ALLELIC DIVERSITY AT THE MEROZOITE SURFACE PROTEIN-1 LOCUS OF PLASMODIUM FALCIPARUM IN CLINICAL ISOLATES FROM THE SOUTHWESTERN BRAZILIAN AMAZON

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Abstract. Nucleotide sequences of each variable block in the Plasmodium falciparum merozoite surface protein-1 gene (PfMSP-1) may be grouped into one of two or three possible allelic types, named after the reference isolates MAD20, K1, and RO33. Allelic diversity at this locus basically results from different combinations of allelic types in variable blocks. We used a polymerase chain reaction (PCR)-based strategy to type the variable blocks 2, 4a, 4b, and 10 of the PfMSP-1 gene of P. falciparum isolates from 54 symptomatic malaria patients living in Rondonia, a hyperendemic area in the southwestern Brazilian Amazon. Ten different PfMSP-1 gene types, defined as unique combinations of allelic types in variable blocks, were identified among the 54 isolates. Twenty-one isolates (39%) harbored more than one gene type and two had at least three genetically distinct clones. Hybrid sequences, with a MAD20-type sequence in the 5' segment (4a) and a K1-type sequence in the 3' segment (4b), were quite common in block 4. Direct sequencing of block 4 PCR products revealed a new putative recombination site in four isolates. In contrast with previous studies, the observed distribution of gene types does not deviate significantly from that expected under the null hypothesis of random association between allelic types detected in each variable block. These contradictory data are discussed with reference to the immunoepidemiologic features prevailing in distinct malaria-endemic areas.

Extensive polymorphism has been revealed by comparing nucleotide sequences of the merozoite surface protein-1 gene of Plasmodium falciparum (PfMSP-1) that codes for a major malaria vaccine candidate. Seven variable blocks, that are separated by either conserved or semiconserved regions, have been described in this molecule (Figure 1a). There are two versions of each PfMSP-1 variable block, represented by the sequences of the isolates MAD20 and K1. Most PfMSP-1 polymorphisms may be generated by intragenic recombination between these two representative types at sites near the 5' end of the gene (blocks 3, 4, and 5). The only major exception to this dimorphic rule is the variable block 2, which has a third polymorphic form represented by the isolate RO33. This third version is relatively infrequent in culture-adapted isolates but quite common in field isolates from endemic areas in South America, Africa, and Oceania. Therefore, major PfMSP-1 gene types can be defined as unique combinations of variable regions derived from 1) either of three possible block 2 allelic types, 2) one or both allelic types in block 4 because recombinant types with sequences derived from both MAD20 and K1 alleles have been found in this region, and 3) either of two allelic types in variable blocks 6, 8, 10, 14, and 16 in the central and 3' portions of the gene.

Immunization experiments in monkeys have been performed with native PfMSP-1 and recombinant peptides derived from different P. falciparum isolates, but it is unclear whether a cross-protection can be induced when the immunizing and challenging parasites exhibit high degrees of amino acid diversity. This issue is particularly relevant when considering PfMSP-1 as a malaria vaccine candidate. Because the prevalence of PfMSP-1 polymorphisms is susceptible to both geographic and temporal variation, a particular immunogen may or may not match the most prevalent gene types in a given endemic area at a given moment. To address this question, more information is required about the genetic diversity in parasites from geographically diverse regions. We used a recently developed polymerase chain reaction (PCR)-based strategy to investigate the extent of PfMSP-1 allelic diversity in P. falciparum isolates from the southwestern Brazilian Amazon. Ten of the 24 possible distinct PfMSP-1 gene types were identified. Most parasites exhibited intragenic recombination within block 4, and a new putative recombination site was found in this variable region. In contrast with previous studies, however, we were unable to detect nonrandom associations between allelic types detected in each variable block. These contradictory data are discussed with reference to the immunoepidemiologic features prevailing in distinct malaria-endemic areas.

Source of P. falciparum isolates and DNA extraction. The field component of this study was conducted between June and July 1995 at the Center for Tropical Medicine of Rondonia, a 80-bed reference hospital situated in Porto Velho, southwestern Brazilian Amazon, after approval from the scientific and ethical committees of its Division of Research. Venous blood samples were collected after informed consent was obtained from 49 outpatients and 5 inpatients (70% males) eight months to 63 years of age (mean age = 27 years) with an acute febrile illness and P. falciparum infection that was confirmed by microscopic examination of Giemsa-stained thick blood smears. Blood samples were immediately frozen at −20°C. Parasitemias ranged from 40 to 64,000 parasites/μL of blood, and two patients (an eight-month-old infant with severe anemia and generalized convulsions and an eight-year-old boy with severe anemia and hemoglobinuria) had severe malaria. All patients lived in urban or rural areas in northern Rondonia (municipalities of
PORTO VELHO, CANDEIAS DO JAMARI, AND JAMARI, A HYPOENDEMIC REGION CHARACTERIZED BY UNSTABLE TRANSMISSION OF BOTH P. FALCIPARUM AND P. VIVAX. 17 After thawing, 200 μl of clotted blood were lysed in 50 μl of 0.15% saponin in phosphate-buffered saline (PBS, pH 7.4), washed in PBS, and resuspended in 200 μl of TEN buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 100 mM NaCl) with 0.5% sodium dodecyl sulfate and 200ng/ml of proteinase K (Boehringer Mannheim, Mannheim, Germany). After overnight incubation at 54°C, DNA was extracted using a standard phenol-chloroform protocol, precipitated with ethanol at −80°C in the presence of 125 mM sodium acetate (pH 5.2), and resuspended in 50 μl of sterile distilled water. The DNA templates were stored at −30°C until tested.

The PfMSP-1 typing strategy. The four-step procedure may be briefly described as follows, and oligonucleotide primer locations are shown in Figure 2b.

First step: block 2 typing. Block 2 was typed by nested PCR essentially as described elsewhere. 9 The first amplification was performed with the common primers C1F (5′-AATCTAGAAGCTTTAGAAGATGCAG-3′) and C3R (5′-ACATATGATTGGTTAAATCAAAGAG-3′), while the second amplification was performed in three separate reaction tubes with the common reverse primer C3R and either of the type-specific forward primers M2F (5′-GGTTCAGGTAATTCAAGACGTAC-3′), K2F (5′-TCTTAAATGAAAGAAATTACTACAAA-3′), or R2F (5′-TAAAGGATTGGAGAAAATTACTACA-3′).
Second step: block 10 typing. A single PCR with the common forward primer C9F (5′-TTGAGAATGAAATCAGTG-3′) and the type-specific reverse primers M10R (5′-GTTGAAATCAGTGATACATCATTGC-3′) and K10R (5′-GTGAGAATGAAATCAGTG-3′) was performed to type block 10.

Third step: preparing the DNA template to type block 4. Two different procedures were used: (3a) amplification of a short fragment between blocks 3 and 5 (about 470 basepairs [bp]) with the common primers C3F (5′-TTGAGAATGAAATCAGTG-3′) and C5R (5′-GGATCAGTAAATAACTATCATTGC-3′),11 or (3b) amplification of a long fragment between blocks 2 and 6 (between 900 and 1150 bp) with one of the type-specific forward primers M2F, K2F or R2F; chosen after the first step, and one of the type-specific reverse primers M6R (5′-ATTGGAACAGATTTTCTGTAGGATCCTTG-3′) or K6R (5′-GATATCTTGGGTCTGAGTGCTG-3′), chosen after the second step.13 Since intragenic recombination in the region between blocks 2 and 6 and 16 has not been observed,15, 18, 19 the allelic type determined for block 10 (either MAD20 or K1) is considered to be the same for block 6, and this information was used to select type-specific reverse primers for the step 3b. When the first or second step revealed the presence of two or more different allelic types, the reaction 3b was used in the third amplification step. Each parasite subpopulation present in mixed infections could be separately typed, because different DNA templates were prepared to type block 4.

Fourth step: block 4 typing. Block 4 was typed by nested PCR in four separate reactions with one of the allelic type-specific forward primers M4F (5′-TTGAGAATGAAATCAGTG-3′) or K4F (5′-AATGGATCCCACTCCACCCG-3′) and one of the allelic type-specific reverse primers M4R (5′-TGCACTTCTTTTTTCTATTCTGAG-3′) or K4R (5′-TCTCTGATTTTTTGTCTTATCAAG-3′).11 Both the 5′ (block 4a) and the 3′ (block 4b) segments of block 4 were typed.11

The 24 gene types distinguishable by the PCR-based strategy used here were assigned numbers (Table 1). Accordingly, a MAD20-like gene type (number 23) has MAD20-type sequences in all variable blocks, while the PfMSP-1 gene. Allelic types are named after the reference isolates MAD20 (M), K1 (K), and RO33 (R).

The proportion of each gene type expected under the null hypothesis of random association between allelic types was computed as its proportion of the expected distribution of PfMSP-1 gene types under the null hypothesis of random association between allelic types.

Table I

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<th>Variable block</th>
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* Each gene type is 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 10 of the PfMSP-1 gene. Allelic types are named after the reference isolates MAD20 (M), K1 (K), and RO33 (R).

† Twenty-one isolates harbored more than one fully typed parasite population; frequencies (%) were computed using the total number of gene types detected in this parasite population (n = 77) in the denominator.

** Expected distribution of PfMSP-1 gene types under the null hypothesis of random association between allelic types.

DNA sequencing of PCR products. Selected block 4 products were sequenced using an ABI PRISM® Dye Terminator Cycle Sequencing kit (Perkin-Elmer Cetus) following the manufacturer’s protocol (P/N 402702 Revision A, August 1995). Briefly, block 4 fragments amplified by the fourth step were excised from the gels, reamplified with the appropriate primers, and the reactions were loaded onto 2% NuSieve® plus 1% SeaKem® agarose gels (FMC BioProducts, Rockland, ME). The products were excised from the gels, purified using the Cleanmix kit (Talent, Trieste, Italy), and sequenced with the forward primers M4F or K4F. Sequences were analyzed by the 373 DNA Sequencing System (Applied Biosystems, Foster City, CA).

Data analysis. For the purpose of this study, the frequency of each gene type was computed as its proportion of the total of typed parasite populations among the isolates tested. The proportion of each gene type expected under the null
hypothesis of random association of variable block allelic types was derived from a simple probability model analogous to those used in population genetics to estimate the expected frequency of multi-locus genotypes. For instance, the expected proportion of MAD20-like gene types in this population is calculated by multiplying the observed proportions of parasites with MAD20-type sequences in blocks 2 (0.195), 4a (0.805), 4b (0.182), and 6-16 (0.961), while the expected proportion of Wellcome-like gene types is calculated by multiplying the observed proportions of parasites with a MAD20-type sequence in block 2 (0.195) and K1-type sequences in blocks 4a (0.195), 4b (0.818), and 6-16 (0.039). To test the null hypothesis of random assortment of allelic types in this parasite population, the expected and observed frequencies were compared using Kolmogorov-Smirnov statistics. The same test was used to compare the distribution of gene types in single and mixed infections. Significance was defined at the 5% level.

RESULTS

Ten of 24 possible different PfMSP-1 gene types were identified, and 77 parasite populations were fully typed among 54 isolates. The observed frequency of each type is shown in Table 1. About 95% of all parasites belonged to the seven most frequent gene types. Five PfMSP-1 gene types identified here have been previously characterized in fully or partially sequenced P. falciparum clones or isolates: 19 type 2 is Wellcome-like, 13 is CAMP-like or Uganda Palo Alto-like, 16 is RO71-like, 23 is MAD20-like, and 24 is RO33-like. Two other combinations described here were also detected in Thai isolates by Southern blot hybridization and partial sequencing: 11 types 17 (Thai isolates 807, 827, 828, 836, 837, 842, and 843) and 22 (Thai isolate 946). Moreover, 22 of the 24 types were recently detected in clinical isolates from southern Vietnam by the same PCR-based strategy used here. The types 4 and 11 were the only combinations of PfMSP-1 allelic types absent in that endemic area. 19 More than one type was found in 21 Amazonian isolates (39%), and two isolates (R3 and R121) harbored at least three genetically distinct clones.

Interestingly, the block 4 type described in isolate T9-101, 10 with a MAD20-type sequence in the 5’ segment (4a) and a K1-type sequence in the 3’ segment (4b), was quite common (62%) in Amazonian isolates. Nevertheless, no parasite population was found to harbor a K1-type sequence in the 5’ segment and a MAD20-type sequence in the 3’ region of block 4, a recombinant version recently described in isolates from Thailand (3 of 23 sequenced isolates), the Solomon Islands (6 of 23 typed isolates), and Vietnam (29 of 136 typed isolates). 11, 15 Furthermore, K1-type sequences in blocks 6-16 were rarely observed here (4%), and no K1-like gene type (number 1 in Table 1) was identified.

Table 1 also shows the frequency of each gene type that is expected under the null hypothesis of random association of allelic types. Despite some seemingly divergent data (for instance, gene types 14, 17, and 23), no significant difference was found between expected and observed distributions (d = 0.076, d = 0.153, P > 0.05 by the Kolmogorov-Smirnov test). All expected prevalences were included in the 95% confidence interval of the observed data. The same data were also compared by the χ² statistic for goodness of fit, with similar results (χ² = 2.87, degrees of freedom = 5, P > 0.05). For χ² analysis, all cells with expected frequencies < 5 were pooled. We also tested the null hypothesis that gene types were present in single and mixed infections at similar frequencies. For this analysis, we classified as harboring mixed infections all isolates with more than one allelic type detected after the first, second, or fourth step, even if some of the subpopulations present in mixed infections could not be fully typed by all amplification steps. Twenty-six isolates met these criteria. Types 13, 15, and 17 were found only in mixed infections, but once again we were unable to reject the null hypothesis (d = 0.120, d = 0.321, P > 0.05 by the Kolmogorov-Smirnov test).

We confirmed the presence of hybrid sequences within block 4 (Figure 3). In two (R39 and R71) of six isolates with recombinant block 4 sequence MAD20 plus K1, the putative site of recombination was the same as that previously described, 16, 11 between nucleotides 1129 and 1135 of the MAD20 sequence. However, a new putative site of recombination was found in four isolates (R18, R106, R119, and R134) between nucleotides 1119 and 1124. Nucleotide sequences were further confirmed by analysis of the fragment...
amplified using primers M4F and C5R with the reverse primer C5R applied to the cycle sequencing reaction. Therefore, we propose that the region upstream of nucleotide 1119 may be defined as the 5' segment of block 4 (block 4a), while the region downstream of nucleotide 1135 may be defined as the 3' segment (block 4b). These boundaries are indicated by arrows in Figure 3.

**DISCUSSION**

We have investigated genetic diversity at the PfMSP-1 locus in field *P. falciparum* isolates from the Brazilian Amazon. A relatively restricted number of PfMSP-1 gene types (10 of 24 major versions) was identified in this area, and a new putative site of recombination was described within the variable block 4 of this gene.

Differential prevalences of dimorphic PfMSP-1 epitopes have been previously investigated by Conway and others in *P. falciparum* isolates from West Africa (Gambia and Nigeria) and Brazil (eastern Amazon). The isolates were serotyped by indirect immunofluorescence with a panel of allelic type-specific monoclonal antibodies after a brief period (24–48 hr) of *in vitro* growth. The low prevalence of K1-type sequences or epitopes in blocks 6-16 was a common feature of this and our studies, and contrasts with that observed in Thai isolates. However, some epitopes were shown to occur more frequently than expected in these serotyped parasite populations. These findings could be explained by genetic drift if rates of intragenic recombination are low, but Conway and others alternatively suggested that certain epitope associations could confer a selective advantage to the parasites. Moreover, there is an indirect statistical evidence, derived from sequence analysis of *in vitro* cultured strains, for positive selection in blocks 2 and 3. Nevertheless, analogous associations between allelic types were not detected in our Amazonian isolates. Our contradictory results may originate from the fact that 1) short-term *in vitro* growth may have induced selection against particular parasite subpopulations in the serotyped isolates, and 2) typed regions of the PfMSP-1 antigen or gene are not exactly the same in both studies, and 3) different epidemiologic patterns of host-parasite interactions that prevail in each endemic area may affect the genetic structure of malaria parasites.

Differences in sample size might theoretically originate the discrepancies between both studies. The number of field isolates typed in Gambia (n = 445) by far exceeds our sample size, and statistically significant deviations from the null hypothesis of random associations of epitopes are more likely to be detected in such large samples. Negative results, therefore, might be due to a relatively low power of the tests in small samples. However, at least some significant associations between dimorphic epitopes were detected by Conway and others in relatively small numbers of field isolates from Nigeria (n = 60) and the eastern Brazilian Amazon (n = 62).

The epitopes recognized by the panel of monoclonal antibodies used by Conway and others are located on the PfMSP-1 blocks 2, 3, 4b, 6-16, and 16-17. The most frequent statistical associations were found between epitopes on blocks 2 and 3 and between epitopes on blocks 3 and 4b. In contrast, the present study was focused on the variable blocks 2, 4a, 4b, and 6-16; semiconserved (block 3) and conserved (block 17) regions were not typed here. As a result, possible nonrandom associations found between epitopes on block 3 and neighboring regions could not be addressed by our methodology.

An extensive PCR-based study of *P. falciparum* isolates from southern Vietnam has provided more information about the genetic diversity and putative selection at the PfMSP-1 locus. Laboratory methods and statistical analyses were similar to the ones we used, except that the sampled parasite population in Vietnam is considerably larger (186 genetically distinct parasite populations from symptomatic patients were typed), and that malaria endemicity, as judged by the parasite rate in this region (about 32%), is quite high if compared with that usually found in Rondonia (usually < 2%). Twenty-two of 24 possible PfMSP-1 gene types were found, suggesting a higher degree of genetic diversity in Vietnam than in the Amazon. Nonrandom associations between allelic types were detected between blocks 4 (including both the 5' and 3' regions) and 6 among Vietnamese isolates. Nonrecombinant types (that is, those characterized by the same allelic type, either MAD20 or K1, in blocks 4a, 4b, and 6) were more common than expected by random recombination in this region. It remains unclear whether similar biases tend to be induced by selective pressure in parasite populations from different endemic areas.

Possible selection mechanisms for PfMSP-1 polymorphisms in natural parasite populations are still conjectural since the function of PfMSP-1 is unknown. The selective role of the host's immune system remains to be fully investigated. The relative importance of variable versus conserved domains as targets of naturally acquired anti-PfMSP-1 immune responses is controversial, and such responses are likely to differ in both quantitative and qualitative features in human populations exposed to varying levels of malaria transmission. It is reasonable to hypothesize that naturally acquired immunity may be more selective in areas of high endemicity than in hypoendemic regions such as the Brazilian Amazon, where effective anti-parasite immune responses rarely develop in the host population. If nonrandom assortment of PfMSP-1 allelic types is essentially a result of immune selection, this phenomenon is expected to be more common under conditions of high malaria transmission. Malaria endemicity in southern Vietnam is considerably lower than in holoendemic Africa, but transmission is still much more intense than in the Brazilian Amazon. Therefore, immune pressure should result in an intermediate pattern in Vietnam (more intense selection than in Brazil but less than in Gambia or Nigeria), which is compatible with the findings by Kaneko and others. Further cross-sectional and longitudinal studies of malaria parasites in different endemic areas are required to elucidate this point.

The development of molecular methods such as that used here has provided field and laboratory researchers with improved tools to type wild parasite populations. Furthermore, the possible effects of naturally acquired and vaccine-induced immunity against conserved and polymorphic antigens on the genetic structure of natural *P. falciparum* populations represent a critical issue to be investigated by these methods in the near future.
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