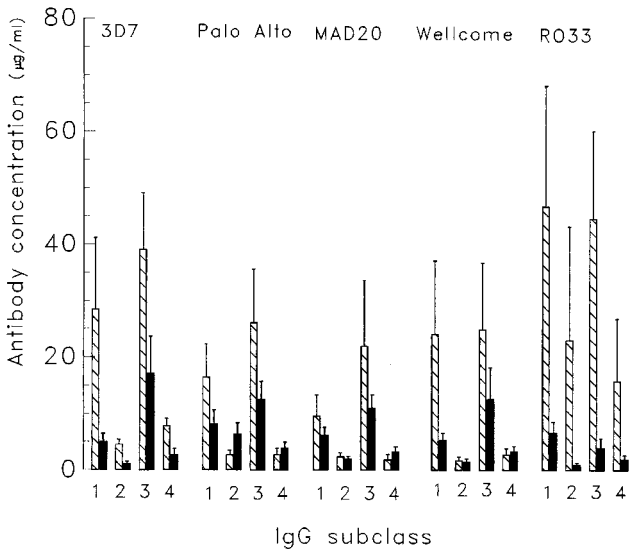

FIG. 8. Association between block 2 allelic type in infecting parasites and mean concentrations of IgG subclass antibodies to block 2 peptides in 72 malaria patients from Rondônia. Bars indicate means, and error bars indicate standard errors of the means. Antibody concentrations are separately shown in homologous (hatched bars) and heterologous (closed bars) combinations. The number of samples in homologous and heterologous combinations are as follows: (i) for 3D7 and Palo Alto peptides: K1-type parasites present, $n = 37$, and K1-type parasites absent, $n = 35$; (ii) for MAD20 and Wellcome peptides: MAD20-type parasites present, $n = 25$, MAD20-type parasites absent, $n = 47$; (iii) for RO33 peptide, RO33-type parasites present, $n = 34$, RO33-type parasites absent, $n = 38$. Statistical analysis compared concentrations of each IgG subclass to each peptide in homologous and heterologous combinations by the Mann-Whitney test. The following statistically significant differences ($P < 0.05$) were found: (i) 3D7:IgG1 and IgG3, (ii) Palo Alto:IgG1, and (iii) RO33:IgG3.

human host that carry distinct *msp-1* variants. The relatively restricted *msp-1* repertoire found in Brazil (Table 2) and in an area of holoendemicity, Tanzania (27), contrasts with previous findings for an area of mesoendemicity, Vietnam (28, 42). Extensive *msp-1* diversity also occurs in other areas with intermediate levels of malaria endemicity, such as Thailand (40). These data are somewhat surprising, since the proportion of isolates expressing more than one *msp-1* type is much higher in Tanzania (60%) than in Brazil (38%) and Vietnam (44%) (27, 28, 42). The seven most common gene types in Brazil accounted for 84% of the parasite populations typed in Tanzania (27) but for only 58% of those in Vietnam (28, 42).

In the framework of the strain theory of malaria transmission, naturally acquired type-specific immunity might select against parasites that express the most-frequent antigen variants, and novel polymorphisms would emerge. Provided that variable epitopes on MSP-1 are immunodominant, frequency-dependent selection might thus explain, at least partially, temporal variations in *msp-1* gene type frequencies in Rondônia (Fig. 3), as well as those described in block 2 allelic-type frequencies in parasites from Sudan (4) and Senegal (44). In contrast, no temporal variation in *msp-1* diversity was found for a period of 6 years in Gambia (17), for 1 year in coastal Kenya (46), and for 1 to 2 years in southern Vietnam (28, 42). Temporal variations may also be explained by human migration across the Amazon Basin (49). New parasite strains may have been introduced into Rondônia between 1985 and 1997. Alternatively, allelic frequencies may fluctuate at random in the absence of selective pressure. One observation of practical interest for multivalent vaccine development is that, despite the temporal variations, the seven most common *msp-1* gene types remained largely predominant at two time points 12 years apart.

Nonrandom associations between allelic types (28, 42) (Fig. 5) and dimorphic epitopes (16) may result from natural selection at the *msp-1* locus or random genetic drift. This distinction has practical implications, since selective pressure suggests that a given protein plays a major role in host-parasite relationships. Further support for the natural selection hypothesis comes from the predominance of nonsynonymous over synonymous nucleotide substitutions in *msp-1* sequences (22, 39). As the function of MSP-1 is unknown, the nature of biological constraints that could select for particular allelic type associations remains to be determined.

Similar proportions of clinically immune Africans and Amazonian patients were found to have antibodies that recognize MSP-1-derived peptides, but the IgG subclass compositions of these antibodies tended to differ. The magnitude of IgG3 responses was higher in African adults than in Amazonians, the

TABLE 3. Association between block 2 allelic types in infecting parasites and IgG antibody recognition of block 2 peptides during primary *P. falciparum* infections in Amazonian patients

Donor	Age (yr)	Allelic type	IgG antibody concn (g/ml) for allelic type[a]:				
			3D7	Palo Alto	MAD20	Wellcome	RO33
R18	63	K1	91.8	92.5	0	0	0
R22	20	RO33	20.3	3.8	3.8	15.2	2.1
R28	24	K1, MAD20, RO33	10.3	0	10.7	0	0
R42	8	K1	36.8	24.5	0	0	0
R45	22	MAD20	24.7	0	0	0	0
R53	41	MAD20	59.5	53.3	26.8	17.1	16.8
R77	11	K1, MAD20, RO33	69.6	20.9	34.0	31.5	2.7
R78	24	K1	70.0	0	54.9	55.0	73.5
R83	6	K1	52.5	10.8	7.3	3.4	3.3
R96	14	MAD20, RO33	31.7	16.4	16.4	36.7	16.7
R107	8	K1	15.4	23.7	24.6	24.4	9.6
R108	<1	K1	61.5	14.1	52.2	4.5	3.5
R117	19	RO33	5.1	0	4.8	5.0	0
R124	2	K1	28.8	23.7	48.5	30.1	27.7
R129	29	MAD20	22.4	28.1	41.9	9.1	0
R143	14	MAD20	0	0	30.1	27.4	0

[a] IgG concentrations were calculated as the sum of concentrations of all IgG subclasses.

TABLE 4. Pairwise correlations between concentrations of IgG antibodies to different block 2 peptides[a]

Block 2 peptides	r_s for:			
	Malaria patients from Pará (n = 20) with indicated disease stage		Clinically immune Africans (n = 24)	Malaria patients from Rondônia (n = 91)
	Acute	Convalescent		
Same allelic family				
3D7 and Palo Alto	0.816	0.507	0.600	0.545
MAD20 and Wellcome	0.521	0.624	0.555	0.229
Different allelic families				
3D7 and MAD20	0.499	0.674	0.337	0.094
3D7 and Wellcome	0.697	0.657	0.296	0.188
3D7 and RO33	0.294	0.118	0.324	0.050
Palo Alto and MAD20	0.500	0.423	0.459	0.198
Palo Alto and Wellcome	0.565	0.518	0.389	0.080
Palo Alto and RO33	0.306	−0.069	0.155	0.162
MAD20 and RO33	0.000	0.264	0.552	0.225
Wellcome and RO33	0.257	−0.039	0.416	0.127

[a] Results are expressed as Spearman's rank correlation coefficients (r_s).

opposite trend being observed in relation to noncytophilic antibodies and IgG1 (Fig. 6). The relative participation of IgG3 in responses to some peptides tended to increase among acutely infected Amazonians with more-frequent past malaria exposure (Fig. 7). The balance between IgG1 and IgG3 responses to the C-terminal part of MSP-1 in subjects exposed to different levels of malaria endemicity in Senegal follows the same overall trend detected in this study (53). Causal relations between high levels of parasite-specific IgG3 and clinical protection from malaria cannot be inferred from crosssectional surveys, but two recent studies from Senegal are suggestive of such an association (3, 57). Since both IgG1 and IgG3 are functionally equivalent (54), the reasons why a preferential switch to IgG3 is found in heavily exposed subjects are unclear, and further analyses are needed to investigate the role of IgG3 antibodies to MSP-1 in clinical immunity to malaria. It remains also unknown why different *P. falciparum* surface antigens (25), and fragments of the same antigen (Fig. 6 and 7), are recognized by naturally acquired antibodies with contrasting IgG subclass compositions.

The levels of antibodies to conserved and dimorphic blocks, but not to polymorphic block 2, decreased in Amazonian patients 2 months after the acute malaria episode (Fig. 6). Indeed, short-lived antibodies to *P. falciparum* and *P. vivax* MSP-1 were previously described in semi-immune subjects (7, 8, 12, 30, 59), but different time scales (1 to 9 months) were used to define short-lived responses. Once again, comparisons are restricted by variations in IgG subclass composition of antibodies to distinct MSP-1 regions, since human IgG subclasses differ in their serum half-lives (54). IgG3 has a serum half-life of only 9 to 10 days, but our data indicate that its preponderance in block 2 recognition does not necessarily result in short-lived responses, as recently suggested in relation to those of other antigens (25).

Repetitive *P. falciparum* antigenic determinants, such as those in block 2 of MSP-1, may be involved in both immune protection and evasion (58). A monoclonal antibody that targets block 2 inhibits parasite growth in vitro, but its fine specificity has not been determined (47). To play a role in immune evasion, block 2 repeats should be immunodominant and induce variant-specific antibodies. The results presented herein suggest that naturally acquired variant-specific responses may be more prevalent than previously supposed (11, 12). Variant-specific recognition of block 2 peptides was relatively common in primary infections (Table 3), and within-type correlations of antibody concentrations were low in most groups of subjects (Table 4). In contrast, variant-specific antibodies were seen in only 5% of *P. falciparum* infections in an area of unstable malaria transmission in Sudan, where within-type correlation coefficients ranged between 0.846 and 0.961 (12). These results indicate that either type-specific or variant-specific antibodies may predominate in block 2 recognition in distinct areas of endemicity. If so, repetitive sequences of block 2 may or may not play a role in immune evasion, depending on the prevailing patterns of malaria transmission.

Some instances of mismatch between anti-block 2 antibody responses and the block 2 type detected by PCR in infecting parasites (reference 12 and Table 3) raise the possibility of selective unresponsiveness to antigenic variants expressed by infecting parasites. However, most probably, these findings simply reflect the presence of parasite populations undetected by PCR. Extensive clonal diversity of *P. falciparum* infections may occur even in areas of very low malaria endemicity (20). If parasitemias of clones expressing different MSP-1 types fluctuate over time (19, 24), PCR may fail to detect parasite subpopulations to which patients' B cells are currently responding but that are momentarily present at very low levels in peripheral blood. Moreover, despite these few discrepancies and the presence of variant-specific antibodies, we found a clear association between infection with parasites expressing a given block 2 type and increased levels of cytophilic antibodies to that type (Fig. 8). If cytophilic antibodies are primarily involved in clinical protection from malaria (6, 32), these findings have obvious implications in the context of strain-specific immunity.

In conclusion, in this study we have shown that polymorphic, dimorphic, and conserved fragments of *P. falciparum* MSP-1 are targeted by naturally acquired antibodies from patients exposed to hypoendemic malaria transmission in the Brazilian Amazon region. High levels of antibodies against the locally prevalent versions of variable peptides were detected, and the IgG subclass composition of these antibodies seems to depend on both exposure-dependent host-driven mechanisms and poorly understood antigen-driven mechanisms. Finally, antibody recognition of the polymorphic block 2 in Amazonian patients involved both type-specific and variant-specific antibodies.

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