Antibody response to the N and C-terminal regions of the *Plasmodium vivax* Merozoite Surface Protein 1 in individuals living in an area of exclusive transmission of *P. vivax* malaria in the north of Brazil

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Abstract

Recently, we found that a recombinant protein based on the 19 kDa C-terminal region of the *Plasmodium vivax* Merozoite Surface Protein 1 (PvMSP1$_{19}$) was recognized by a large proportion of individuals naturally infected. The present study was designed to determine the prevalence of antibody to PvMSP1$_{19}$ in individuals from the village of Cotijuba, northern Brazil, where only *P. vivax* transmission occurs. Immuno-epidemiological studies on the prevalence of antibody to the C-terminus of PvMSP1 are of particular importance as this region of MSP1 is being intensively studied as a prime candidate for development of a vaccine against malaria. We evaluated the antibody response to PvMSP1$_{19}$, and compared it to the N-terminal region of PvMSP1 and to blood stage antigens. The total frequencies of individuals with IgG to blood stages, PvMSP1$_{19}$ or the N-terminal region of PvMSP1 were 76.6, 42.3 and 29.8%, respectively. The frequency of responders to PvMSP1$_{19}$ did not

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increase with age. However, the frequency of responders to this recombinant protein was significantly higher (77.4%) in individuals with a recent (< 6 months) history of malaria, when compared to subjects whose last malaria attack occurred more than 6 months before (43.9%), or to individuals without a past history of symptomatic malaria (6.25%). These results confirm earlier studies by demonstrating that the PvMSP1$_{19}$ is highly immunogenic in individuals recently exposed to *P. vivax* malaria. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: P. vivax; Malaria; Merozoite

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1. Introduction

In the last 30 years, malaria became one of the major causes of human morbidity in the north of Brazil as the number of cases increased from less than 40 000 to more than 500 000 cases a year (National Health Foundation, NHF–Brazilian Ministry of Health). *Plasmodium vivax* and *Plasmodium falciparum* are the main species that affect humans, and *P. vivax* is responsible for 76.8% of the 405 051 cases reported in 1997. In most of endemic sites, simultaneous transmission of *P. falciparum* and *P. vivax* is reported (Marques, 1987). However, in the state of Pará, there are localities where only *P. vivax* can be found. Transmission of *P. vivax* malaria in these villages is not recent, however, in the last few years, they have experienced a significant increase in the number of cases, a fact that made them important to be studied. Also, these sites provide the opportunity to perform epidemiological and immunological studies of *P. vivax* malaria without the influence of *P. falciparum* transmission. Several studies have shown that the presence of *P. falciparum* has a profound effect on the transmission and development of immunity to *P. vivax* (Maitland et al., 1997).

In spite of being highly prevalent in Brazil as in many parts of the world, the immunological mechanisms operating in individuals exposed to *P. vivax* have been very poorly explored. Recently, we have characterized serum antibody and T-cell reactivity of individuals from the city of Belém in the north of Brazil recently exposed to *P. vivax* malaria with 11 recombinant proteins representing the N and C-terminal regions of the Merozoite Surface Protein 1 of *P. vivax* (PvMSP1). We found that a high frequency of individuals had IgG antibodies and T-cell reactivity to at least one recombinant protein derived from PvMSP1. The recombinant protein based on the 19 kDa C-terminal region of PvMSP1 (PvMSP1$_{19}$) containing the two epidermal growth factor-like regions was the most immunogenic during natural infection in humans. This recombinant protein was recognized by antibodies or T-cells of 83.8% of the individuals (Soares et al., 1997). This high frequency of responders was also described in an independent survey performed in Papua New Guinea, where the sera of more than 80% of the individuals reacted with a recombinant PvMSP1$_{19}$ (Fraser et al., 1997). These immuno-epidemiological studies on naturally acquired immunity to the C-terminal of PvMSP1 are of particular

importance as this region of MSP1 is being intensively studied as a prime candidate for the development of a vaccine against malaria (Daly and Long, 1993, 1995; Ling et al., 1994; Nussenzweig and Long, 1994; Kumar et al., 1995; Chang et al., 1996; Galinski and Barnwell, 1996; Holder and Riley, 1996; Tian et al., 1997).

In our earlier study, we evaluated the antibody and T-cell reactivity in individuals who lived in the city of Belém, a site where malaria transmission very rarely occurs. All the individuals had contact with *P. vivax* infected mosquitoes during short stays in the areas surrounding the city where malaria transmission is frequent. The purpose of this study was to extend our earlier observation on the immunity to the PvMSP1 to the village of Cotijuba in the state of Pará where transmission of *P. vivax* occurs. We characterized the antibody response of individuals living in this village to recombinant proteins representing the N and C-terminal regions of PvMSP1 as well as, to blood stages antigen of *P. vivax* and *P. falciparum*.

2. Materials and methods

2.1. Study area and population

This study was performed in Cotijuba (48°33′W, 01°15′S), a ∼16 km² island situated 25 km from Belém, the capital of the state of Pará (eastern Brazilian Amazon). Reports of malaria transmission in this region date from several years ago. However, in the last 2 years, the village has experienced a significant increase in the number of cases. The region is characterized by a tropical wet climate. The annual temperature ranges from 23 to 32°C. Rainfall is the main climatic feature which shows some seasonality, with a rainy season from December to May and a 'dry' season from June to November. The average index of relative humidity ranges between 85 and 90%. Only *P. vivax* transmission is reported, and the main vector is *Anopheles aquasalis*. *An. oswaldoi*, *An. nuneztovari* and *An. triannulatus* have also been found. The village contains approximately 1386 inhabitants. Part of the population lives in the area of the island where the ferryboat pier is located. This area has a church, a health center and a school. One part of the inhabitants live in small communities dispersed in several beaches and one part resides in areas that are routes to the different beaches. The adults are engaged in agriculture or fishing activities. Health services are provided through one government health center and local NHF service. In addition, mobile teams of the NHF visit most houses regularly. Most individuals are aware of the cause of malaria and are advised to seek treatment. Records of cases of malaria in the area, kept by the NHF, were the source of our data for the years 1994–1996 concerning monthly incidence, sex and age distribution. Data on the population composition by age and sex were obtained from Instituto Brasileiro de Geografia e Estatística (IBGE).

2.2. Parasitologic procedures

Microscopic diagnosis and treatment of malaria cases were done at a clinical outpost of NHF in Cotijuba. Giemsa-stained thick blood smears from all individuals were examined by NHF technicians at $1000 \times$ magnification for the detection of malaria parasites. The slides were re-examined by technicians of the Instituto Evandro Chagas, in Belém. The individuals were treated for malaria in the day that followed the diagnosis. The standard treatment consisted of one oral dose of 600 mg of chloroquine and 30 mg of primaquine administered at the day following the diagnosis. In the subsequent 6 days, these patients received daily doses of 30 mg of primaquine.

2.3. Sera

Blood samples were obtained between November 1996 and January 1997 with informed consent by all individuals. We collected venous blood samples (10 ml) from 104 individuals for serology. Forty nine individuals were males and 55 females. Their average age was 25 years ranging from 4 to 63 years. Clotted blood samples were centrifuged and serum aliquots were stored at $-20°C$. A history of the past and present experience with malaria was obtained from each individual and confirmed by the local NHF service. Subjects were divided into three groups:
1. individuals with a recent (< 6 months) history of *P. vivax* malaria ($n = 31$);
2. individuals whose last malaria attack due to *P. vivax* occurred more than 6 months before ($n = 41$);
3. individuals without a history of symptomatic malaria infection and negative by standard Giemsa-stained thick smears ($n = 32$).

2.4. Recombinant PvMSP1 proteins

The N-terminal region of PvMSP1 protein from the Belém strain was expressed as glutathione *S*-transferase (GST) fusion protein. The detailed construction has been described elsewhere (Levitus et al., 1994; Soares et al., 1997). The recombinant protein ICB2-5 contains 506 aa located at the N-terminal region of PvMSP1. Recombinant protein ICB10 (PvMSP1$_{19}$) encodes 111 aa and contains the two EGF-like motifs described for the C-terminal region of other MSP1 molecules (del Portillo et al., 1991). As control, GST was produced alone. Recombinant proteins and GST were affinity purified on glutathione-sepharose 4B columns (Pharmacia, Uppsala, Sweden), their purity determined by SDS-PAGE and protein concentration measured by the Bradford method (Bradford, 1976).

2.5. Immunoassays

2.5.1. Immunofluorescence antibody assay (IFA)
IFA was performed using blood stages (trophozoites and schizonts) of *P. vivax* and *P. falciparum*. Red blood cells were collected from donors with a high patent

parasitemia. These individuals had never had a previous episode of the disease. After several washes in medium, these cells were dried onto imunofluorescence slides and stored at $-70°C$ until used. After they were removed from the $-70°C$, cells were fixed with acetone for 2 min. Serum samples were first assayed at 1:40 dilution in phosphate buffered saline (PBS), and the positive samples were analyzed in a 2-fold serial dilution from 1:40 until 1:10 240. After 30 min at 37°C, the slides were washed with PBS, and incubated with fluorescein-labeled goat anti-human IgG (Fc specific, Biolabs, São Paulo, Brazil) diluted 1:100 in PBS containing 1% Evans blue. After 30 min at 37°C, several washes were performed with PBS. Buffered glycerin and a cover slip were added on top of each slide. The immunofluorescence was observed at magnifications of $100 \times$ and $400 \times$ with the aid of a Zeiss microscope.

2.5.2. Detection of antigen specific IgG antibodies by ELISA

Sera of 104 individuals were tested for reactivity with the PvMSP1 recombinant proteins by ELISA. Microtiter plates (Costar, Cambridge, MA) were coated with 200 ng/well of affinity-purified fusion proteins or GST and incubated overnight at 4°C, after that time they were washed three times with 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 $PvMSP1_{19}$ were titrated with subsequent 2-fold serial dilution until 1:102 400. After 2 h incubation at rt, unbound material was washed away, and peroxidase-conjugated goat anti-human IgG (Fc specific) (Sigma, St. Louis, MO), diluted 1:10 000, was added to each well. After a 1 h incubation at rt, 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). All the OD_{492} 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_{492} values were averaged. Cutoff points were set at three standard deviations above the mean OD_{492} value of sera from 30 healthy individuals from the city of São Paulo never exposed to malaria.

2.6. Statistical methods

Frequency of responders in the different groups of serum donors, was analyzed by comparison of proportions. $p < 0.05$ was considered to be significant. Kruskal–Wallis one-way analysis of variance test was used for comparison between absorbancies and antibody titers values of different groups. Results were obtained with the aid of the True Epistat software program.

3. Results

3.1. Incidence of malaria in the study area

A monthly passive survey of malaria cases for 1994–1996 is presented in Fig. 1.

The incidence of malaria increased from 60 cases reported in 1994 to 823 cases in 1996. Analysis of data of Fig. 1 identifies at least one clear peak around April–May, coincident with end of the rainy season. The incidence of malaria in different sex and age groups was not significantly different when compared to the demographic profile of the population for 1996 (Fig. 2). Thus, it seems that there was no specific group of age or sex with a higher risk of infection. Analysis of the parasite species in the passive malariometric NHF survey revealed that *P. vivax* was the only species of malaria prevalent in the area. The active survey for microscopical examination of Giemsa-stained thick smears and serological studies were performed from November 1996 to January 1997 and included 104 subjects. Only one individual (0.96%) had a blood sample that contained *P. vivax* parasites. This individual had fever on that day.

3.2. Prevalence and levels of antibodies against P. vivax and P. falciparum blood stages

A total of 104 sera collected in the study area were examined for the presence of antibodies to blood stages by IFA. Eighty subjects (76.7%) had antibodies against *P. vivax* and 26 (25%) against *P. falciparum* blood stages. The frequency of individuals with antibodies to *P. vivax* was significantly higher when compared to
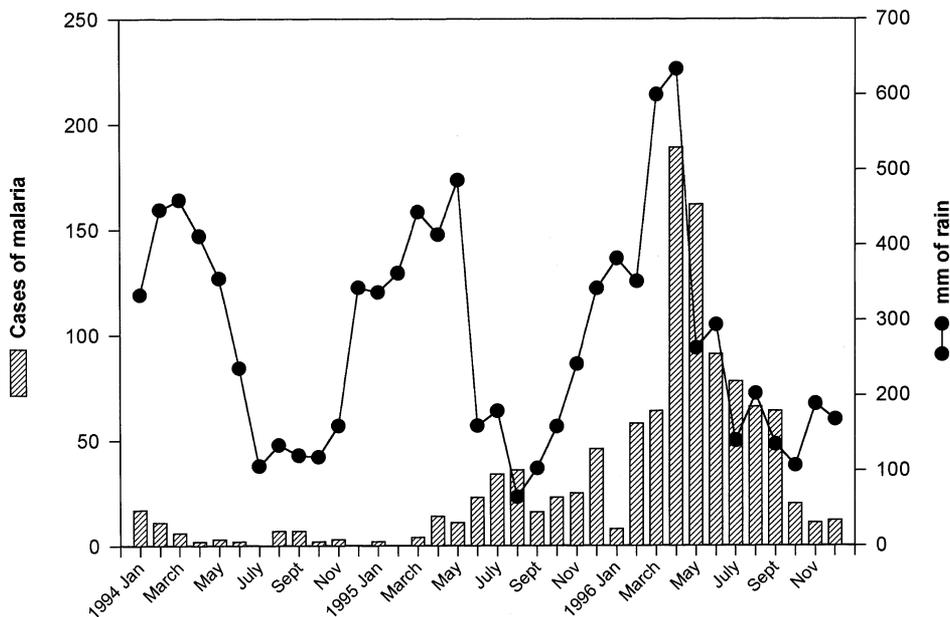

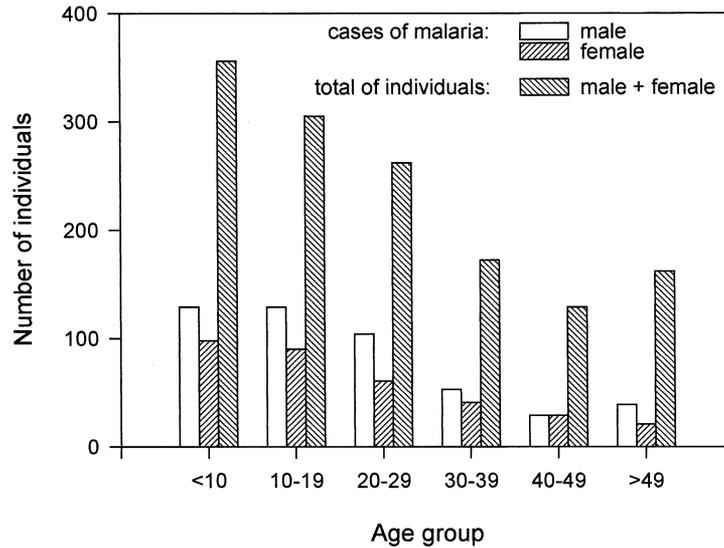Fig. 1. Rainfall and the incidence of malaria in the village of Cotijuba, Brazil from 1994 to 1996.

Fig. 2. Passive case detection of malaria in the village of Cotijuba according to sex and age (year 1996), in comparison with the demographic profile of the population.

the frequency of individuals positive to *P. falciparum* antigens ($p < 0.05$, comparison of proportions). Also, the antibody titers to *P. vivax* were in average 7.5-fold higher than those observed to *P. falciparum* ($p < 0.0001$, Kruskall–Wallis).

The frequency of subjects with IgG to *P. vivax* blood stage antigens was higher in individuals with a recent (< 6 months) history of *P. vivax* malaria (group A) than in individuals whose last malaria attack occurred more than 6 months before (group B) or individuals without a history of symptomatic malaria infection (group C, Fig. 3, $p < 0.05$). Interestingly, 50% of the individuals in group C had antibodies to blood stages of *P. vivax* as determined by IFA. Comparison of IFA titers showed that the antibody titers in individuals of group A were higher than in individuals from groups B and C ($p < 0.0001$).

3.3. Prevalence and titers of IgG antibodies to recombinant proteins derived from PvMSP1

The overall IgG antibody prevalence for $PvMSP1_{19}$ or protein ICB2-5 were 42.3 and 29.8%, respectively. As shown in Fig. 4, the frequency of individuals with antibody to $PvMSP1_{19}$ was significantly higher in individuals from group A (recent history of malaria) when compared to individuals of group B (77.4 and 43.9%, respectively, $p < 0.05$). Only, a small proportion of individuals without a history of symptomatic malaria were positive to $PvMSP1_{19}$ (6.25%). Comparison of absorbance values did not show a statistically significant difference among responders of the three groups.

Similar results were obtained with the recombinant protein ICB2-5 (Fig. 4). The frequency of responders in group A (recent history of malaria) was higher than in group B (58.1 and 24.4%, respectively, $p < 0.05$) and a small proportion of individuals without a history of symptomatic malaria were positive to recombinant ICB2-5 (9.4%). Absorbance values were not statistically significant among responders to protein ICB2-5 of the three groups.

Titration curves were performed with each serum sample to determine precisely the antibody titer to each recombinant protein. The antibody titers to $PvMSP1_{19}$ and protein ICB2-5 ranged from 100 to 102 400 and 100 to 6400, respectively (data not shown). Although the average titer to $PvMSP1_{19}$ was higher, the difference was not statistically significant ($p = 0.108$). Also, no difference was found when we compared the antibody titers to each recombinant protein among subjects of groups A, B and C (data not shown).

3.4. Comparative analysis of antibody responses in different age groups

The prevalence of antibodies to protein ICB2-5 increased significantly with age ($r^2 = 0.85$). However, no correlation was found between the prevalence of antibodies to $PvMSP1_{19}$ or to *P. vivax* blood stages and age of the subjects ($PvMSP1_{19}$, $r^2 = 0.02$; *P. vivax* blood stages, $r^2 = 0.20$). The highest prevalence of antibodies to $PvMSP1_{19}$ occurred in the group of people of 31–40 years old, whereas antibodies to blood stages of *P. vivax* were observed most frequently in individuals of 41–50 years old (Fig. 5).
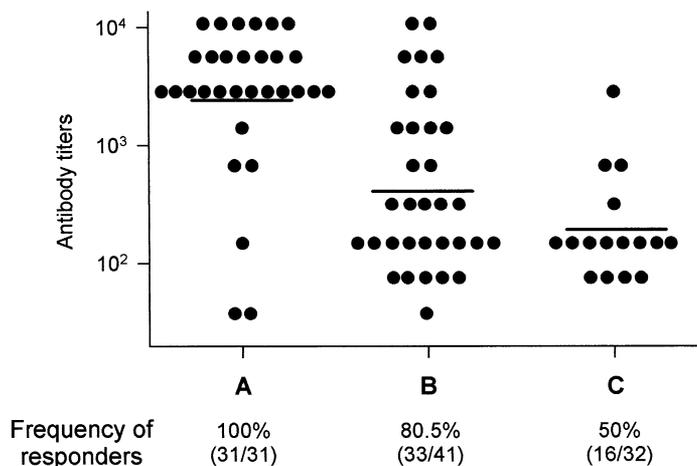
Fig. 3. Antibody titers and frequencies of responders to blood stages of *P. vivax* among individuals living in Cotijuba. Antibodies to *P. vivax* were estimated by IFA. The bars represent the geometric mean of antibody titers in each group. Group A, individuals with a recent (< 6 months) history of *P. vivax* malaria. Group B, individuals whose last malaria attack due to *P.vivax* occurred more than 6 months before. Group C, individuals without a past history of symptomatic malaria infection. Statistical comparisons are shown in the Section 3.
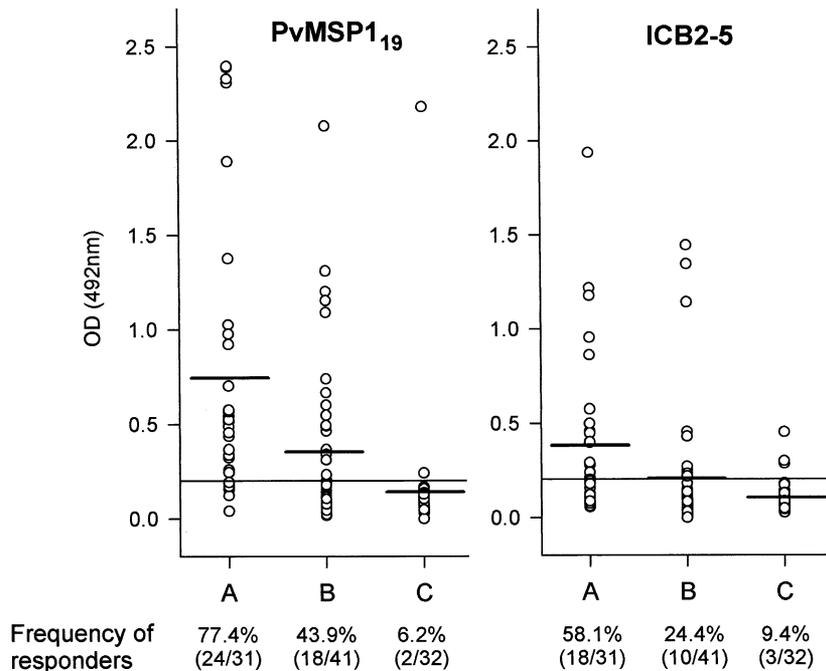
Fig. 4. Comparative analysis of the optical densities and frequencies of serum samples with antibodies to PvMSP1$_{19}$ and ICB2-5 among individuals living in Cotijuba. Serum samples were tested by ELISA at a dilution of 1:100. All of the OD$_{492}$ values represent binding of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. The line at 0.2 OD in each panel represents the cutoff value. The bars represent the average OD in each group. For definition of A, B and C see legend to Fig. 3.

4. Discussion

In the present study we analyzed the profile of IgG responses to *P. vivax* blood stage antigens and to recombinant proteins representing the N and C-terminal regions of PvMSP1 in the village of Cotijuba, an island located in the state of Pará, north of Brazil, where only *P. vivax* transmission is reported.

We found that during the low transmission season, a high frequency of individuals who had experienced at least one episode of patent *P. vivax* infection in their life had antibodies to PvMSP1$_{19}$. This result confirms and extends earlier studies performed by us and others suggesting that the PvMSP1$_{19}$ is highly immunogenic during natural human infection with *P. vivax* (Fraser et al., 1997; Soares et al., 1997). The presence of antibodies to PvMSP1$_{19}$ as well as to recombinant protein ICB2-5 seems to be related to previous symptomatic episodes of malaria, as only very few individuals without a past history of the disease displayed antibodies to these polypeptides. In contrast, 50% of individuals without a past history of the disease had antibodies to blood stages of *P. vivax*. The precise explanation for such

high reactivity to blood stage is unknown at present. It can be due to imprecise malaria histories reported by individuals, or to past malaria episodes misdiagnosed or asymptomatic cases of the disease. The presence of asymptomatic malaria in Brazil is a matter of controversy. Some groups have described asymptomatic malaria as infrequent (Prata et al., 1988; Camargo et al., 1996). However, recently, it has been described areas with a high prevalence of asymptomatic cases of the disease (Andrade et al., 1995). Whether in the island of Cotijuba, during the peak of transmission, there are individuals with asymptomatic *P. vivax* malaria will require further studies.

Our study also confirmed that the C-terminal region is more immunogenic than the N-terminal region of PvMSP1 during natural infection in humans. The reason for the higher degree of recognition of $PvMSP1_{19}$ can be related to the fact that $PvMSP1_{19}$ is less polymorphic than the N-terminal region of PvMSP1. Recently, we evaluated the extension of the polymorphism of the N and C-terminal regions of PvMSP1 in this area. We confirmed that while the N-terminal region is polymorphic, we could not detect a single modification in amino acid deduced sequences from polymerase chain reaction (PCR) products derived from the C-terminal region of PvMSP1 (Soares et al., in preparation). Alternatively, the lower level of recognition can be due to the fact that the recombinant protein ICB2-5 might have a different conformation than the N-terminal region of native PvMSP1.

Whether the immune response to the N and C-terminal of PvMSP1 participate in the protective immunity to *P. vivax* blood stages is unknown. In earlier studies we have observed that in individuals with patent infection or after treatment, the main subclasses of IgG that react with the PvMSP1 recombinant proteins are IgG1 and IgG3 (Soares et al., 1997). These subclasses mediate opsonization and complement fixation of micro-organisms, in general, and have been implicated in antibody
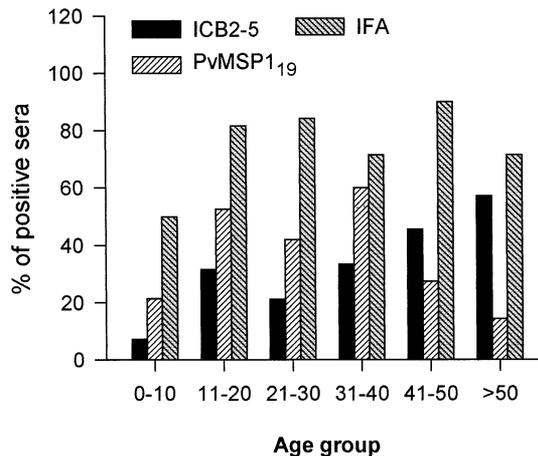

Fig. 5. Prevalence of IgG antibodies against recombinant proteins of PvMSP1 (ICB2-5 and $PvMSP1_{19}$) and *P. vivax* blood stages in different age groups. The number of individuals in each age group was: 0–10, $n = 14$; 11–20, $n = 39$; 21–30, $n = 18$; 31–40, $n = 15$; 41–50, $n = 11$; > 50, $n = 7$.

mediated protective immunity against *P. falciparum* blood stages (Bouharoun-Tay-oun et al., 1990). Also, B-cells play an important role in the resistance to malaria blood stage infection as Ig μ-chain gene knockout mice are unable to eliminate *P. chaubaudi chabaudi* parasites (von der Weid et al., 1996). Most relevant, several studies using recombinant proteins representing the $MSP1_{19}$ of *P. yoelii*, *P. falci-parum* or *P. vivax* could demonstrate experimentally induced immune protection against malaria in rodents and non-human primates (Daly and Long, 1993; Ling et al., 1994; Kumar et al., 1995; Chang et al., 1996; Galinski and Barnwell, 1996; Tian et al., 1997). This protective immunity has been shown to be dependent mainly on antibodies (Daly and Long, 1995). Therefore, it is plausible to believe that antibod-ies to MSP1 may participate at some extension in the elimination of blood stages of *P. vivax*.

Finally, the present study on the characterization of the transmission pattern and naturally acquired immune responses to the PvMSP1 in the village of Cotijuba will allow us to perform more detailed immunological studies in vivax malaria.

Acknowledgements

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