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Original Articles

A *Plasmodium vivax* Vaccine Candidate Displays Limited Allele Polymorphism, Which Does Not Restrict Recognition by Antibodies

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Abstract

Background: The 19 kDa C-terminal region of the merozoite surface protein 1 ($MSP1_{19}$) has been suggested as candidate for part of a subunit vaccine against malaria. A major concern in vaccine development is the polymorphism observed in different plasmodial strains. The present study examined the extension and immunological relevance of the allelic polymorphism of the $MSP1_{19}$ from *Plasmodium vivax*, a major human malaria parasite.
Materials and Methods: We cloned and sequenced 88 gene fragments representing the $MSP1_{19}$ from 28 Brazilian isolates of *P. vivax*. Subsequently, we evaluated the reactivity of rabbit polyclonal antibodies, a monoclonal antibody, and a panel of 80 human sera to bacterial and yeast recombinant proteins representing the two allelic forms of *P. vivax* $MSP1_{19}$ described thus far.
Results: We observed that DNA sequences encoding $MSP1_{19}$ were not as variable as the equivalent region of other species of *Plasmodium*, being conserved among Brazilian isolates of *P. vivax*. Also, we found that antibodies are directed mainly to conserved epitopes present in both allelic forms of the protein.
Conclusions: Our findings suggest that the use of $MSP1_{19}$ as part of a subunit vaccine against *P. vivax* might be greatly facilitated by the limited genetic polymorphism and predominant recognition of conserved epitopes by antibodies.

Introduction

Malaria remains the most prevalent and devastating parasitic disease worldwide, with a yearly estimate of more than 300 million cases due to *Plasmodium falciparum* and at least 40 million cases due to *P. vivax*. The population at risk is estimated at billions of individuals living in tropical regions of the world. The disease is most prevalent and deadly in Africa, where *P. falciparum* is endemic. Although the patterns of disease transmission, infection, and mortality are quite diverse, malaria transmission also occurs in large areas of South and Southeast Asia, Latin Amer-

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ica, and Oceania, where malaria caused by *P. vivax* is widespread.

Although malaria control was successful in many countries in the past, it is now very difficult to eradicate the disease in developing countries, mainly because of the presence of drug-resistant parasites and mosquito vectors. In view of these problems, the development of new strategies for prevention and control of the disease are in great need.

Mass vaccination is considered a feasible objective that in the future may be added to other strategies such as chemotherapy, vector control, etc., for the prevention and control of malaria. One of the most promising vaccine candidates against the erythrocytic forms of malaria is the merozoite surface protein 1 (MSP1, reviewed in ref. 1). This protein is synthesized as a large-molecular-weight precursor (180–230 kDa) during schizogony and is later processed into several smaller fragments corresponding to the major merozoite surface proteins. During the invasion process, a proteolytic cleavage step releases most of the molecule from the merozoite membrane and only a 19 kDa glycerolphosphatidylinositol-anchored fragment of the C-terminus ($MSP1_{19}$) is carried into the invaded red cells (2,3). The biological importance of MSP1 for parasite survival is unknown; however, it is well established that antibodies that recognize its C-terminal region inhibit merozoite invasion in vitro (3–5) and confer passive immunity on naive mice (6,7). Most relevant to malaria control is the fact that immunization with recombinant proteins based on the sequence of $MSP1_{19}$ in human, non-human primate, and rodent species malaria can provide remarkable protection against experimental infection with blood stages of *Plasmodium* (8–13).

$MSP1_{19}$ of different species consists of two domains, each with six cysteine residues configured as an epidermal growth factor (EGF)-like domain. EGF-like structures are immunogenically very important. Epitopes recognized by protective antibodies are dependent on the conformation provided by the disulfide bonds and the presence of both EGF-like domains (9,14).

Despite the structural conservation, there are several amino acid modifications among $MSP1_{19}$ of different species of malaria. Most relevant for vaccine development is the polymorphism observed in different strains of the same species. A series of amino acid substitutions were detected in the $MSP1_{19}$ gene of *P. yoelii*, a rodent malaria parasite (15). These substitutions are immunologically relevant because protective antibodies generated by vaccination are directed at these polymorphic epitopes (16).

In *P. falciparum*, a human malaria parasite, five amino acid substitutions have been described in the 19 kDa fragment of MSP1. On the basis of these five possible substitutions, seven natural allelic forms have been described (17–19). It is well established that there are strain-specific as well as conserved epitopes detected by mouse monoclonal antibodies or human polyclonal antibodies from individuals who had been infected with malaria (20–24). It is currently unknown whether protective antibodies induced by vaccination with *P. falciparum* $MSP1_{19}$ recognize polymorphic or conserved epitopes, or both.

Sequence data for the $MSP1_{19}$ gene from *P. vivax* (*PvMSP1*$_{19}$), the second-most important human malaria parasite, are more limited. Deduced amino acid sequences of $PvMSP1_{19}$ from 20 isolates from Southeast Asia and two strains from Latin America showed only a single amino acid substitution (25). However, it is unknown whether this single substitution is of immunological significance.

Recently, we have evaluated several immunoepidemiological aspects of the naturally acquired human immune responses to the MSP1 of *P. vivax*, the parasite that accounted for 75% of the 405,000 cases of malaria reported in Brazil in 1997. We found that $PvMSP1_{19}$ was the most immunogenic of 11 recombinant proteins during natural infection in humans, being recognized by antibodies or T cells of 83.8% of individuals recently exposed to *P. vivax*. Even more important was the finding that the antibody titers to $PvMSP1_{19}$ were higher than the titers to other recombinant proteins representing the N-terminal region of PvMSP1 (26,27). This high frequency of responders to $PvMSP1_{19}$ was also described in an independent survey performed in an area of *P. vivax* transmission in New Guinea (28). The fact that more than 70% of the individuals had IgG antibodies suggests that $PvMSP1_{19}$ is highly conserved among the different isolates of *P. vivax*. Nevertheless, some individuals fail to develop antibodies to $PvMSP1_{19}$ even in the presence of high antibody titers to other polypeptides of PvMSP1 (26,27).

The lack of reactivity observed in some individuals could be explained at least in part by allelic variation. Given this possibility, the present study was designed to examine the extent of $PvMSP1_{19}$ polymorphism in Brazil and its immunological relevance for recognition by an-

tibodies. Initially, we sequenced the DNA corresponding to $PvMSP1_{19}$ of 88 gene fragments obtained from 28 Brazilian isolates. Subsequently, we evaluated the reactivity of rabbit polyclonal antibodies, a monoclonal antibody, and a panel of 80 human sera with bacterial and yeast-derived recombinant proteins representing the two allelic forms of $PvMSP1_{19}$ described thus far.

Materials and Methods

Polyclonal and Monoclonal Antibodies

The polyclonal rabbit antiserum was obtained by immunization of a rabbit with a baculovirus-derived recombinant protein representing the 19 kDa region of PvMSP1 (29,30) in Freund's adjuvant (J. Barnwell, unpublished data). The IgG fraction was purified by affinity chromatography using a protein A–agarose matrix (Pharmacia). Antibody concentration was estimated by optic density at 260 nm. The initial concentration was 1 mg/ml. Murine anti-$PvMSP1_{19}$ monoclonal antibody (MAb) 3F8.A2 and mouse ascites were produced as described by Barnwell et al. (31).

Parasites and Human Blood Samples

After receiving each patient's informed consent, we obtained blood samples from 80 patients with vivax malaria who were diagnosed at Instituto Evandro Chagas in Belém, state of Pará, in the northern part of Brazil. Details on the study area, patient age, and diagnosis have been described by Soares et al. (26,27). Eighteen additional blood samples were collected at the Tropical Medical Center (CEMETRON), Porto Velho, state of Rondônia, in the northwest part of Brazil. Five milliliters of peripheral blood were collected from each patient directly into EDTA tubes before drug treatment. Plasma and infected erythrocytes were removed after centrifugation, stored frozen at −20°C, and transported to São Paulo for further study.

Cloning of PCR Products and Sequencing

Genomic DNA was purified from infected blood samples as described earlier (32). Briefly, 1 ml of TE (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, pH 8.0) was added to each blood sample. After centrifugation at 12,500 × *g* for 10 sec, the pellet was resuspended a second time in TE, and the procedure was repeated twice. The colorless pellets were resuspended in 200 μl of PK buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, and 10 mg/ml proteinase K). Samples were incubated at 65°C for 45 min and then at 95°C for 15 min to inactivate proteinase K. Samples were stored at 4°C until needed. Amplification reactions of the C-terminal region of the PvMSP1 gene (314 bp) were performed using primers 5′-GCTAAATGTGCAAACTCAGTTATTA-3′ (forward) and 5′-AGCTTAGGAAGCTGGAGGAGCTACA-3′ (reverse) purchased from Life Technologies. For the N-terminal region, we used the primers described by Porto et al. (33), kindly provided by Dr. H. A. del Portillo (ICB, University of São Paulo). Polymerase chain reaction (PCR) mixtures were as follows: 5 μl of blood DNA; 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM dATP, dCTP, dGTP, and dTTP; 2.5 U of *Taq* polymerase (Life Technologies); and 20 pmol of each primer. Amplification reactions were performed with the aid of a Pharmacia thermal cycler (95°C/1 min, 50°C/1 min, 72°C/2 min for 35 cycles). As positive control, PCR reactions were also performed using a cloned $PvMSP1_{19}$ sequence as the PCR template. The PCR products were separated on agarose gel. The 314 bp band was excised from the gel and the DNA purified with glass beads of a commercially available kit (Nucleiclean, Sigma) according to the specifications of the manufacturer. The DNA concentration was estimated in agarose gel. PCR products were cloned directly using a commercially prepared cloning plasmid with a single T-base overhang (*pMOS* Blue, Amersham). Eighty-eight amplified gene fragments cloned from 28 isolates were selected for sequencing studies. Two to four plasmids were sequenced from each isolate. Before sequencing, all 88 plasmids were checked for the presence of an insert that hybridized by Southern blot with a radiolabeled DNA representing the $PvMSP1_{19}$ (Belém strain). Nucleotide sequence analysis was done with the thermosequenase cycle sequencing kit (Amersham) according to the manufacturer's instructions. Plasmid-specific primers (M13 Universal and T7) were labeled with [γ-^{32}P] ATP (Amersham) using T4 polynucleotide kinase (Life Technologies).

Generation of Plasmids Coding for GST-Fusion Proteins Representing Allelic Forms of $PvMSP1_{19}$

A recombinant protein containing amino acids 1615 to 1704 of $PvMSP1_{19}$ (Belém strain) was generated. The C-terminal region of PvMSP1 was amplified by PCR, using the cloned

$PvMSP1_{19}$ (26) sequence as a substrate for primers: 5′-GGGATCCCCACTATGAGCTC-CGAGCAC-3′ (forward) and 5′-TGAATTC-CCGCTACAGAAAACTCCCTCAAA-3′ (reverse). The amplified fragment was purified and cloned into the *pMOS* Blue T-vector, and both strands were sequenced as described above. The insert was removed by treatment with *Bam*HI and *Eco*RI and ligated into the pGEX-3X expression vector (Pharmacia) treated with these same enzymes. The recombinant GST-$PvMSP1_{19}$ differs from the recombinant protein ICB10 (26) in that it does not contain the amino acids that are most likely to be removed for insertion of the glycerolphosphatidylinositol anchor. This recombinant protein presented a much better solubility and improved yield for purification.

The coding sequence of $PvMSP1_{19}$ (Belém strain) was modified by PCR mutagenesis as follows: a single substitution was introduced, a G for an A (nt 5050), resulting in an amino acid change from Lys_{1684} to Glu_{1684} substitution toward the C-terminal of the second epidermal growth factor (EGF) domain. Two PCR amplifications were performed using the following oligonucleotide primers: (*i*) 5′-GGGATCCCCAC-TATGAGCTCCGAGCAC-3′ (forward) and 5′-AGACGATTTCATTGCTGTCC-3′ (reverse); (*ii*) 5′-GGACAGCAATGAAATCGTCT-3′ (forward) and 5′-TGAATTCCCGCTACAGAAAACTCCCT-CAAA-3′ (reverse). Products of these two PCR amplifications were purified on agarose gel and used as targets for a second amplification. The primers for the amplification were 5′-GGGATC-CCCACTATGAGCTCCGAGCAC-3′ (forward) and 5′-TGAATTCCCGCTACAGAAAACTCCCT-CAAA-3′ (reverse). The product of this PCR reaction (1615_{Thr} to 1704_{Ser}) was purified and cloned into the *pMOS* Blue T-vector and both strands were sequenced to confirm the single base pair mutation. The insert corresponding to the PCR product was excised by treatment with *Bam*HI and *Eco*RI and ligated into the pGEX-3X expression vector treated with these same enzymes.

The DNA representing the $PvMSP1_{19}$ fragments of Belém and Asian strains were removed from the pGEX plasmid by treatment with the restriction enzymes *Msc*I and *Pst*I. This DNA fragment was purified and ligated in the presence of dNTPs and Klenow polymerase into the yeast expression plasmid pEG(KT) (34) previously digested with *Msc*I and *Hin*dIII. Recombinant plasmids were screened for the presence of the insert by hybridization using [α-^{32}P] ATP-labeled insert. The correct orientation was determined by restriction analysis.

Expression and Purification of Recombinant Proteins

$PvMSP1_{19}$ representing the Belém and Asian strains were expressed in *E. coli* (DH5α) as GST fusion proteins essentially as described by Soares et al. (26,27). Expression in the yeast *Saccharomyces cerevisiae* was performed essentially as described by Mitchell et al. (34). Recombinant proteins and GST were affinity purified on glutathione-Sepharose 4B columns (Pharmacia), their purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was estimated by the Bradford method (Biorad).

Immunoassays

Detection of antigen-specific IgG antibodies was done by ELISA. Plasma or serum samples were tested for reactivity with the $PvMSP1_{19}$ recombinant proteins (Belém and Asia) by ELISA as described previously (26,27). Briefly, microtiter plates (Costar, Cambridge, MA) were coated with 200 ng/well of affinity-purified bacterial fusion proteins or GST. Because of restrictions in the amount of yeast-derived recombinant proteins, we used 100 ng/well of these fusion proteins or GST. Human plasma samples were added to wells at 1:200 dilution with subsequent 2-fold serial dilutions until 1:102,400 was attained. Rabbit and mouse serum samples were also tested at the indicated dilutions. After 2 hr incubation at room temperature, unbound material was washed away, and peroxidase-conjugated goat anti-human IgG (Fc-specific, 1:10,000) (Sigma), or anti-rabbit IgG or anti-mouse IgG (both diluted 1:4000, from Kirkegaard and Perry, Gaithersburg, MD) was added to each well. After 1 hr incubation at room temperature, excess labeled antibody was removed during washing, and the reaction was developed with o-phenylenediamine (Sigma). Plates were read at 492 nm on an ELISA reader (Labsystems Multiskan MS). The OD_{492} values in Figure 4 represent binding of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone.

The inhibition assay was performed as described by Devey et al. (35). ELISA plates were coated with the recombinant bacterial $PvMSP1_{19}$ representing the Belém allele. In Figure 5a, the indicated recombinant fusion protein or GST was added at different concentrations (2-fold dilu-

tions ranging from 10 μg/ml to 3 ng/ml) in each well. Human serum at the indicated final dilution was added after the soluble protein. The ELISA was then performed as described above. In Figure 5B, serum samples were diluted to provide 0.75 to 1.25 OD_{492}. The soluble recombinant proteins of $PvMSP1_{19}$ or GST were added to reach a final concentration of 10 μg/ml. The percentage of inhibition for each serum sample was calculated as follows:

$$1 - \frac{OD_{492} \text{ in the presence of soluble recombinant protein}}{OD_{492} \text{ in the absence of soluble recombinant protein}} \times 100.$$

Results

Analysis of Polymorphism of $PvMSP1_{19}$ Sequences in 28 Isolates of P. vivax *Collected in Different Endemic Areas of Brazil*

To estimate the polymorphism of $PvMSP1_{19}$ in Brazil, total DNA was extracted from blood samples of 28 individuals during patent *P. vivax* infection. Seven of these 28 individuals were permanent residents of the city of Belém, Pará, where they acquired the disease (26,27). Three individuals were infected in the state of Maranhão, and they were diagnosed with malaria in the city of Belém. The states of Pará and Maranhão are located contiguously in the northern part of the Amazon region. Eighteen blood samples were collected from residents of Porto Velho in the state of Rondônia. This area is located in the northwest section of Brazil, more than 1000 miles from the other endemic areas (36).

A 314 bp fragment representing the C-terminus of the *P. vivax MSP1* gene was amplified by PCR as described in Materials and Methods. Two to four clones derived from each isolate were sequenced. A total of 88 amplified cloned gene fragments were compared with the original Belém sequence of the *P. vivax* MSP1 gene. We found that the sequences encoding the $PvMSP1_{19}$ in Brazil were conserved. The dimorphism of $PvMSP1_{19}$ reported in isolates from Asia (25) was not detected in the 28 isolates obtained in Brazil. Also, we were unable to find any other nucleotide substitution.

In parallel, as a control, we amplified the DNA portion coding for the highly polymorphic region P5 located in the N-terminal portion of PvMSP1 (33). According to the size of the PCR products, we found two distinct alleles described earlier in Brazilian isolates (33). Also relevant is the fact that in approximately 40% of the blood samples, we observed the presence of both alleles, indicating that these individuals had mixed infection with at least two strains of *P. vivax* (data not shown).

Recognition by Antibodies of Two Allelic Forms of $PvMSP1_{19}$ Expressed as Recombinant Bacterial or Yeast GST Fusion Proteins

To determine whether the allelic polymorphism described for $PvMSP1_{19}$ could modify recognition by specific antibodies, a gene encoding $PvMSP1_{19}$ was generated by introducing a single base pair substitution, a G for an A, resulting in an amino acid change from Lys_{1684} to Glu_{1684} toward the C-terminus of the second EGF-like domain (Fig. 1A). This gene represents the Asian dimorphic sequence of $PvMSP1_{19}$ (25) and was designated $PvMSP1_{19}$ Asia.

$PvMSP1_{19}$ recombinant proteins representing the Belém and Asia strains were expressed as soluble GST fusion proteins of ~38 kDa in *E. coli* and *S. cerevisiae*. As determined by SDS-PAGE, the recombinant GST-$MSP1_{19}$ representing the Asian allele migrates with a molecular weight that is slightly higher than that of the GST fusion protein representing the $MSP1_{19}$ allele from Belém strain (Fig. 1B, C).

Initially, these four fusion proteins and GST were used as antigens for recognition by a murine MAb specific for $PvMSP1_{19}$ (31) or by IgG purified from a rabbit immunized with a baculovirus-expressed recombinant $PvMSP1_{19}$ [Belém strain (29,30)]. Both the polyclonal antiserum and the MAb strongly inhibited the in vitro invasion of reticulocytes by *P. vivax* merozoites of the Belém strain (J. Barnwell, unpublished results). We found that rabbit polyclonal antibodies recognized similarly the recombinant proteins representing each allelic form of $PvMSP1_{19}$ generated in *E. coli* (Fig. 2A). Comparable recognition was also observed when we used yeast-derived recombinant proteins in the ELISA (Fig. 2B). The MAb was also unable to differentiate the two allelic forms of $PvMSP1_{19}$ produced either in *E. coli* or in yeast (Fig. 2C, D, respectively).

Next, we evaluated the serological recognition of the recombinant proteins representing the two allelic forms of $PvMSP1_{19}$ by antibodies collected from humans who were infected with *P. vivax*. In our initial analysis we selected two individuals from Belém. Serum samples were collected from these two individuals during their

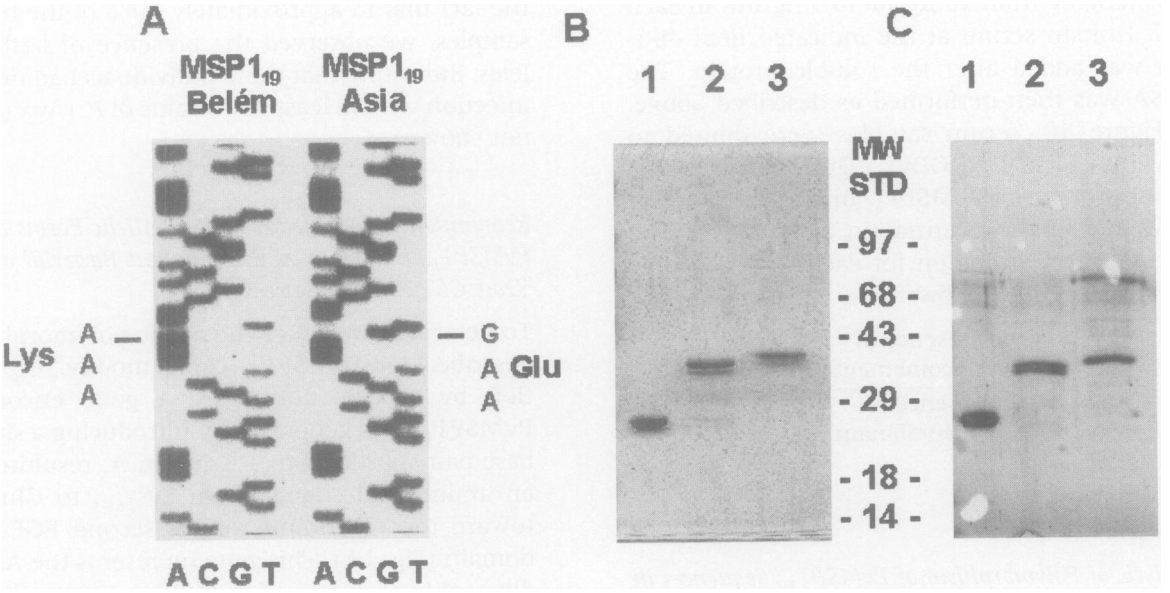

Fig. 1. Plasmid DNA sequence analysis and SDS-PAGE of recombinant proteins representing the two allelic forms of PvMSP1$_{19}$. (A) The single base pair difference between the two allelic forms of PvMSP1$_{19}$ is indicated in a sequencing gel. (B, C) SDS-PAGE of purified recombinant fusion proteins or GST produced in *E. coli* and *S. cerevisiae*, respectively. Lane 1, GST protein; lane 2, GST fusion protein representing PvMSP1$_{19}$ Belém; lane 3, GST fusion protein representing PvMSP1$_{19}$ Asia. Molecular weight standard (MWSTD) values are expressed in kDa.

first episode of *P. vivax* malaria. These individuals had never been infected with *P. vivax* or *P. falciparum* before. From a single blood sample, we obtained serum as well as DNA. The DNA was used as a target for amplification and four cloned PCR products from each individual were sequenced. In both cases, the DNA sequence matched the Belém allele of PvMSP1$_{19}$. By ELISA, we observed that sera collected from each of these two individuals reacted equally well with recombinant proteins representing both allelic forms of PvMSP1$_{19}$ (Fig. 3). The same results were obtained when we used bacterial (Fig. 3A, B) or yeast-derived (Fig. 3C, D) recombinant proteins.

We then extended this same type of analysis to 78 other serum samples collected from individuals with patent *P. vivax* infections. In the serum of 77 of the 80 individuals (96.25%), no difference was detected in ELISA titers to each recombinant protein representing the two allelic forms of PvMSP1$_{19}$. When the recognition of the two proteins was directly compared at different serum dilutions, there was a high degree of correlation between them ($r^2 > 0.97$ in all cases; Fig. 4). Only 3 of the 80 serum samples (3.75%) displayed slightly increased reactivity with a recombinant protein representing one of the allelic forms. One of them recognized the Belém allele better than the Asian allele and the other two displayed a slightly higher reactivity with protein representing the Asian allele. Although we reproduced this analysis on different occasions, the difference in antibody titers represented only a single, 2-fold dilution (data not shown).

Similar analysis was also performed with yeast-derived recombinant proteins in the ELISA. We found a very high correlation between both proteins representing the two allelic forms of PvMSP1$_{19}$ in their serum recognition ($r^2 = 0.96$, $n = 30$, data not shown). ELISAs were also performed using subclass-specific antibodies to human IgG1 and IgG3, the predominant subclasses of IgG to PvMSP1$_{19}$ (26,27). The correlation observed was also high ($r^2 = 0.936$ and 0.875 for IgG1 and IgG3, respectively, $n = 34$; data not shown).

Finally, we performed inhibition assays with bacterial recombinant proteins representing both allelic forms of PvMSP1$_{19}$. An example of the inhibitory curve obtained with each recombinant protein is shown in Figure 5A. Different concentrations of both proteins inhibited human antibody binding to PvMSP1$_{19}$ Belém at exactly the same extension. Inhibitory assays were performed with 50 serum samples from *P. vivax*–

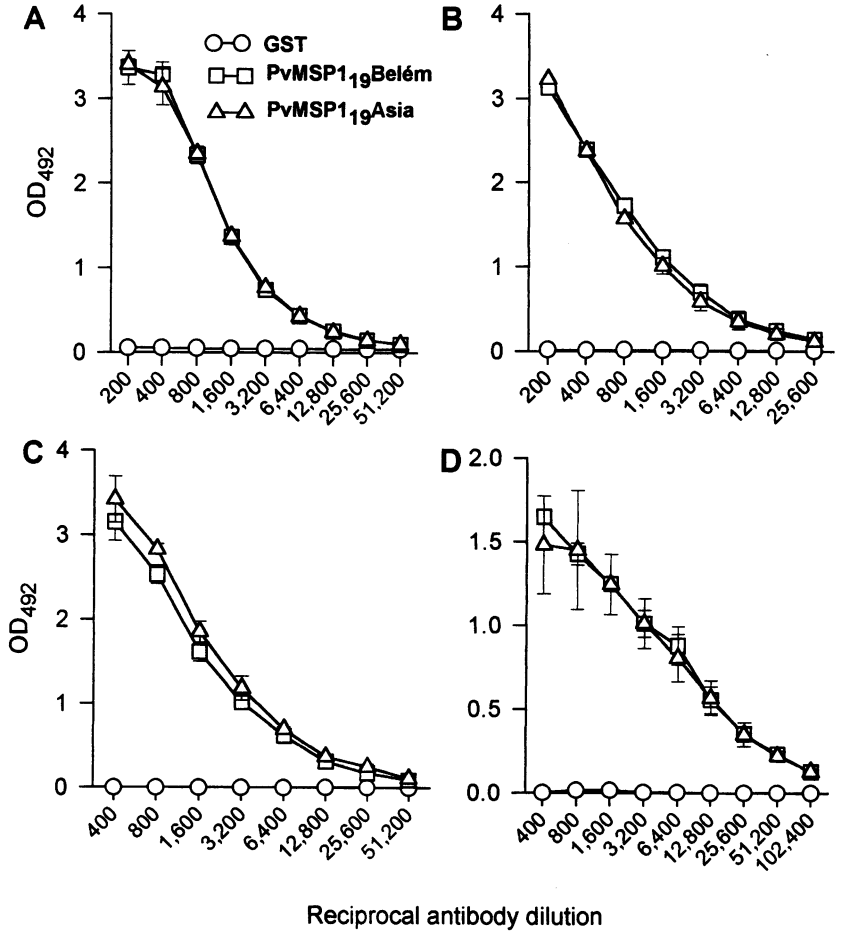

Fig. 2. Recognition of recombinant proteins representing both allelic forms of PvMSP1$_{19}$ by rabbit polyclonal antibodies and a mouse MAb. (A, B) Antibody titration curves using rabbit polyclonal antibodies specific for PvMSP1$_{19}$. ELISA plates were coated with recombinant proteins produced in bacteria (A) or yeast (B). (C, D) Antibody titration curves using mouse MAb specific for PvMSP1$_{19}$. ELISA plates were coated with recombinant proteins produced in bacteria (C) or yeast (D). Results are shown as the average of triplicate samples ± standard deviation (SD).

infected individuals. In most individuals (64%), inhibition of binding was very high (>90%) for soluble recombinant proteins representing each allele of PvMSP1$_{19}$. These individuals are indicated in Figure 5B as group I. In a smaller group of individuals (36%), we observed the presence of allele-specific antibodies. In these cases, the recombinant protein PvMSP1$_{19}$ Asia was unable to inhibit the binding of human antibodies to the same extension as the protein PvMSP1$_{19}$ Belém (group II). Nevertheless, in these individuals the average inhibition obtained by proteins PvMSP1$_{19}$ Belém and PvMSP1$_{19}$ Asia were 94.30 ± 6.0 and 77.09 ± 7.7%, respectively, indicating that most of the antibodies were directed to conserved epitopes present in both allelic forms. To confirm the presence of allele-specific antibodies, we inhibited the binding of sera from individuals of group II to wells coated with PvMSP1$_{19}$ Asia. We observed that both recombinant proteins inhibited the binding at a similar extension. The values of inhibition to GST, PvMSP1$_{19}$ Asia, and PvMSP1$_{19}$ Belém were 2.3 ± 3.8, 91.4 ± 7.8, and 89.5 ± 9.7%, respectively. These findings confirm that PvMSP1$_{19}$ Belém displays allele-specific epitopes.

In similar experiments using the rabbit polyclonal antibodies to PvMSP1$_{19}$, we observed that both proteins inhibited more than 96% of the antibody binding to PvMSP1$_{19}$ Belém or to PvMSP1$_{19}$ Asia (data not shown).

Discussion

Our study on the variability of the DNA sequences encoding MSP1$_{19}$ in Brazilian isolates of *P. vivax* failed to identify allelic forms distinct from the originally described Belém allele. Our data contrasted with the results from studies performed in Southeast Asia where two allelic forms of PvMSP1$_{19}$ were found (25). It is possible that this variant also exists in Brazil. However, the Belém allele predominated in isolates collected in the two major endemic areas of the country. In

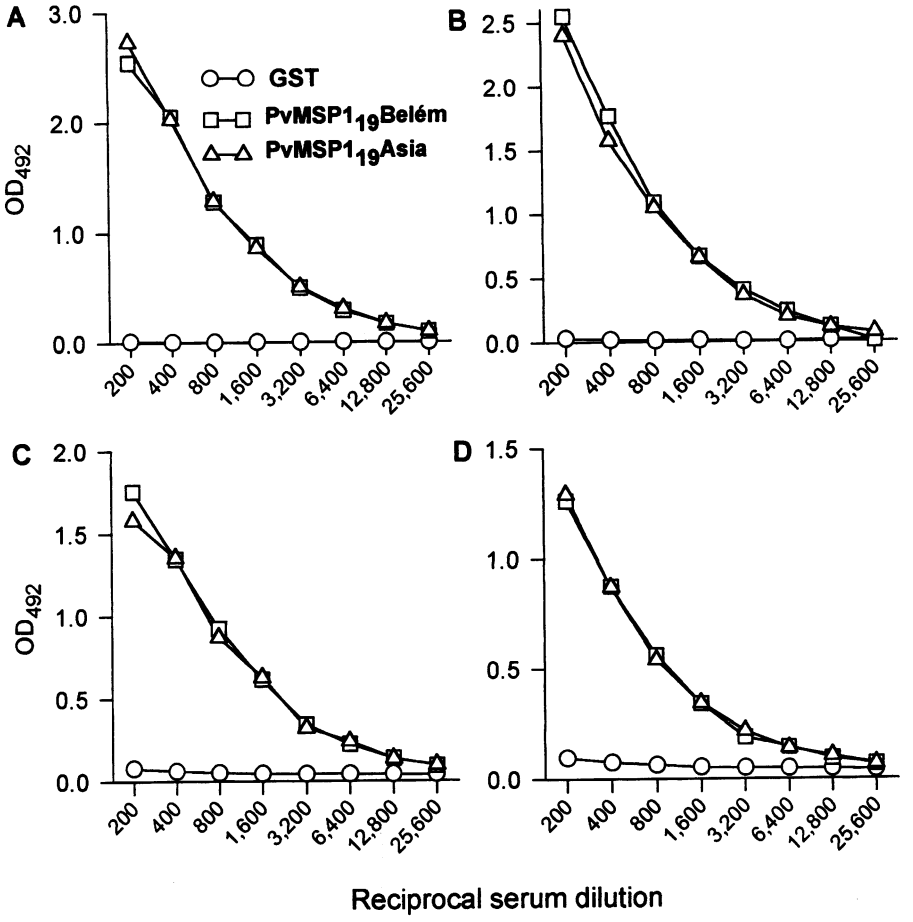

Fig. 3. Recognition of recombinant proteins representing both allelic forms of PvMSP1$_{19}$ by antibodies from two individuals during their first infection with *P. vivax*. Antibody titration curves were calculated for serum samples from two individuals during their first episode of malaria (A, C: individual 40; B, D: individual 51). ELISA plates were coated with recombinant proteins produced in bacteria (A, B) or yeast (C, D). Results are shown as the average of duplicate samples.

Asia, the variant allelic form was very frequent, representing 9 of the 20 isolates sequenced.

The conservation found thus far in the sequences of PvMSP1$_{19}$ is unexpected. It seems to be restricted to the C-terminal region because a much wider sequence variation has been described for the N-terminal region of PvMSP1 (37–39). Also, this conservation is restricted to *P. vivax*, since distinct allelic forms of MSP1$_{19}$ have been found in Brazilian isolates of *P. falciparum* (M.U. Ferreira, unpublished results). The PvMSP1$_{19}$ sequence conservation contrasts sharply with data of MSP1$_{19}$ sequences obtained from other species of malaria parasites such as *P. yoelii* and *P. falciparum*. In both cases, several allelic forms have been found in natural isolates. In view of the variation found in other species, the conservation in PvMSP1$_{19}$ may suggest a strong selective pressure to maintain the structure and imply a function for this part of the molecule.

The reason for the allelic dimorphism described for PvMSP1$_{19}$ in Asia is presently unknown. A reasonable explanation for such variability is that PvMSP1$_{19}$ provides a mechanism for the parasite to evade the antibody response. If this were the case, the polymorphism would raise doubts about the possible use of PvMSP1$_{19}$ as part of subunit vaccines against *P. vivax*. We assessed this question by studying the recognition by antibodies of recombinant proteins representing the two allelic forms of the protein. We found that recombinant proteins representing both allelic forms produced in two distinct expression systems (bacterial and yeast) were equally well recognized by specific antibodies that inhibit merozoite invasion in vitro. Also, a strikingly similar recognition was observed with a panel of 80 human sera from infected individuals who were exposed to *P. vivax*. These results suggest that the single amino acid modification found thus far in nature does not impair the recognition of PvMSP1$_{19}$ by IgG antibodies of most naturally infected individuals.

Because we used recombinant proteins, it is not possible to completely rule out that allele specific epitopes are expressed more in the native forms of PvMSP1$_{19}$. This possibility can be cor-

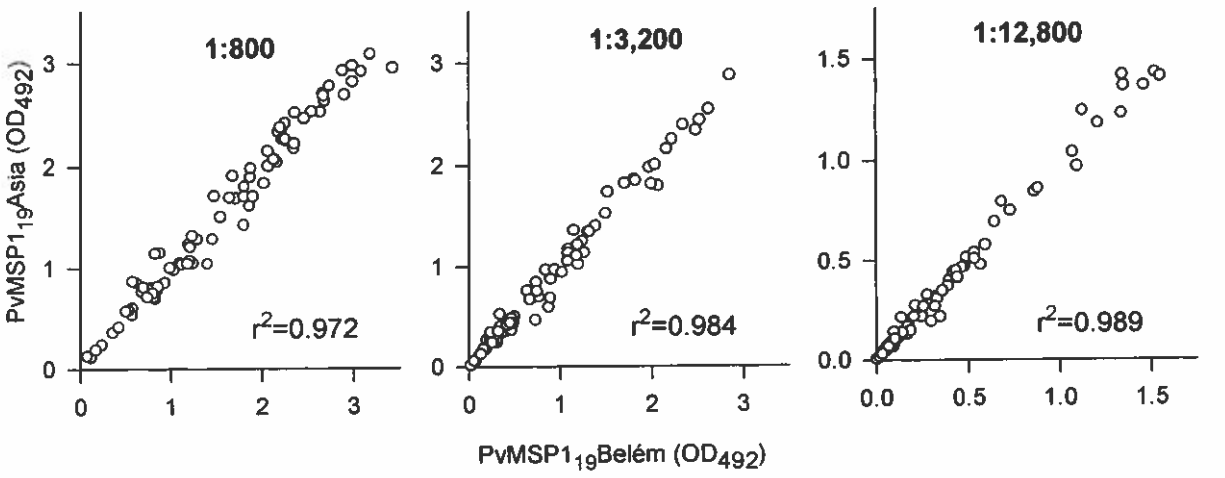

Fig. 4. Comparison of antibody recognition of bacterial recombinant proteins representing both allelic forms of PvMSP1$_{19}$. Each panel represents the reactivity at different dilutions of serum samples obtained from 80 individuals who were infected with *P. vivax*. Results are shown as the average of duplicate samples. The correlation coefficient (r^2) for each graph is shown at the bottom.

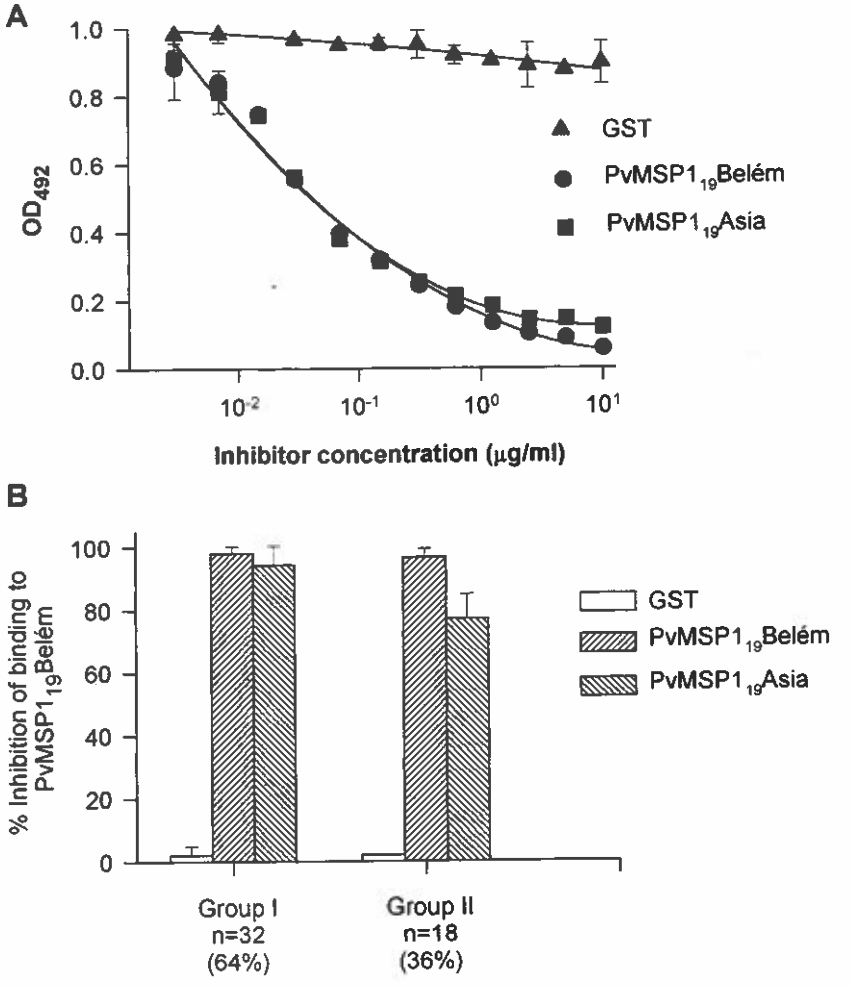

Fig. 5. Inhibition ELISA carried out with recombinant proteins representing each allelic form of PvMSP1$_{19}$. ELISA plates were coated with a bacterial recombinant protein representing PvMSP1$_{19}$ Belém allele. (A) The indicated recombinant fusion protein or GST was added at different concentrations in each well. Human serum from individual 40 was added at a dilution of 1:800. Results are expressed as the average of triplicate samples ± SD. (B) Average inhibition of the binding of human sera to recombinant protein PvMSP1$_{19}$ Belém. The indicated soluble recombinant proteins or GST was added to a final concentration of 10 μg/ml. The percentage of inhibition was calculated as described in Materials and Methods. Individuals were selected for group I when inhibition values with either recombinant protein were >90%. Group II represents individuals who had inhibition of <90% when the recombinant protein PvMSP1$_{19}$ Asia was used. Results are expressed as the average of the indicated number of individuals ± SD.

rectly addressed in the future by active immunization of experimental animals with each one of the allelic forms followed by a challenge with blood stages of *P. vivax* (Belém or Asia strains).

Earlier studies on the recognition of different allelic forms of $MSP1_{19}$ were performed in two other species of *Plasmodium*. The results obtained with the rodent malaria agent *P. yoelii* unequivocally demonstrated that protective antibodies are directed mainly at the polymorphic residues of $MSP1_{19}$. Antibodies from mice immunized with a bacterial recombinant protein representing a certain allele of $MSP1_{19}$ only recognized parasites of a homologous strain. Protective immunity against *P. yoelii* was also strain-specific (16). This work suggested that similar strain-specific protective antibodies could be generated in human malaria.

Studies on the recognition of allelic forms of $MSP1_{19}$ of *P. falciparum*, a human malaria parasite, were performed with yeast-derived recombinant proteins representing four alleles (E-KNG, Q-KNG, E-TSR, and Q-TSR). They demonstrated that there are multiple conserved epitopes among these different allelic forms recognized by human and non-human primate antibodies (23,24,40–42). The recognition by human antibodies of recombinant proteins representing allelic forms with a single amino acid modification (E-KNG and Q-KNG) was quite similar (23,40). These results resemble the findings reported here, and they may suggest that single amino acid substitutions may not be sufficient to impair recognition by antibodies. The recognition of a third allelic form (E-TSR), which has three or four amino acid modifications compared to E-KNG or Q-KNG, respectively, was not as similar, and the presence of strain-specific human antibodies was observed (23,24,40). To our knowledge, no comparisons between other allelic forms found in nature have been reported thus far (19).

Alternatively, the single amino acid substitution found in the two allelic forms of $PvMSP1_{19}$ may represent a strategy to evade cell-mediated immune responses. It is well documented that few amino acid changes can dramatically impair the binding of peptides to MHC class I or class II (43–46). They may also reduce recognition by T cells or generate antagonistic peptides that inhibit activation of specific T cells by the MHC–peptide complex (47–49). It is possible that in Asia there may be individuals with a particular MHC that recognize exclusively one of the two allelic forms. In this case, the dimorphism could be an advantage to the parasite. Although we have described the presence of T cell epitopes in $PvMSP1_{19}$ (26), this possibility will have to be further explored in transmission areas where both allelic forms of $PvMSP1_{19}$ are found.

Finally, the dimorphism may represent an adaptation to host polymorphism, for example, allowing the MSP1 molecule to bind to a polymorphic reticulocyte receptor. This possibility cannot be immediately addressed because an MSP1 receptor has not been described.

In conclusion, the present findings suggest that the use of $PvMSP1_{19}$ as part of a subunit vaccine against *P. vivax* might be greatly facilitated by the limited genetic polymorphism and the predominant recognition of conserved epitopes by antibodies.

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