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HUMAN IgG RESPONSES AGAINST THE N-TERMINAL REGION OF THE MEROZOITE SURFACE PROTEIN 1 OF *PLASMODIUM VIVAX*

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The complete primary structure of the gene encoding the Merozoite Surface Protein 1 of Plasmodium vivax *(PvMSP-1) revealed the existence of interspecies conserved regions among the analogous proteins of other* Plasmodia *species. Here, three DNA recombinant clones expressing 50, 200 and 500 amino acids from the N-terminal region of the PvMSP-1 protein were used on ELISA and protein immunoblotting assays to look at the IgG antibody responses of malaria patients from the Brazilian amazon region of Rondônia. The results showed the existance of* P. vivax *and* P. falciparum *IgG antibodies directed against PvMSP-1 antigenic determinants expressed in the clones containing the first 200 and the following 500 amino acids of the molecule, but not within the one expressing the most N-terminal 50 amino acids. Interestingly, there was no correlation between the levels of these IgG antibodies and the previous number of malaria infections.*

Key words: vivax malaria – merozoite surface protein 1 – GST-recombinant clones – human IgG – ELISA – protein immunoblotting

Plasmodium vivax has the widest global distribution and is the most prevalent human malaria species in the Americas and Asia; yet, we are just beginning to understand its antigenic composition (David et al., 1988). This lack of information is mainly due to the impossibility of culturing the parasite *in vitro*, the low parasitemias obtained from individual patients, and the difficulties of adapting parasite isolates into monkeys. To partially circumvent these problems, recombinant DNA technology should facilitate the study of *P. vivax* proteins.

The Merozoite Surface Protein 1 (MSP-1) is a blood-stage and schizont-specific malaria antigen synthesized as a precursor and later processed into four of the major merozoite surface proteins (Holder & Freeman, 1982). Significantly, number of immunization trials with this molecule in rodent and primate models (reviewed in Holder, 1988) as well as in man (Patarroyo et al, 1988), point to the MSP-1 protein as a vaccine candidate against the malaria blood stages. Unfortunately, *P. falciparum* is the only human malaria for which the protective properties of the MSP-1 molecule have been assessed.

Recently, the complete, primary structure of the MSP-1 gene from the *P. vivax* Belém strain revealed the existence of Interspecies Conserved Blocks (ICBs) among the analogous proteins of other *Plasmodia* species (del Portillo et al., 1991). In this work, three different glutathione transferase (GST) fusion proteins expressing 50 (ICB1), 200 (ICB1-2) and 500 (ICB2-5) amino acids (aa) from the N-terminal portion of the *P. vivax* MSP-1 (PvMSP-1) molecule were produced. The recombinant products were affinity purified and tested on ELISA and protein immunoblotting against sera of malaria patients from the state of Rondônia (Brazil). Sera from giemsa-positive *P. vivax* and *P. falciparum* patients contained IgG antibodies capable of recognizing antigenic deter-

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minants in the fusion proteins containing the first 200 and the following 500 aa of the N-terminal portion of the PvMSP-1. In contrast, none of the immune sera recognized, neither by ELISA nor by protein immunoblotting, the GST-fusion protein encoding the first 50 aa of the molecule. Interestingly, there was no correlation between the IgG antibody levels against the PvMSP-1 recombinant products and the previous number of malaria infections.

MATERIALS AND METHODS

Cloning and expression of the GST-PvMSP-1 recombinant proteins – Three different DNA inserts of 150, 600 and 1500 bp from the 5'-end of the PvMSP-1 gene and encoding 50 (ICB1), 200 (ICB1-2) and 500 (ICB2-5) aa from the N-terminal portion of the molecule, respectively, were amplified by PCR. To do so, six oligomers (18 bases long) delimiting these regions (del Portillo et al, 1991) were synthesized and used in amplifications of the original clones containing the entire PvMSP-1 gene. The amplifications were done on a DNA thermal cycler (Perkin Elmer Cetus) at 95°C/1 min, 55°C/1 min and 72°C/2 min for 25 cycles and using the reagents of the Gene-Amp kit (Perkin Elmer Cetus). PCR products were filled - in by Klenow (Sambrook et al, 1989), phosphorylated (Current Protocols in Mol. Biol., 1989) and subcloned into the Sma I dephosphorylated site of the pGEX-3X vector (Pharmacia). Ligated products were transformed into $CaCl_2$ competent *E. coli* DH5-alpha cells and plated on LB-amp plates. Colonies were screened immunologically (Sambrook et al., 1989) using a rabbit polyclonal monospecific antiserum produced against an N-terminal portion of the MSP-1 protein of *P. vivax* including all regions comprised within ICB2-5 (not shown). Positive colonies were grown overnight on 4 ml LB-amp cultures and a 1 ml aliquot centrifuged for 2 min. Pellets were resuspended in 100 µl of PBS/1.0% (v/v) Triton X-100 plus 100 µl of Laemmli cracking buffer and 10 µl aliquotes resolved on 10% sodium dodecyl sulfate polyacrilamide gel electrophoresis SDS-PAGE (Laemmli, 1970). Since no antibodies against ICB1 were available, colonies containing pGEX-3X-ICB1 constructs were identified by nucleic acid hybridizations (Sambrook et al., 1989) using ^{32}P labelled ICB-1 PCR products as a probe. A positive clone expressing GST-ICB1 fusion products was subsequently identified by SDS-PAGE as above. The open reading frame and copy number of this clone were confirmed by DNA sequencing (Sanger et al., 1977).

Affinity purification and protein concentration – GST and GST-recombinant products were one-step affinity purified on glutathione Sepharose 4B columns (Analects, 1991). The protein concentration was measured by the Bradford method (Bradford, 1976) and the purity of the products determined by SDS-PAGE (Laemmli, 1970).

Sera – A total of 120 sera divided into four groups (n = 30) were used in this study. Sera from normal individuals and malaria patients were collected at the blood-center (Hemeron) located in the capital city of Porto-Velho, Rondônia. The malaria sampling included invididuals from both sexes, different ages, first-infected as well as multiple-infected individuals. Group I corresponded to normal individuals who had no previous clinical history of malaria. Group II corresponded to giemsa-positive *P. vivax* patients whose blood was collected on the day of this diagnosis, excepting for two first-infected vivax patients who had been diagnosed two and six weeks earlier from the day of the blood collection. Group III corresponded to giemsa-positive *P. falciparum* patients whose blood was collected the same day of this diagnosis. Group IV corresponded to chronic chagasic patients from Uruguay, a country where malaria is not endemic, and who had never left this country.

Protein immunoblotting – Affinity purified recombinant products were resolved on 10% SDS-PAGE (Laemmli, 1970), blotted (Tow-bin et al., 1979) onto Hybond-C membranes (Amersham) and blocked overnight at 4 °C with TBS-milk (50 mM Tris-HCl pH 7.5, 0.9% (w/v) NaCl, 4% (w/v) non-fat dry milk). After this, the membranes were incubated for two h at room temperature with sera diluted 1:100 in TBS-milk containing 0.1% (v/v) Tween 20 and 20% (v/v) bacterial wild type extracts. Bound IgGs were detected using the ABC Vectastain kit for human, rabbit or mouse IgGs, as needed (Burlingame, CA).

Enzyme Linked Immuno-Assay (ELISA) – ELISA assays were performed essentially as described (Guilbert et al., 1982). Each individual serum, diluted 1:100, was tested twice in independent assays against 400 ng of affinity purified GST, 400 ng of affinity purified

GST-fusion protein and coating buffer (0.1 M carbonate pH 9.6) alone. Bound IgGs were detected with peroxidase conjugated goat anti-human IgG (BioSys) followed by the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry, Gaithersburg, MD) according to the manufacturers' instructions. Average individual OD_{630} values of the GST-fusion products were subtracted from the highest average OD_{630} value of either the GST or coating buffer wells. OD_{630} values above the mean plus three standard deviations of the normal control Group I were considered positives.

Statistical Analyses – The chi-square test described in Zar (1984) was used to compare the percentage values of the ELISA positive sera against ICB1-2 and ICB2-5.

RESULTS

Three different overlapping DNA inserts (150, 600 and 1500 bp) expanding 2100 bp from the ATG initiation codon of the PvMSP-1 gene and encoding 50 (ICB1), 200 (ICB1-2) and 500 (ICB2-5) aa from the N-terminal region of the PvMSP-1 antigen were specifically amplified by PCR (Fig. 1A). The amplified products were subcloned into the pGEX-3X vector and GST-fusion proteins from individual positive clones were affinity purified on Glutathione Sepharose 4B columns. Coomassie-stained protein bands of the predicted molecular weights 26 kDa GST, 31 kDa ICB1, 46 kDa ICB1-2 (lower MW bands represent degradation products) and 76 kDa ICB2-5 were observed (Fig. 1B). To confirm the specificity of these results, a gel containing similar quantities of these samples was blotted and reacted against a rabbit polyclonal monospecific anti-PvMSP-1 antibody. As expected, this serum did not contain IgG antibodies recognizing the GST alone or the GST-ICB1 fusion products (Fig. 1C). In contrast, they did contain IgG antibodies against antigenic determinants encoded in the GST-ICB1-2 and GST-ICB2-5 recombinant polypeptides (Fig. 1C).

These affinity purified GST-fusion proteins were used on ELISA to study the IgG immune responses of malaria patients from the Rondônia amazon region of Brazil. Fig. 2 shows the results obtained with a total of 120 sera divided into four groups of 30 samples each. None of the malaria (vivax or falciparum) immune sera contained IgG antibodies recognizing vivax antigenic determinants on the GST-fusion protein expressing the first 50 aa of the PvMSP-1 molecule (Fig. 2-ICB1). In contrast, these same sera contained IgG antibodies in 47% and 20% of the vivax and falciparum immune sera, respectively, specifically recongnizing vivax antigenic determinants on the GST fusion protein exprssing the first 200 aa of the molecule (Fig. 2-ICB1-2). Similarly, 33% and 47% of the vivax and falciparum immune sera, respectively, contained IgG antibodies reacting against vivax antigenic determinants within the ICB2-5 region (Fig. 2-ICB2-5). In most instances, the same immune serum recognizing ICB1-2 did also recognize ICB2-5; yet, there were two instances in which two individual immune sera recognized ICB2-5 and failed to recognize ICB1-2. Control sera from normal individuals or chagasic patients from Uruguay, all failed to recognize to any significant extent the GST vivax fusion proteins confirming the specificity of all the above results (Fig. 2).

A chi-square test comparing the percentage of *P. vivax* ELISA positive sera recognizing ICB1-2 (47%) and that one recognizing ICB2-5 (33%), revealed that this difference was not significant. In contrast, the percentage of *P. falciparum* ELISA positive sera recognizing ICB1-2 (20%) and that one recognizing ICB2-5 (47%), was significantly different ($p < 0.028$). The differences between the percentages of vivax and falciparum positive sera were also significantly different for ICB1-2 (47% and 20% respectively, $p < 0.028$), whereas for ICB2-5 they were not.

No significant correlation ($p > 0.1$, student-t test) was found between the ELISA values against either ICB1-2 or ICB2-5 and age, sex or the number of previous malaria infections for groups II and III.

These same sera were tested by protein immunoblotting against affinity purified GST and the GST-fusion proteins. Fig. 3 shows a representative result of these experiences. Ten positive vivax sera presenting the highest OD_{630} values obtained by ELISA against ICB1-2 were pooled into two groups of five sera containing increasing OD values: group 1 ($\overline{OD}_{630} = 0.439$); group 2 ($\overline{OD}_{630} = 0.663$). Similarly, two groups of the 10 falciparum sera with the highest ELISA values against ICB2-5 were pooled: group 1 ($\overline{OD}_{630} = 0.546$); group 2 ($\overline{OD}_{630} = 0.826$). All groups from vivax (Fig. 3A) and falciparum (Fig. 3B) sera specifically recognized GST-ICB1-2 and GST-ICB2-5, whereas they failed to recognize GST-ICB1 or GST alone. Moreover, the groups of pooled

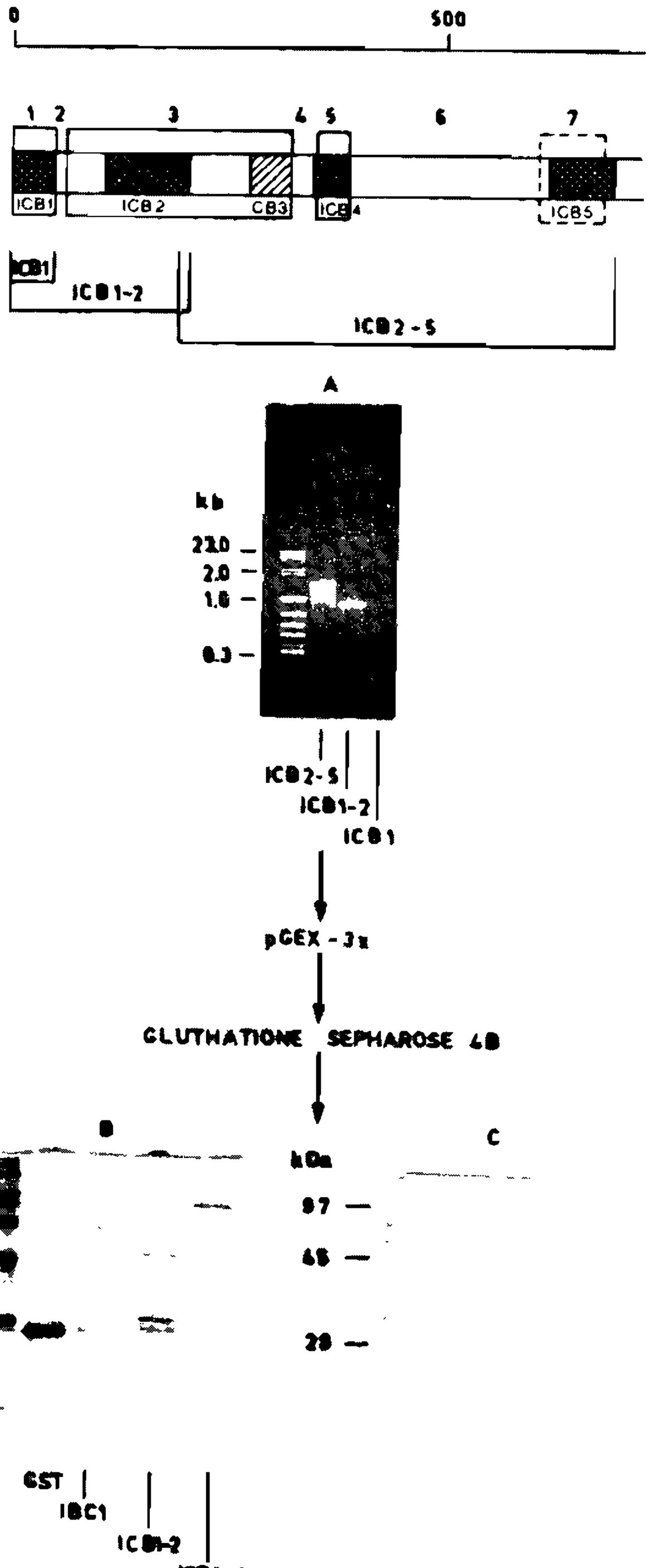

Fig. 1: cloning and expression of GST-PvMSP-1 recombinant proteins. The entire N-terminal region of the PvMSP-1 protein was expressed in three different clones encoding 50 (ICB1), 200 (ICB1-2) and 500 (ICB2-5) aa (upper diagram). To do so, DNA inserts encoding these regions were amplified by PCR and their products visualized by ethidium bromide staining on a 1% agarose gel (A). These amplified products were cloned into the pGEX-3x vector and expressed as GST fusion proteins. After one-step affinity chromatography, Coomassie blue stained proteins of expected molecular weights were observed by SDS-PAGE (B). Protein immunoblots of these products probed with a rabbit monospecific anti-PvMSP-1 antiserum, specifically recognized ICB1-2 and ICB2-5 but, as expected (see materials and methods), failed to recognize either GST or ICB1 (C). Molecular weight markers in Kb (A) or kDa (B and C) are shown to the left.

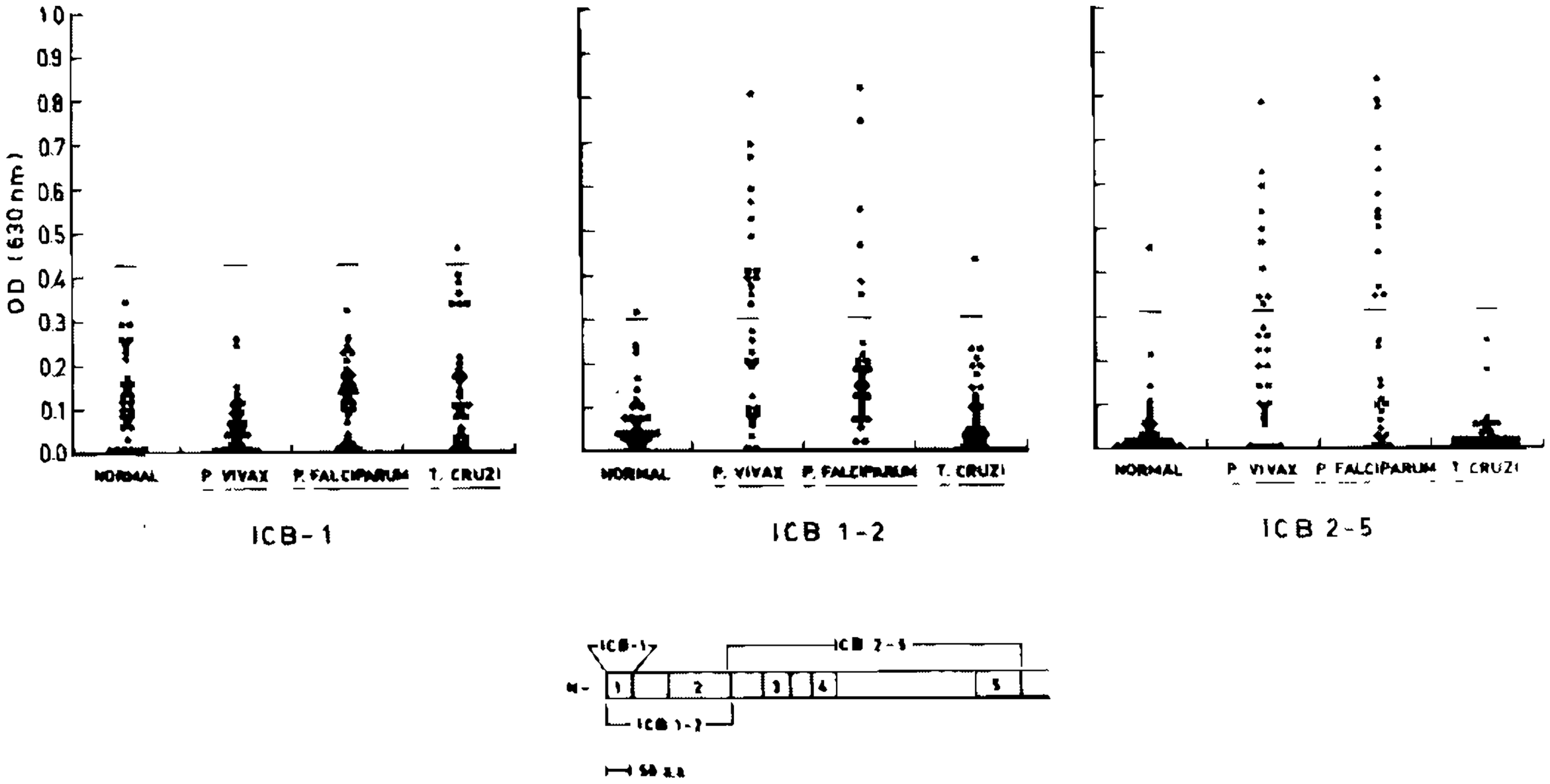

Fig. 2: human IgG responses against recombinant proteins expressing the N-terminal region of the PvMSP-1 molecule. Anti-GST-recombinant antibodies were measured by ELISA using affinity purified products from clones expressing 50 (ICB1), 200 (ICB1-2) and 500 (ICB2-5) aa from the N-terminus of the MSP-1 protein of *Plasmodium vivax* (lower diagram). Horizontal lines indicate the mean plus three standard deviation values obtained from normal control sera and the individual dots represent individual serum samples. In all groups (n=30); normal (Group I), *P. vivax* (Group II), *P. falciparum* (Group III), *Trypanosoma cruzi* (Group IV) as described in materials and methods.

sera containing the highest OD values, recognized stronger the fusion proteins confirming the results obtained by ELISA.

DISCUSSION

Immunity in malaria is known to be strictly species-specific. Indeed, many aspects of the molecular and cellular biology, in particular the receptors-ligands involved in the red blood cell-merozoite interactions of the two major human malarias, *P. vivax* and *P. falciparum*, are markedly different (Barnwell & Galinski, 1991). Thus, studies with merozoite surface proteins of *P. vivax, per se,* should be reinforced in relation to vaccine development fo this parasite species.

The specific objective of this work was to initiate studies on the IgG responses of malaria patients from Rondônia against recombinant proteins expressing the complete N-terminal region of the Merozoite Surface Protein-1 of *P. vivax* (PvMSP-1). The Rondônia state is responsible for about 70% of the reported million yearly malaria cases from the brazilian amazon region with about 50% of the infections due to *P. vivax*, approximately 48% to *P. falciparum* and the remaining 2% to *P. malariae* (TDR, 1988).

The PvMSP-1 recombinant proteins were produced as glutathione transferase (GST) fusion proteins since this bacterial expression system have proved most versatile and simple (Analects, 1991). The affinity purified GST-fusion polypeptides were used on ELISA and protein immunoblotting assays using a total of 120 sera from normal individuals, chagasic, falciparum and vivax patients.

Neither by ELISA nor by protein immunoblotting we were able to detect IgG antibodies from vivax or falciparum immune sera that specifically recognized vivax antigenic determinants within the first 50 aa of the PvMSP-1 molecule. This region corresponds to the most N-terminal portion of the molecule and to the Interspecies Conserved Block 1 (ICB1) described by del Portillo et al. (1991). Significantly, vaccination trials using two synthetic peptides derived from this region of the PfMSP-1, have altered the course of experimental infections in non-human primates (Cheung et al, 1986; Patarroyo et al., 1987) and man (Patarroyo et al., 1988). The 11 aa peptide used in the human vaccination trial has a 45% interspersed homology to the analogous region of the PvMSP-1 protein and of the six aa changes, two are non-conservative (del Portillo et al., 1991). Unfortunately, results on the humoral IgG responses of naturally infected patients against IgG falciparum peptide are not presently available and the literature with regard to the IgG responses against this peptide

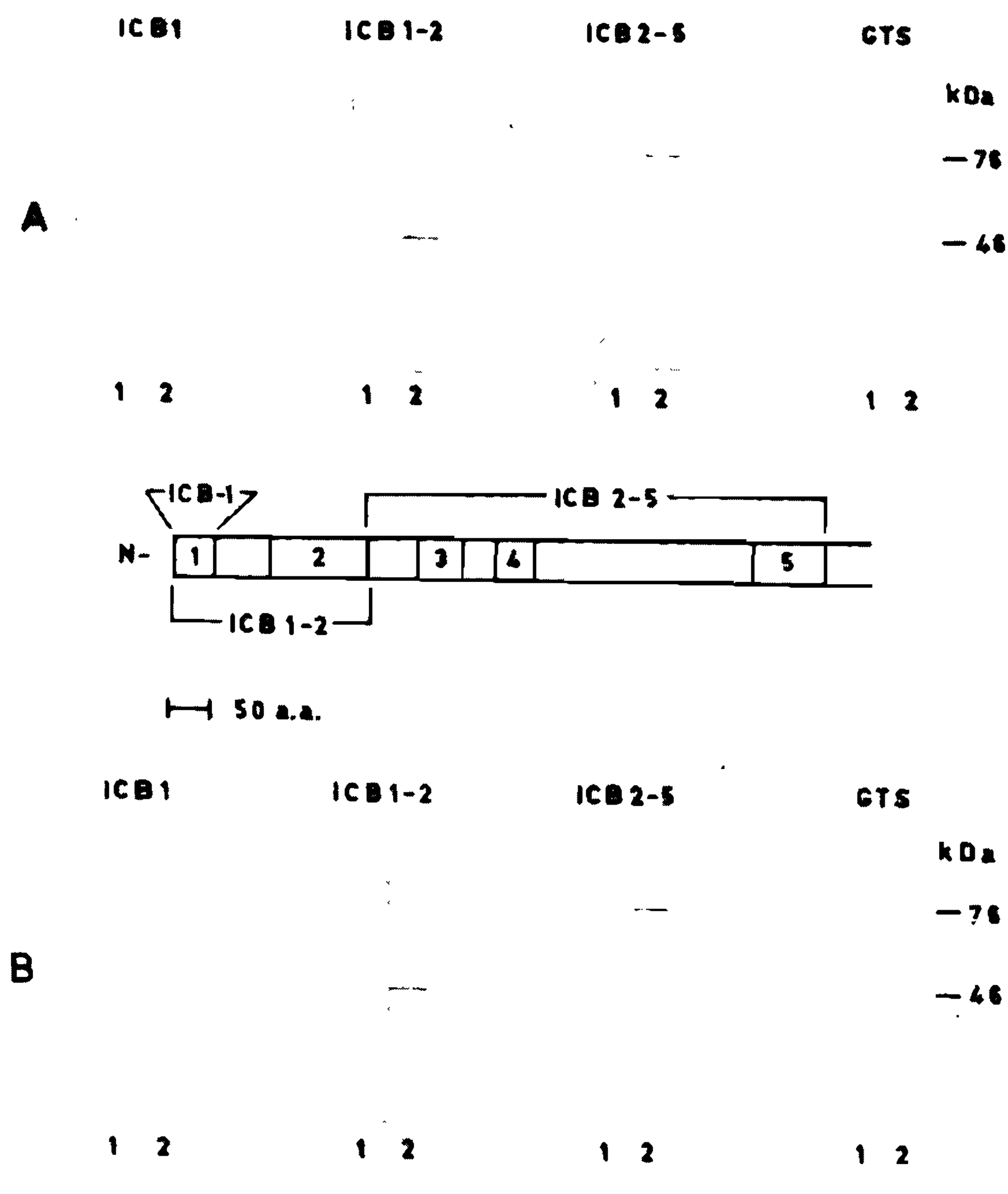

Fig. 3: reactivity of vivax and falciparum immune sera against PvMSP-1 recombinant proteins by immunoblotting. Five µg of affinity purified glutathione transferase (GST) and GST-recombinant proteins expressing N-terminal regions (ICB1, ICB1-2 and ICB2-5) of the PvMSP-1 protein (diagram), were resolved on SDS-PAGE and blotted. Nitrocellulose strips were probed with the sera from 10 vivax (A) or 10 falciparum (B) patients presenting the highest OD_{630} ELISA values against ICB1-2 and ICB2-5, respectively. Groups 1 and 2 represent 5 pooled sera each of increasing OD_{630} values: A1 $\overline{OD}_{630} = 0.439$, A2 $\overline{OD}_{630} = 0.663$, B1 $\overline{OD}_{630} = 0.546$ and B2 $\overline{OD}_{630} = 0.826$. Molecular weight markers are shown to the right.

in vaccine trials is contradictory (Rodriguez et al., 1990; Ruebush II et al., 1990; Herrera et al., 1991). Regardless, unless the presentation of GST-ICB-1, both by ELISA and protein immunoblotting, is completely different from the one presented by the native molecule, it is possible that a vaccine based on this region will not be boosted by natural infections.

In contrast, 47% and 20% of the vivax and falciparum sera, respectively, contained IgG antibodies recognizing vivax antigenic determinants within ICB1-2. Other PfMSP-1 recombinant clones expressing this analogous ICB1-2 region, were similarly shown to be recognized by falciparum IgG antibodies (Gentz et al., 1987; Müller et al., 1989).

Within ICB2-5, 33% of the vivax and 47% of the falciparum immune sera recognized vivax antigenic determinants; yet, these values were not significantly different. In contrast, the falciparum sera contained IgG levels significantly higher against ICB2-5 than against ICB1-2. In this con-

text, it is important to recall that this construct expresses three conserved regions including CB3, a region only conserved between the human malarias (del Portillo et al., 1991) and that there are IgG antibodies in falciparum patients recognizing these analogous regions in the PfMSP-1 molecule (Gentz et al., 1987; Müller et al., 1989).

Two aspects of the data presented here are lastly worth discussing; i) since many of the falciparum patients used in this study could have been previously infected with vivax malaria, one might argue that the IgG antibodies detecting the vivax fusion proteins were of vivax origin and not due to the existence of truly cross-reacting falciparum antibodies. Some of the falciparum patients used in here and elsewhere (unpublished) however, were well documented first-infected falciparum individuals thus clearly demonstrating the existence of such cross-reactive antibodies; ii) another interesting aspect of these results was the lack of correlation between the antibody levels and the previous number of malaria infections. We are presently investigating this aspect by looking at the IgM and IgG responses on a longitudinal study of sera from the same patients that had been infected several times throughout a year and using these recombinant vivax products.

In conclusion, the cloning and expression of the entire N-terminal region of the MSP-1 protein of *P. vivax* has demonstrated the existence of IgG antibodies among vivax and falciparum patients specifically reacting against the MSP-1 protein of *P. vivax*. To the best of our knowledge, this is the first report that shows the existence of antigenic determinants on specific regions of the PvMSP-1 protein being recognized by falciparum cross-reacting antibodies. Future studies should clarify wheter these IgG responses are predominantly recognizing polymorphic or conserved regions, the extent of immunological memory against these regions in natural infections, B and T-cell epitope maps, and the protective properties of these PvMSP-1 GST-fusion proteins in monkey immunization trials.

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