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Acquired Immune Responses to the N- and C-Terminal Regions of *Plasmodium vivax* Merozoite Surface Protein 1 in Individuals Exposed to Malaria

IRENE S. SOARES,[1,2]* GABRIELA LEVITUS,[3] JOSÉ M. SOUZA,[4] HERNANDO A. DEL PORTILLO,[3] AND MAURICIO M. RODRIGUES[2]

Departamento de Patologia, Centro de Ciências Biológicas, Universidade Federal do Pará, 66075-900,[1] and Instituto Evandro Chagas, 66090-000,[4] Belém, Pará, and Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, 04023-062,[2] and Departamento de Parasitologia, Universidade de São Paulo, 05508-900,[3] São Paulo, São Paulo, Brazil

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In this study, we evaluated the naturally acquired immune response to *Plasmodium vivax* merozoite surface protein 1 (PvMSP1) in individuals with recent clinical episodes of malaria from the state of Pará, Brazil. Ten recombinant proteins representing the first 682 amino acids (aa) of the N-terminal region and one representing the final 111 aa of the C-terminal region were expressed in *Escherichia coli* as glutathione *S*-transferase fusion proteins. Both of these regions have been suggested as candidates for development of a vaccine against *Plasmodium* sp. The total frequencies of individuals with antibodies and cellular immune responses to PvMSP1 were high (83.8 and 75%, respectively). The recombinant proteins representing the N- and C-terminal regions were recognized by 51.4 and 64.1% of sera, respectively. The frequency of responders to the C-terminal region increased according to the number of previous malaria episodes, reaching 83.3% after four episodes. Cellular immune response was measured by in vitro proliferation and gamma interferon production. Peripheral blood mononuclear cells of 75 and 47.2% of individuals proliferated in response to stimulation by the N- and C-terminal regions, respectively. Also, we found that one protein representing the N terminus and a second representing the C terminus of PvMSP1 stimulated 54.5% of individuals to secrete gamma interferon. We concluded that PvMSP1 is immunogenic to a large proportion of individuals exposed to malaria. Our results also suggested that the C-terminal region of PvMSP1 containing the two epidermal growth factor-like domains is particularly immunogenic to antibodies and T cells during natural infection in humans.

Malaria remains the most prevalent and devastating parasitic disease worldwide, with a yearly estimate of 200 million cases due to *Plasmodium falciparum* and 35 million cases due to *P. vivax*. Vaccination is considered an approach that will complement other strategies for prevention and control of the disease. Multiple antigens expressed at distinct stages of the parasite have been described as targets for protective immunity and therefore candidates to be part of a subunit vaccine against malaria (reviewed in reference 32).

Among the proteins of blood stages of *Plasmodium*, merozoite surface protein 1 (MSP1) has been the most intensively studied as a potential target for protective immunity. This protein is synthesized as a precursor with a high molecular mass (180 to 230 kDa) during schizogony, and it is later processed into some of the major merozoite surface proteins (reviewed in reference 24). During the invasion process, proteolytic cleavage releases most of the molecule from the merozoite surface, and only a 19-kDa fragment of the C-terminal region ($MSP1_{19}$) is carried into the invaded erythrocytes (5, 6). 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 (10, 12, 34) and confer passive immunity to naive mice (9). Initial studies of immunization with MSP1 purified from blood stages found that rodents or monkeys could be successfully vaccinated against *P. yoelii* or *P. falciparum* infection, respectively (23, 40). The potential of this molecule for vaccine development motivated studies aimed at the generation of recombinant proteins containing portions of MSP1. Several recombinant proteins based on the MSP1 sequence of different *Plasmodium* species were used to immunize rodents and monkeys. However, it was not until recently that remarkable protective immunity could be elicited by immunization with a few of such recombinant proteins. Immunization of mice with an *Escherichia coli*-derived recombinant protein containing $MSP1_{19}$ resulted in very effective protection upon lethal challenge with *P. yoelii* blood stages (14, 28). The induction of protective immunity requires both epidermal growth factor (EGF)-like motifs present in $MSP1_{19}$ (29), and it is mediated predominantly by antibodies (15). Most relevant is the fact that a certain degree of protective immunity was also achieved against *P. falciparum* infection, by vaccinating *Aotus* monkeys with either a baculovirus-derived recombinant protein containing a 42-kDa fragment of the C-terminal region of *P. falciparum* MSP1 (PfMSP1) (11) or a yeast-derived $MSP1_{19}$ recombinant protein (26).

These studies in experimental models were complemented by the finding that humans living in an area where malaria is endemic also develop immune response to PfMSP1. These studies established unequivocally that there are epitopes recognized by human antibodies or T cells located at both the N- and C-terminal regions of PfMSP1 (reviewed in reference 25). An association between immune response (antibody and T cell mediated) to PfMSP1 and reduced susceptibility to clinical malaria has been found in some studies (2, 19, 37, 39) but not

* Corresponding author. Mailing address: Departamento de Microbiologia, Immunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Botucatu 862, 04023-062, São Paulo, SP, Brazil.

in others (3, 31, 42). These results, most likely, are not contradictory but rather reflect the wide variations found in distinct areas where malaria is endemic regarding the transmission pattern, parasite polymorphism, and genetic background of human populations.

Unfortunately, there is no evidence available as to whether a recombinant or synthetic vaccine against *P. falciparum* will cross-protect against *P. vivax*. Accordingly, several groups are making an effort to characterize, at the molecular level, antigens that are expressed by merozoites of *P. vivax* (1, 4, 16, 21). The number of antigens expressed by blood stages for which the genes have been cloned and sequenced is limited. However, the possibility for designing experiments to study naturally acquired immunity of humans to defined antigens of *P. vivax* and vaccination in the experimental models now exists.

Despite being a potential target for vaccine development, the MSP1 protein of *P. vivax* (PvMSP1) has been the subject of relatively few studies. The N-terminal region of PvMSP1 was expressed in *Escherichia coli* (17, 27, 30) and yeast cells (22), and the products were tested by enzyme-linked immunosorbent assay (ELISA) or Western blot analyses with sera of individuals with patent malaria infections from Brazil. The results demonstrated that similarly to PfMSP1, the N-terminal region of PvMSP1 is immunogenic in natural infections; however, 40% of the individuals with patent infections did not contain detectable levels of immunoglobulin (IgG) antibodies to the recombinant proteins representing the N-terminal region of PvMSP1, even after multiple attacks of malaria. A high proportion of these sera reacted against recombinant proteins expressing interspecies conserved and polymorphic blocks, as previously defined by del Portillo et al. (16). In contrast, the recombinant proteins expressing exclusively conserved blocks were poorly recognized by these antibodies. Naturally recognized T- or B-cell epitopes have not been mapped for PvMSP1, and no studies on the naturally acquired immunity of malaria patients against the 19-kDa C-terminal region of PvMSP1 have been reported to date.

In this study, we evaluated antibody and cellular immune responses to PvMSP1 in individuals recently exposed to malaria from the state of Pará, Brazil, including those elicited by the 19-kDa fragment of the C-terminal portion of this molecule. Ten glutathione *S*-transferase (GST) fusion proteins spanning 682 amino acids (aa) from the N-terminal region and the most C-terminal 111 aa of the PvMSP1 protein from the Belém strain were used. To investigate B-cell responses against these recombinant proteins, serum samples were tested for the presence of IgG antibodies and its isotypes. This study was complemented by estimating the in vitro peripheral blood mononuclear cell (PBMC) proliferative responses as well as gamma interferon (IFN-γ) production upon stimulation with these recombinant proteins.

MATERIALS AND METHODS

Subjects. After verbal consent, blood samples from 94 individuals were collected for this study. One milliliter of venous blood was collected from each individual in nonheparinized tubes and used as a source of serum. Fifteen to 20 ml of venous blood of each of 66 individuals was collected aseptically in heparinized tubes and transported at room temperature (RT), within 20 h, for lymphoblastic proliferation assays. The first group of individuals were healthy adult volunteers selected from among blood donors from the city of São Paulo, state of São Paulo, in southeastern Brazil. Malaria is not present in this part of the country, and these individuals had never had malaria or traveled to areas where malaria is endemic. The total number of volunteers was 30, their average age was 35.3 ± 10.5 years, and they were all male. The second group was composed of 37 adults from the area of Belém, state of Pará, in the north of Brazil, and they had been treated for *P. vivax* malaria in the previous 4 months. The standard treatment consisted of one oral dose of 600 mg of chloroquine and 30 mg of primaquine administered on the day the diagnosis was made. In the subsequent 6 days, these patients received daily doses of 30 mg of primaquine. The mean age of this group was 37.5 ± 11.9 years, and 91.66% of the subjects were male. The third group was composed of 27 subjects (22 adults and 5 children), and their blood samples were used only for serological studies. They came from the area of Belém, and when the blood samples were collected, they had patent *P. vivax* infection as determined by microscopic analysis of Giemsa-stained blood drops. Blood samples were collected before treatment. The mean age of these subjects was 23.6 ± 10.0 years, and 77.8% were male. Most individuals of the last two groups are lifelong residents of the city of Belém. These subjects were exposed to *P. vivax* malaria-infected mosquitoes during short-term stays in regions surrounding the city where transmission occurs. Only cases of *P. vivax* are reported in these areas, and low levels of transmission are observed during the entire year (31a, 40a). By the end of the rainy season (March through May), a higher number of cases is observed. Blood samples from these patients were collected at Instituto Evandro Chagas, where they report for diagnosis and treatment. Also, because these individuals do not live in a transmission zone and always reported to Instituto Evandro Chagas, we could determine precisely the number of previous clinical malaria episodes. Only the individuals for whom we had precise information on the number of *P. vivax* malaria episodes were used to establish a correlation between the number of malaria episodes and the frequency of responders to recombinant proteins ICB2-5 and ICB10 (see Fig. 3).

Recombinant PvMSP1 proteins. The N-terminal region of PvMSP1 from the Belém strain was expressed as 10 different GST fusion proteins; detailed construction has been described elsewhere (27). The recombinant proteins encoded 52 aa (ICB1), 94 aa (ICB2), 45 aa (ICB3), 41 aa (ICB4), 63 aa (ICB5), 200 aa (ICB1-2), 212 aa (ICB2-3), 98 aa (ICB3-4), 506 aa (ICB2-5), and 293 aa (P4-ICB5). The C-terminal region (ICB10) was amplified by PCR, using the original clone from the Belém strain as a substrate for primers, and encodes aa 1615 to 1726 (16). The amplified fragment was cloned by standard methods (38) into the pGEX-3X expression vector. Recombinant protein ICB10 thus encodes 111 aa and contains the two EGF-like motifs described for other MSP1 molecules. As a control, GST was produced alone. Recombinant proteins and GST were affinity purified on glutathione-Sepharose 4B columns (Pharmacia, Uppsala, Sweden), their purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein concentration was measured by the Bradford method (8).

Immunoassays. (i) Detection of antigen-specific IgG antibodies by ELISA. Sera from 94 individuals were tested for reactivity with the PvMSP1 recombinant proteins by ELISA essentially as earlier described (27). Briefly, each well of microtiter plates (Costar, Cambridge, Mass.) was coated with 200 ng of affinity-purified fusion proteins or GST, incubated overnight at 4°C, and then washed three times with phosphate-buffered saline (PBS)–0.05% Tween 20. The plates were blocked at 37°C for 2 h with 5% nonfat milk in PBS. In the first test, serum samples were added to duplicate wells at 1:100 dilution. The sera that were positive for recombinant protein ICB2-5 or ICB10 were titrated with subsequent twofold serial dilution to 1:102,400. After 2 h of incubation at RT, unbound material was washed away, and peroxidase-conjugated goat anti-human IgG (Fc specific; Sigma), diluted 1:40,000, was added to each well. After 1 h of incubation at RT, excess labeled antibody was removed during washing, and the reaction was developed by using the TMB microwell peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, Md.). Plates were read at 450 nm on an ELISA reader (Labsystems Multiskan MS). All OD_{450} (optical density at 450 nm) values represent binding of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. Each serum was tested in duplicate, and the OD_{450} values were averaged. Cutoff points were set at 3 standard deviations (SD) above the mean OD_{450} value of sera from 30 healthy individuals from the city of São Paulo who had never been exposed to malaria.

(ii) ELISA to determine antigen-specific antibodies of distinct IgG subclasses. ELISA was performed as described above except that subclass-specific mouse anti-human IgG monoclonal antibodies were used as the second-step reagents. These monoclonal antibodies recognize human IgG1, IgG2, IgG3, or IgG4 (Sigma), and they were diluted 1:2,000 in PBS–5% nonfat milk. After 1 h of incubation at RT, plates were washed and peroxidase-labeled anti-mouse IgG (heavy and light chains; Kirkegaard & Perry) was added to a final concentration of 1:4,000. The plates were washed and developed as described above. The OD_{450} values represent binding of each subclass of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. Only serum samples that had IgG specific for recombinant protein ICB2-5 or ICB10 were tested in this assay. Each serum was tested in duplicate, and the OD_{450} values were averaged. Cutoff points were set at 3 SD above the mean OD_{450} value of sera from eight healthy individuals from the city of São Paulo who had never been exposed to malaria.

(iii) Detection of antibodies to reduced recombinant proteins ICB10 and ICB2-5. Reduced and carboxymethylated protein was prepared exactly as described in reference 18. Briefly, 0.25 mg of recombinant protein ICB10 or ICB2-5 or GST was incubated at 37°C for 1 h in 0.5 M Tris-HCl (pH 8.0) containing 2 mM EDTA and 60 mM dithiothreitol (DTT; Sigma). Iodoacetamide (Sigma) was added in a 2.5-fold molar excess over DTT. The samples were kept in the dark for 30 min, and the proteins were dialyzed overnight at 4°C against PBS. After dialysis, the integrity of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein concentration was estimated by the Bradford method (8). Nonreduced and reduced proteins were

used in the same concentration to coat ELISA plates. ELISA was performed as described for the detection of antigen-specific IgG.

PBMC proliferation assay and IFN-γ production. Blood samples were collected in heparinized tubes and transported to the laboratory within 20 h. After being diluted with the same volume of PBS, PBMC were isolated on Ficoll-Paque (Pharmacia) by centrifugation. The PBMC at the interface were collected, washed three times in Hanks balanced salt solution (Life Technologies GIBCO-BRL), and resuspended in complete media. Viable PBMC counts were made under a phase-contrast microscope by the trypan blue dye exclusion test. The complete medium was RPMI 1640 medium (Life Technologies) supplemented with 10% normal human AB serum (CLB, Amsterdam, The Netherlands), 2 mM L-glutamine, 10 mM HEPES, 0.22% sodium bicarbonate, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 1 mM nonessential amino acid solution, and 100 U of penicillin and streptomycin per ml. A total of 1.5×10^5 cells in 200 μl of culture medium were added to each well of a flat-bottom 96-well plate (Corning Co., Corning, N.Y.). Each recombinant protein of PvMSP1 or GST was added in 20 μl. The final concentration of recombinant protein or GST was 2 or 10 μg/ml. The assay was performed in triplicate cultures, one of which contained only culture medium. Concanavalin A (Sigma) was used as a positive control in all experiments. Cultures were incubated in a humid environment at 37°C in a 5% CO_2 atmosphere for 6 days, and then 25 μl of supernatant was collected and frozen for IFN-γ determination. Subsequently, 25 μl of complete medium containing 1 μCi of [^{3}H]thymidine (Amersham, Little Chalfont, England) was added to the cultures. After 20 to 24 h, cultures were harvested with a semiautomatic cell harvester, and radioactivity was determined in a liquid scintillation counter. The geometric mean counts per minute for each set of triplicate wells was calculated, and the stimulation index (SI) was determined as the geometric mean counts per minute of recombinant protein-stimulated culture divided by the geometric mean counts per minute of the GST-stimulated culture. The background counts for GST varied from 264 to 2,126 cpm. PBMC of three healthy individuals had more than 2,800 cpm when stimulated with GST. IFN-γ concentrations were estimated in triplicate samples with the aid of a commercially available ELISA kit (Genzyme, Cambridge, Mass.) with a threshold of 3 pg/ml. The assay was performed as recommended by the manufacturer.

Statistical analysis. Kruskal-Wallis one-way analysis of variance and rank correlation test were performed by using the True Epistat software package.

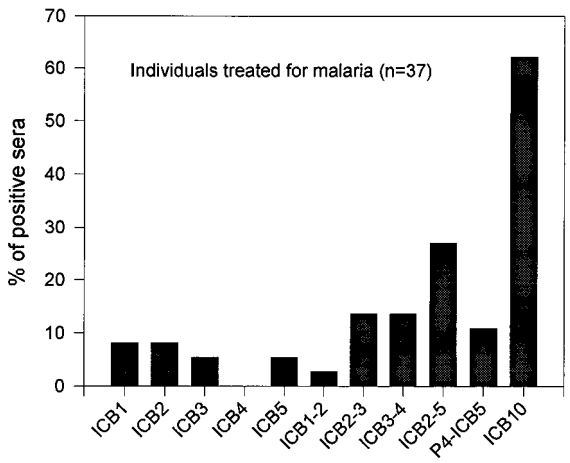

FIG. 1. Percentages of serum samples from individuals treated for *P. vivax* malaria who recognize PvMSP1 recombinant proteins. The percentage of responders was calculated as those serum samples that presented OD_{450} values 3 SD above the average OD_{450} obtained from serum samples of 30 healthy individuals never exposed to malaria. Serum samples were tested at a dilution of 1:100. All OD_{450} values represent binding of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. The cutoff values for the recombinant proteins were as follows: ICB1, 0.345; ICB2, 0.239; ICB3, 0.354; ICB4, 0.123; ICB5, 0.234; ICB1-2, 0.734; ICB2-3, 0.240; ICB3-4, 0.100; ICB2-5, 0.331; P4-ICB5, 0.365; and ICB10, 0.396.

RESULTS

Recognition of recombinant proteins expressing the N- and C-terminal regions of PvMSP1 by antibodies of individuals recently exposed to *P. vivax*. Serum IgG antibodies from individuals of the area of Belém, where only cases of *P. vivax* malaria are reported, were tested for recognition of PvMSP1 recombinant proteins. Initially, 37 serum samples collected from individuals who had been treated for *P. vivax* malaria in the previous four months were tested by ELISA, using as antigens 11 GST fusion proteins representing different portions of PvMSP1. Ten of these 11 recombinant proteins cover different portions of the first 682 aa from the N-terminal region of PvMSP1. A single recombinant protein, ICB10, represented the final 111 aa of the C-terminal region of PvMSP1 and included the two EGF-like motifs described for other MSP1 proteins.

The results show that 83.8% recognized at least one of these recombinant proteins, indicating that PvMSP1 is highly immunogenic for humans. The percentage of sera recognizing each recombinant protein is shown in Fig. 1. As has been described previously (27), recombinant proteins ICB2-3, ICB3-4, ICB2-5, and P4-ICB5 were preferentially recognized by antibodies to the N-terminal region. A total of 51.4% of subjects recognized at least one of the recombinant proteins representing the N-terminal region of PvMSP1. Most relevant was the fact that a recombinant protein representing the conserved C-terminal region of PvMSP1 (ICB10) was recognized by 62.2% of individuals. Considering that this recombinant protein represents only 111 aa of PvMSP1, these results suggest that this portion of the molecule can be highly immunogenic during natural human infections.

Because recombinant protein ICB10 was the polypeptide most frequently recognized by antibodies of malaria-exposed individuals, it was selected for subsequent serological studies. We also used recombinant protein ICB2-5 to compare the reactivities of serum samples to the N- and C-terminal regions of PvMSP1. First, we determined whether these two proteins were equally recognized by serum antibodies from subjects during an ongoing *P. vivax* infection compared to the group of individuals treated for malaria. For that purpose, the sera of 27 individuals with patent *P. vivax* infections were tested. The results revealed that similar proportions of individuals recognized recombinant protein ICB10 during infection and in the period of 4 months after treatment (Fig. 2A). Similar results were obtained with recombinant protein ICB2-5. Titration curves were done with each serum sample to determine precisely the antibody titers to both recombinant proteins. The antibody titers in both cases ranged from 1:100 to 1:102,400 (Fig. 2B). However, antibody titers to recombinant protein ICB10 were significantly higher than titers to recombinant protein ICB2-5 ($P = 0.005$, Kruskal-Wallis one-way analysis of variance). Noteworthy was the fact that during infection, the antibody titers to recombinant protein ICB10 were significantly higher than the titers found in individuals after treatment ($P = 0.0019$, Kruskal-Wallis one-way analysis of variance). This result suggests that the infection provides a boost to the production of antibodies specific for the C-terminal portion of PvMSP1 that is not observed in the case of the N-terminal recombinant protein ICB2-5 ($P = 0.825$).

As detailed in Materials and Methods, most individuals studied lived in the city of Belém. These subjects were exposed to *P. vivax*-infected mosquitoes during short stays in the areas surrounding the city. Because these individuals always reported to the Instituto Evandro Chagas for diagnosis and treatment, it was possible to estimate, at the time their blood samples were taken, the precise number of previous clinical malaria episodes of 57 individuals. Based on that information, we estimated the frequency of responders to the recombinant protein ICB10 or ICB2-5 after each contact with the parasite. As depicted in Fig. 3, the frequency of individuals with antibody to ICB2-5 or ICB10 during or after the first malaria episode is not low (38.5 or 46.2%, respectively). A positive

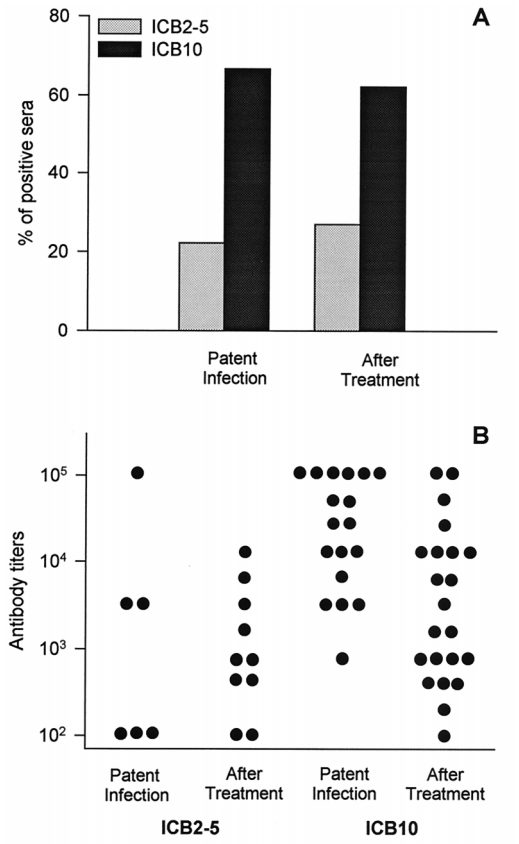

FIG. 2. Comparison of the frequencies and antibody titers of serum samples that recognize recombinant proteins ICB10 and ICB2-5 among individuals with patent *P. vivax* infection and after treatment. (A) The percentage of responders was estimated exactly as described for Fig. 1. The numbers of individuals with patent infection and after treatment were 27 and 37, respectively. The cutoff values were the same as for Fig. 1. (B) The antibody titer of each individual represents the last dilution that provided an OD_{450} value of 0.1/ml after subtraction of binding of the same serum antibodies to GST alone. All groups were statistically compared as described in Materials and Methods and in Results. The results of the comparison are as follows: (i) antibody titers to recombinant ICB10 were higher than the titers to recombinant protein ICB2-5 ($P = 0.005$); (ii) in the group of individuals with patent infection, antibody titers to recombinant ICB10 were higher than in all other groups (ICB10 [after treatment], $P = 0.0019$; ICB2-5 [patent infection], $P = 0.021$; ICB2-5 [after treatment], $P = 0.001$); and (iii) no difference was observed when we compared the antibody titers to ICB2-5 of individuals with patent infection and after treatment ($P = 0.825$).

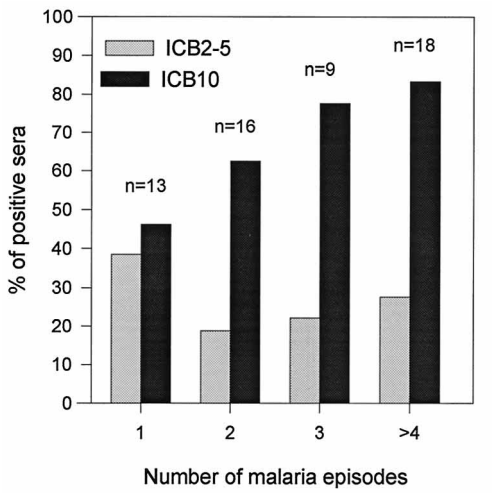

FIG. 3. Relationship between the percentage of serum samples that recognize recombinant protein ICB2-5 or ICB10 and the number of malaria episodes. The percentage of responders was estimated exactly as described for Fig. 1. Malaria-exposed individuals were grouped according to the number of times they had *P. vivax* patent infection. The number of individuals belonging to each group is shown above the columns. The cutoff values were the same as used for Fig. 1. A positive correlation was observed between the increase in the frequency of responders to ICB10 and the number of malaria episodes ($r = 0.985$, $P = 0.019$, correlation rank test). In contrast, the frequency of individuals that recognized recombinant protein ICB2-5 did not correlate with the increase in the number of malaria episodes ($r = 0.423$, $P = 0.577$).

correlation ($r = 0.985$, $P = 0.019$, correlation rank test) was observed between the increase in the frequency of responders to ICB10 and the number of malaria episodes, reaching 83.3% of individuals after the fourth contact with the parasite. In contrast, the frequency of individuals that recognized recombinant protein ICB2-5 decreased after the first malaria episode, and it did not correlate with the increase in the number of malaria episodes ($r = -0.423$, $P = 0.577$). We decide to group the individuals with four or more episodes of malaria to avoid samples that were too small. This group of individual varied from 4 to 12 episodes. No correlation was found between the frequency of responders to both recombinant proteins and their age (ICB10, $r = -0.811$ and $P = 0.051$; ICB2-5, $r = 0.656$ and $P = 0.157$).

The 19-kDa C-terminal region of plasmodial MSP1 is believed to contain as many as three disulfide bonds in each of the two EGF-like regions (6). To determine whether the recognition of recombinant protein ICB10 by human antibodies was dependent on the presence of intact disulfide bonds, an ELISA comparing the reactivities of reduced and nonreduced forms of the recombinant protein was performed. We also used, in the same assay, the reduced and nonreduced forms of recombinant protein ICB2-5 as a control, since this polypeptide is not suspected to contain disulfide bonds. The recognition of recombinant protein ICB10 by human polyclonal antibodies is partially dependent on the presence of intact disulfide bonds, since the reactivity of antibodies was decreased by an average of 42.6% ± 13.1% ($n = 41$) after reduction with DTT and carboxymethylation of this recombinant protein. The decrease of the reactivity varied from as little as 18.9% to as much as 73.8%, depending on the serum sample. In contrast, the reactivity to the protein ICB2-5 decreased by only 4.9% ± 9.7% ($n = 9$) after reduction with DTT. These results demonstrated that while there are antibodies to protein ICB10 specific for the nonreduced form, most individuals also had a certain degree of reactivity to the reduced form.

Because the subclass of IgG produced in response to a given antigen may determine the function of the antibody, the IgG subclasses were estimated by subclass-specific ELISA using as antigens recombinant proteins ICB10 and ICB2-5. In most sera positive for ICB10, the IgG1 and IgG3 subclasses were predominant (Table 1). Smaller proportions of individuals presented IgG2 or IgG4 antibodies specific for this polypeptide. Interestingly, twice as many individuals had detectable IgG1 and IgG3 antibodies during patent infection as after treatment, which was not the case with the other two subclasses. In contrast to the pattern observed with recombinant protein ICB10, the IgG subclasses that predominate in response to ICB2-5 were IgG2 and IgG3. The frequency of individuals with IgG2 or IgG3 antibodies was higher among individuals with patent infection. The predominant IgG subclasses to both recombinant proteins did not vary among persons with different numbers of malaria episodes.

TABLE 1. Frequencies of malaria-exposed individuals with antibodies of distinct IgG subclasses specific for recombinant protein ICB2-5 or ICB10[a]

IgG subclass	% Responders			
	Recombinant protein ICB2-5		Recombinant protein ICB10	
	Patent infection (n = 6)	After treatment (n = 10)	Patent infection (n = 18)	After treatment (n = 23)
IgG1	50.0	0	100.0	47.8
IgG2	83.3	20.0	27.8	39.1
IgG3	83.3	60.0	77.8	34.8
IgG4	33.3	0	16.7	13.0

[a] ELISA was performed with subclass-specific, mouse anti-human IgG1, IgG2, IgG3, or IgG4 monoclonal antibodies. Only serum samples that had total IgG specific for recombinant protein ICB2-5 or ICB10 were tested in this assay. The percentage of responders was calculated as those serum samples that presented OD_{450} values 3 SD above the average OD_{450} obtained from serum samples of eight healthy individuals never exposed to malaria. Serum samples were tested at a dilution of 1:100. The OD_{450} values represent binding of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. The cutoff values for the recombinant proteins were 0.164, 1.187, 0.109, and 0.100 for ICB2-5 IgG1, IgG2, IgG3, and IgG4, respectively, and 0.278, 0.430, 0.204, and 0.100 for ICB10 IgG1, IgG2, IgG3, and IgG4, respectively.

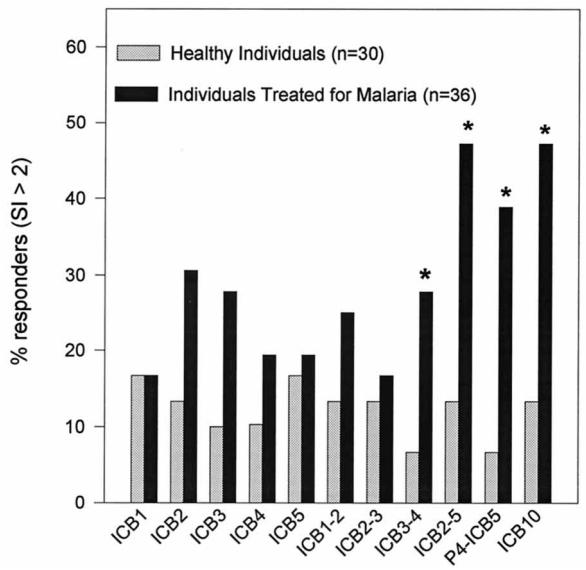

FIG. 4. PBMC proliferative responses to PvMSP1 recombinant proteins from individuals exposed or not exposed to *P. vivax* malaria. The PBMC proliferation assay was performed as described in Materials and Methods. Each recombinant protein of PvMSP1 or GST was tested in final concentrations of 2 and 10 μg/ml. Individuals were considered positive responders when they showed SIs of ≥2 for at least one of the two concentrations of recombinant protein compared to PBMC cultures containing the GST protein alone. Asterisks indicate a statistically significantly higher proportion of responders among individuals treated for malaria in relation to the frequency found in healthy individuals never exposed to malaria ($P < 0.05$, comparison of proportions). PBMC were also stimulated with concanavalin A as a positive control. The SIs obtained after stimulation with this mitogen varied from 11.7 to 129.7.

PBMC proliferative response and IFN-γ production after in vitro culture with different recombinant proteins of PvMSP1. PBMC were collected from 36 of the 37 individuals recently treated for malaria infection. These cells were tested for proliferation upon culture in the presence of each of the 11 recombinant proteins of PvMSP1 or GST. Recombinant proteins or GST were tested, in triplicate, at the two final concentrations, 2 and 10 μg/ml. Individuals who showed SIs of ≥2 for at least one of the two concentrations of recombinant protein, compared to PBMC cultures containing the GST protein, were considered positive responders. The frequency of individuals who responded to each recombinant protein was estimated. Because there are several reports that healthy individuals never exposed to malarial antigens can present PBMC proliferative responses to malarial recombinant antigens or synthetic peptides (20, 45), we also estimated, as a control, the frequency of responders to each recombinant protein among 30 healthy subjects resident in the city of São Paulo. These individuals had never had malaria or traveled to areas where malaria is endemic.

The results from these studies are summarized in Fig. 4. The frequency of responders among individuals never exposed to malaria ranged from 6.7% (proteins ICB3-4 and P4-ICB5) to 16.7% (protein ICB1). The frequency of responders among individuals exposed to malaria ranged from 16.7% (protein ICB1 or ICB2-3) to 47.2% (protein ICB2-5 or ICB10). The proportions of responders among the two groups of individuals were compared statistically. The frequency of responders to ICB3-4, ICB2-5, P4-ICB5, and ICB10 was significantly higher among individuals exposed to malaria than among nonexposed subjects ($P < 0.05$, comparison of proportions). The frequency of responders to the other recombinant proteins was not statistically significantly different in the two groups. The SI varied in different individuals for each of the recombinant proteins. Although most of the positive responders had SIs between 2 and 5, some donors had SIs higher than 10. PBMC obtained from 25% of the individuals treated for malaria failed to proliferate in response to any recombinant protein of PvMSP1. Recombinant proteins ICB2-5 and ICB10 were the antigens most frequently recognized by PBMC of individuals exposed to malaria, showing, in both cases, a positive response in 47.2% of subjects.

Because recombinant proteins ICB2-5 and ICB10 were most frequently recognized by malaria-exposed individuals, we estimated the concentration of IFN-γ in the culture supernatants of PBMC stimulated with each of these recombinant proteins or with GST. The supernatants were collected at day 6 after stimulation with 2 or 10 μg of the recombinant antigens or GST per ml. Due to logistical problems, culture supernatants were collected from only 22 of the 36 individuals whose PBMC were used for the proliferation assay. A significant response was considered only when we observed concentrations of IFN-γ higher than 3 SD above the concentration detected in the supernatant of PBMC cultures of 10 healthy individuals never exposed to malaria stimulated with the protein at the same concentration. Using this parameter, we found that both recombinant proteins (ICB2-5 or ICB10) stimulated 54.5% of the individuals' PBMC to secrete IFN-γ (Fig. 5). The IFN-γ production did not correlate clearly with the proliferative response to either recombinant protein.

Because earlier studies have shown that IFN-γ production by PBMC stimulated by malarial antigens may occur in the absence of specific antibodies or PBMC in vitro proliferative responses for that particular antigen (43), we estimated the frequency of individuals that responded to recombinant ICB10 by either antibody or IFN-γ production. Of the 22 individuals, 45.5% had specific antibodies and 54.5% produced IFN-γ upon stimulation with recombinant protein ICB10. The total of individuals that had at least one type of response reached 77.3%. We also compared the antibody titers to ICB10 with the levels of IFN-γ secretion upon stimulation with this antigen. We observed that 7 of 22 individuals produced IFN-γ without

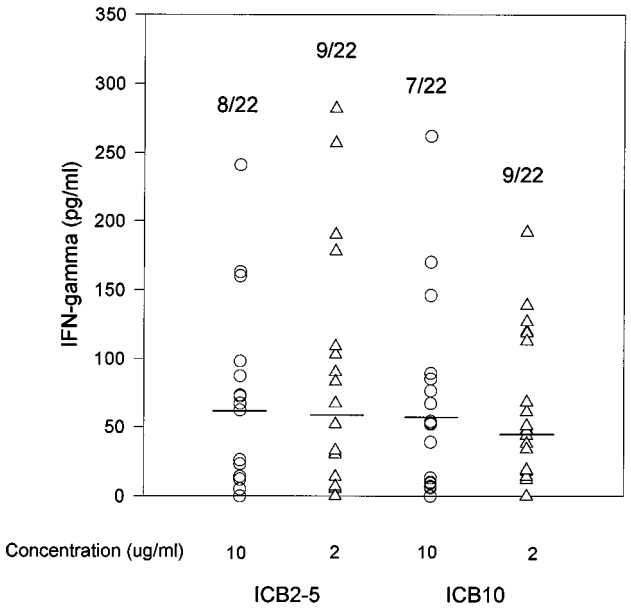

FIG. 5. IFN-γ production by PBMC of individuals treated for malaria upon stimulation with recombinant proteins ICB2-5 and ICB10. Symbols represent the concentrations of IFN-γ in the supernatants of PBMC cultures stimulated for 6 days with each antigen at the indicated final concentration. The values were subtracted from cultures stimulated with GST alone. Individuals were considered positive responders when PBMC supernatant stimulated with the recombinant proteins had IFN-γ production more than 3 SD above the average of 10 healthy individuals stimulated with the same antigen concentration.

having detectable serum antibodies, and 5 of 22 subjects had antibodies to ICB10 without having detectable IFN-γ production. A third group, composed of five individuals, produced both antibodies and IFN-γ in response to ICB10 (data not shown).

DISCUSSION

In this study, we characterized and compared the serum antibody and PBMC reactivities of individuals recently exposed to *P. vivax* malaria with recombinant proteins representing the N- and C-terminal portions of PvMSP1.

Initially, we determined the frequency of individual serum samples that contained IgG antibodies to each of the 11 recombinant proteins. A total of 83.8% of the individuals displayed antibodies to at least one of the recombinant proteins. Among the polypeptides representing the N-terminal region of PvMSP1, the GST fusion proteins containing interspecies conserved and polymorphic blocks, as defined by del Portillo et al. (16), were most frequently recognized by human antibodies. These findings confirm and extend earlier studies performed in the area of the state of Rondonia, Brazil, where malaria is endemic by using 10 recombinant proteins representing the first 682 aa from the N-terminal region (27). Nevertheless, it should be mentioned that recombinant proteins ICB2-5 and P4-ICB5 contain long stretches of amino acids that are conserved among the *P. vivax* strains Belém and Salvador (16, 22). The amino acid conservation in this region may be a factor that explains the higher frequency of responders to these two polypeptides.

Most relevant was the fact that a higher proportion of serum samples reacted with recombinant protein ICB10, which contains only the most C-terminal 111 aa of PvMSP1. When we combined all individuals who had IgG antibodies to at least one of the recombinant proteins of the N-terminal region, we found a total 51.4%, a frequency that is lower than the 64.1% found for the recombinant protein ICB10. Also, it is noteworthy that the frequency of individuals with IgG antibodies to recombinant protein ICB10 increased with the number of *P. vivax* malaria episodes, reaching 83.3% after four episodes. In contrast, the frequency of responders to recombinant ICB2-5 did not follow such a pattern. The antibody titers to recombinant protein ICB10 were also significantly higher than titers to recombinant protein ICB2-5.

The reasons why the C-terminal region of PvMSP1 is recognized by IgG antibodies from a larger percentage of individuals who have higher antibody titers are unknown. These results contrast with our previous studies performed in a distinct area in the state of Rondonia, Brazil, where malaria is endemic. At that time, we found that more than 60% of the individuals had serum IgG antibodies to at least one of the recombinant proteins and that the frequency of serum samples with IgG antibodies to recombinant proteins ICB2-3, ICB3-4, and P4-ICB5 was higher than 35%. Most relevant was the finding that recombinant protein ICB2-5 was recognized by more than 60% of the serum samples (27). In a recent analysis, we found that 67% of these serum samples had IgG antibodies to recombinant protein ICB10, a frequency similar to the one found in this study (data not shown).

Several reasons, not mutually exclusive, may account for the fact that a larger proportion of sera reacted with recombinant protein ICB10. The C-terminal region of PvMSP1 is known to be far less polymorphic than the N-terminal region. Deduced amino acid sequences of this portion of MSP1 from 22 isolates showed only a single amino acid change (33). Two of these 22 strains (Belém and Salvador) derived from South America, and the others were from Southeast Asia. On other hand, extensive polymorphism in the N-terminal portion of PvMSP1 has been described (13, 16, 22, 35, 36). The extension of the polymorphism of the N-terminal and C-terminal regions of PvMSP1 in the area of Belém is unknown, and it will be important to carry out molecular epidemiological studies in this area.

Second, as these antigens are expressed as GST fusion proteins in *E. coli*, it is possible that the folding of recombinant protein ICB10 is more like that of native MSP1 than that of the other fusion proteins. The MSP1 C-terminal regions of different species of *Plasmodium* are composed of amino acid sequences that resemble two EGF motifs. Each EGF-like motif may contain as many as three disulfide bonds that have been shown to be important for the recognition of antibodies from humans exposed to *P. falciparum*. Reduction of the disulfide bond of $PfMSP1_{19}$ with DTT significantly impaired the recognition by human antibodies (18). In agreement with these observations, we found that similar treatment of recombinant protein ICB10 diminished by an average of 42.6% the binding of IgG antibodies of *P. vivax*-exposed individuals, indicating that this GST fusion protein retains a certain degree of secondary and/or tertiary protein structure. Also, this result indicates that some of the naturally acquired IgG antibodies recognize secondary and/or tertiary structures and some recognize epitopes that are independent of the disulfide bonds. In contrast, very little information is available regarding the possible folding of the N-terminal region of native plasmodial MSP1, which could explain why in our study these recombinant proteins are less recognized by human antibodies.

A possible explanation for the discrepancies observed in two distinct endemic areas of Brazil in the recognition of recombinant proteins representing the N-terminal region of PvMSP1 is that in the state of Rondonia, there is also transmission of *P.*

falciparum. Although sera collected from African individuals with patent *P. falciparum* infection do not recognize these recombinant proteins (27), a combination of *P. vivax* and *P. falciparum* infections may have an unpredictable outcome in the human antibody immune response to PvMSP1. It is also possible that the genetic compositions of individuals in these two areas are different, which may account for the differences in the frequency of responders. In fact, in longitudinal serological studies performed earlier with recombinant protein ICB2-5, we found that some individuals did not switch to IgG antibodies even after multiples attacks of malaria. These individuals produced specific, albeit low, levels of IgM during the entire study (30).

Although we found that PvMSP1$_{19}$ is immunogenic during natural infections, 35.9% of the individuals did not present IgG antibodies to recombinant protein ICB10. A possible explanation for the absence of these antibodies could be that certain individuals had only a brief exposure to parasite antigens, insufficient to stimulate a measurable immune response. However, this explanation does not seem appropriate for 65.2% of these individuals. A total of 47.8% of these subjects had detectable antibodies to at least one of the recombinant proteins representing the N-terminal region of PvMSP1, and 17.4% of them displayed antibodies to recombinant proteins representing two other *P. vivax* blood stage antigens (data not shown). The frequency of responders to recombinant protein ICB10 after a single malaria episode is not low (43.3%), and it increased with the number of infections, reaching 83.3% after four episodes. This result indicates that most of the individuals who do not present antibodies had have only one or two contacts with the parasite. Subsequent infections significantly reduced the frequency of nonresponders, indicating that most individuals may become responders. It is also possible that in some cases, the antibody response is too low in the first or second malaria episode and is boosted with subsequent infections. In agreement with this hypothesis is the fact that during patent infection, the antibody titers are significantly higher, suggesting that the contact with the parasite provides a boost to the antibody response. However, it is worth mentioning that three individuals did not present antibodies to recombinant protein ICB10 even after four exposures to *P. vivax* asexual stages (Fig. 3). All three of these individuals had antibodies to recombinant proteins of the PvMSP1 N-terminal region. Also, it is significant that two of these individuals had antibody titers higher than 1:10,000. The lack of antibodies to recombinant protein ICB10 can be explained by the absence of certain conformational epitopes in the fusion protein or different genetic backgrounds of the nonresponder individuals. In addition, it is also possible that some individuals present a different type of immune response to the C-terminal region of the molecule. In fact, we observed that PBMC of several individuals who did not have antibody to recombinant protein ICB10 secreted IFN-γ when stimulated with this protein, resulting in a significant increase in the frequency of individuals who responded to recombinant protein ICB10.

In the second part of the study, we examined whether the recombinant proteins of PvMSP1 elicited in vitro proliferative responses of PBMC obtained from human subjects recently treated for *P. vivax* infection and normal healthy individuals. We found that recombinant proteins ICB3-4, ICB2-5, P4-ICB5, and ICB10 were recognized by a significantly higher frequency of individuals recently exposed to malaria compared with normal healthy individuals. The highest frequency of individuals recognized recombinant proteins ICB2-5 and ICB10. The fact that recombinant protein ICB2-5 was recognized by more individuals than the other recombinant proteins was not unexpected, because this protein is larger (506 aa). In contrast, recombinant protein ICB10 has less than 25% of the size of recombinant protein ICB2-5, and yet it was recognized by a similar frequency of individuals, suggesting that this region may be particularly immunogenic for humans PBMC. The frequency of responders found in our study (47.2%) is not very different from the frequency found in a similar study performed in Africa. In this study, the authors found that 54% of individuals had a positive proliferative response to a recombinant protein of PfMSP1$_{19}$ (39). These studies confirm and extend earlier studies describing the presence of T-cell epitopes in this region as demonstrated by in vitro proliferative responses of PBMC to synthetic peptides (44).

We then determined whether recombinant proteins ICB2-5 and ICB10 could elicit IFN-γ production in vitro by PBMC of 22 individuals exposed to malaria and 10 healthy subjects. We found that several malaria-exposed individuals produced IFN-γ upon stimulation with either one of those polypeptides. Earlier studies have shown IFN-γ production of PBMC upon stimulation with recombinant proteins representing the N-terminal region and the 42-kDa portion of the C-terminal region of PfMSP1 (37). However, our report is the first to demonstrate production of this cytokine by PBMC after stimulation with MSP1$_{19}$. These findings can be relevant for protective immunity, since this cytokine has been implicated in the control of *P. chabaudi* parasitemia by TH1 CD4 T cells in mice (41).

The crucial question related to the potential use of PvMSP1 epitopes as part of a subunit vaccine is whether specific antibodies or T cells participate in the protective immunity to *P. vivax* blood stages. This question is difficult to answer because the mechanisms that mediate protective immunity against *P. vivax* blood stages are unknown. However, it is well established that IgG1 and IgG3 subclasses mediate opsonization and complement fixation of microorganisms, in general, and have been implicated in antibody-mediated protective immunity against *P. falciparum* blood stages (7). Therefore, the fact that recombinant protein ICB2-5 is recognized by IgG3 and anti-ICB10 antibodies are predominately IgG1 and IgG3 may argue in favor of participation of these antibodies in the elimination of blood stages. Also, several studies using recombinant proteins of MSP1$_{19}$ of *P. yoelii* or *P. falciparum* were capable of inducing protective immunity, suggesting that immune responses to MSP1$_{19}$ of other species can be protective (14, 15, 26, 28, 29). In fact, immunization of saimiri monkeys with a baculovirus-derived recombinant protein of PvMSP1$_{19}$ elicited strong protective immunity to *P. vivax* blood stages, significantly reducing the parasitemia in this experimental model (3a).

To properly address the question of whether the anti-PvMSP1 immune response is protective in humans, longitudinal immunoepidemiological studies to determine the association of immunity to PvMSP1 or its portions and resistance to *P. vivax* infection will have to be performed. These studies cannot be carried out in the area of Belém, where the rate of malaria transmission is very low and the individuals have contact with the parasite only during sporadic stays in the areas surrounding the city. Future studies should be carried out in areas with a much higher transmission rate.

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