Advances Toward the Development of an Asexual Blood Stage MSP-1 Vaccine of *Plasmodium vivax*

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Plasmodium vivax is the most widely distributed human malaria parasite and the most prevalent in the Americas and Asia. Unfortunately, studies on *P. vivax* have long been neglected because of the difficulties in obtaining large amounts of this parasite: *P. vivax* cannot be continuously cultured *in vitro* and the parasitemias reached in infected patients are very low. The use of recombinant DNA technology has led to a better knowledge of *P. vivax* thus allowing to initiate vaccine studies of this prevalent human parasite species.

The Merozoite Surface Protein-1 (MSP-1) of *Plasmodium* is a glycoprotein synthesized by intracellular schizonts as a large (180-230 kDa) precursor and later processed to produce a complex of polypeptides on the merozoite surface (rev. in Holder 1988). Although some data have suggested that the MSP-1 might be involved in erythrocyte invasion, the function of this protein in natural infections remains presently unknown. Regardless, the success of several immunization experiments performed with different preparations of the MSP-1 of *P. falciparum* (PfMSP-1) in monkeys as well in humans has emphasized the importance of this antigen as a vaccine candidate against the malaria asexual blood-stages (rev. in Romero 1992). Unfortunately, immunity in malaria appears to be species-specific and thus it is unlikely that a vaccine against one species will protect against others. Indeed, results on a recent human vaccination trial have shown that immunization with peptides derived from sequences of *P. falciparum* proteins including MSP-1 does not induce protection against natural infections of *P. vivax* (Valero et al. 1993). These latter data clearly reinforce the importance of developing *P. vivax* MSP-1 vaccine candidates *per se*.

Preliminary studies using monoclonal antibodies against unmapped epitopes of the MSP-1 protein of *P. vivax* (PvMSP-1) demonstrated that it is polymorphic among wild isolates (Udagama et al. 1990). The genetic basis for such polymorphism was elucidated after the cloning and sequencing of two different PvMSP-1 alleles (del Portillo et al. 1991, Gibson et al. 1992). Thus, nucleotide and amino acid comparisons between the two completely characterized PvMSP-1 alleles (the Belem and Salvador alleles) indicated that the PvMSP-1 gene, like the PfMSP-1 one, consists of conserved, semi-conserved and variable blocks. In an attempt to determine if these polymorphic blocks followed the "allele dimorphic rule" reported for the PfMSP-1 gene (Tanabe et al. 1987), a variant segment of the PvMSP-1 gene has been amplified from *P. vivax* genomic DNA obtained from infected patients. This gene segment was shown to be highly variable not only between *P. vivax* alleles (Gibson et al. 1992) but also among *Plasmodia* species (del Portillo et al. 1991). Furthermore, this segment encodes a stretch of repetitive glutamine residues that should facilitate intragenic recombination events.

In the first study, genomic parasite DNA obtained from 25 infected patients from Rondonia, Brazil, was amplified using specific oligomers flanking the variant PvMSP-1 gene segment as above (Porto et al. 1992). PCR analysis and Southern blot hybridizations strongly suggested that two predominant allele forms, corresponding to the ones characterized in the PvMSP-1 genes from the Belem and Salvador strains, were present in this PvMSP-1 gene segment. Interestingly, these two forms were frequently (48%) found in a single patient indicating the existence of mixed infections.

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Similar studies performed by others (Premawansa et al. 1993) with Sri Lankan isolates revealed the existence of the same two allele forms of this gene segment. These results prompted the authors to consider these two forms as "parental allele forms" named type 1 and type 2. Interestingly, a third form likely produced by intragenic recombination was also detected in this study. More recently, we have further demonstrated the existence of the type 1 and type 2 parental allele forms among 18 natural isolates from the West Coast of Colombia (Mancilla et al. submitted for publication). The existence of two allele forms within this fragment of the PvMSP-1 gene in natural isolates from Rondonia was further demonstrated using a very simple and reliable method to prepare DNA samples from only two drops of infected blood (del Portillo et al. 1993). Analysis of 16 DNA samples obtained by this method also indicated the presence of the type 1 and type 2 allele forms among these wild isolates. More recently, another segment of the PvMSP-1 gene has been amplified from isolates from Philippines, China, the Solomon Islands and Papua New Guinea (Cheng et al. 1993). This fragment however, resides within a conserved block of the molecule as defined by allele comparisons and consequently no dimorphism was detected among these isolates.

Taken together, all these results clearly demonstrate the existence of two parental allele forms in this variant PvMSP-1 gene segment capable of limited genetic exchange during the mosquito stages. Furthermore, the nucleotide and amino acid sequences from these parental allele forms have been highly conserved in isolates from four countries and two continents. The extreme amino acid conservation of these sequences in these very different geographical regions could be explained if intragenic recombination of this gene segment is a rare event. Unfortunately, genetic crosses from *P. vivax* cannot be performed since this species cannot be cultured *in vitro* and no clones of it are presently available. Alternatively, the type 1 and type 2 amino acid sequences are positively selected because they are critical for the PvMSP-1 function and/or structure.

In parallel with the analysis on allele polymorphism of the PvMSP-1 gene, we have also initiated studies on the characterization of the humoral immune responses to the PvMSP-1 in naturally infected malaria patients. The cloning and characterization of the PvMSP-1 gene from the Belem strain allowed the comparison of the deduced amino acid sequences of the MSP-1 molecules from the human malarias *P. vivax* and *P. falciparum* and the rodent malaria *P. yoelii* (del Portillo et al. 1991). This study revealed that the MSP-1 molecule can be divided into interspecies conserved (ICBs) and polymorphic blocks, which reside within the analogous regions of the PfMSP-1 (del Portillo et al. 1991).The N-terminal portion of the molecule was chosen for these studies since many vaccine candidates against *P. falciparum* malaria were based on the analogous region of the PfMSP-1 protein. Moreover, naturally acquired antibodies against the N-terminal region of the PfMSP-1 have been detected in sera of *P. falciparum* infected patients in meso and hyper endemic areas.

In a preliminary study, the N-terminal region of the PvMSP-1 protein was expressed as three GST-recombinant proteins (del Portillo et al. 1992). These fusion products, corresponding to aa 1 to 52 (ICB1), 1 to 200 (ICB1-2) and 170 to 675 (ICB2-5) from the PvMSP-1 molecule of the Belem strain (del Portillo et al., 1991), were affinity purified and tested by ELISA against sera from *P. vivax* and *P. falciparum* multiple-infected patients from Rondonia, Brazil. These initial results indicated that the N-terminal region of the PvMSP-1 molecule was immunogenic in natural infections since a large number of these malaria sera specifically recognized the ICB1-2 and ICB2-5 fusion proteins (del Portillo et al. 1992).

We then analyzed the natural acquisition of antibodies against the N-terminal region of the PvMSP-1 of the Belem strain in a one-year longitudinal study conducted at a farm in the Amazon region of Rondonia (Mertens et al. 1993). Sera from 34 patients who had at least one malaria infection during the study period were tested by ELISA against the affinity purified ICB2-5 recombinant protein (see above) and *P. vivax* and *P. falciparum* total blood-stage antigens. We observed that close to 90% of the patients who had a minimum of three previous episodes developed humoral immune responses to all three antigens at the time of a new malaria exposure. These results were obtained when both IgG and IgM isotypes were analyzed together and are consistent with the idea that the acquisition of antibodies against malaria is a low process that reflects the need for several exposures to parasite antigens. However, when the IgG and IgM responses were considered separately, a different subclass

distribution was observed against the total blood-stage antigens and the N-terminal region of the PvMSP-1. The antibody responses against total blood-stage antigens were those commonly observed for T-cell dependent antigens with the development of a humoral immune response dominated by the IgG isotype as a consequence of consecutive infections. In contrast, in approximately 50% of the sera from patients who had experienced more than three previous malaria infections, the predominant or single detected isotype against the N-terminal portion of the PvMSP-1 was IgM. The lack of predominance of the IgG subclass in the sera from such a significant fraction of multiple-infected patients prompted us to analyze how a new malaria infection affected the individual IgG response to this region of the PvMSP-1 molecule. To be sure that we were looking at the boosting effect of a new infection, we chose patients with no detectable levels of IgG antibodies against the PvMSP-1 N-terminal portion before the malaria infection; most of these patients however, had IgG antibodies to total blood-stage antigens. We found that new infections stimulated the production of anti-PvMSP-1 N-terminal region IgG antibodies in only 50% of the patients who had experienced a minimum of four previous malaria attacks and who were infected 1-3 times during the study period.

One possible explanation for the above results is that a high level of allele polymorphism of the PvMSP-1 molecule in natural populations from Rondonia might have prevented the development of a memory IgG response in a fraction of the patients. The ICB2-5 fusion protein expressing the N-terminal portion from the PvMSP-1 and used in this study, contains one polymorphic and one semi-conserved small region as defined by allele comparisons (Gibson et al. 1992). Therefore, provided that B-cell epitopes within these regions are immunodominant, this slight possibility can still explain the lack of IgG antibodies against this portion of the molecule in multiple-infected patients. As was discussed above however, our results demonstrated the existence of only two parental PvMSP-1 alleles among parasite populations from Rondonia. It is also important to recall here that these results are preliminary and limited to a small number of patients; yet, they underlay the need for a better understanding of the boosting effect of the MSP-1 in natural infections.

Finally, we have been particularly interested in determining if the naturally acquired IgG responses of the malaria patients are directed against the interspecies conserved (ICBs) or polymorphic blocks of the PvMSP-1, as were defined by del Portillo et al. (1991). To do so, seven other DNA fragments expanding the 5' end of the PvMSP-1 gene of the Belem strain were amplified by PCR, cloned in frame into the pGEX-3X vector and expressed as Glutathione-S-transferase (GST)-fusion proteins (Levitus et al., submitted for publication). These recombinant proteins encoded aa 107 to 318 (ICB2-3), 290 to 388 (ICB3-4) and 389 to 611 (P4) and the interspecies conserved blocks ICB2, ICB3, ICB4, and ICB5. The fusion products, together with the ICB1, ICB1-2 and ICB2-5 ones (see above) were further affinity purified on Glutathione Sepharose 4B columns and tested by ELISA and Western blot against different human sera. Malaria serum samples corresponded to 30 *P. vivax* and 30 *P. falciparum* multiple-infected patients from Rondonia, Brazil, whose blood was collected on the day of the diagnosis. Control sera corresponded to 30 normal individuals from Rondonia and 30 chagasic patients who had never left Uruguay, a country where malaria is not endemic. Results of these experiments showed that 57% of the *vivax* sera contained IgG antibodies against the ICB1-2 fusion protein, 63% recognized ICB2-5 and 37% reacted with the ICB2-3, ICB3-4 and P4 recombinant proteins. In contrast, none of them contained IgG antibodies against the conserved regions ICB1, ICB2 and ICB3, while one and two reacted with ICB4 and ICB5, respectively. Similarly, 63%, 67%, 37%, 50% and 30% of the *falciparum* sera contained anti-ICB1-2, anti-ICB2-5, anti-ICB2-3, anti-ICB3-4 and anti-P4 IgG antibodies, whereas none, 10% 3%, 13% and 13% recognized ICB1, ICB2, ICB3, ICB4 and ICB5. None of the control sera recognized the GST-fusion proteins, confirming the specificity of the above results. The differences between the percentages of *vivax* and *falciparum* positive sera were not significant for all recombinant proteins tested. Moreover, no significant correlation was observed between the reactivities against the fusion polypeptides and age and number of previous malaria infections for *vivax* and *falciparum* sera patients.

These results strongly suggest that the IgG responses in these patients are predominantly directed against the variable portions of the PvMSP-1 molecule and that the interspecies conserved blocks of the PvMSP-1 protein are poorly im-

munogenic in natural infections. However, one cannot exclude the possibility that relevant epitopes mapping within ICBs could be either missing or in an altered conformation in these recombinant proteins. Another explanation is that these fusion proteins might contain B-cell epitopes only recognized by IgM antibodies (Mertens et al. 1993). Regardless, the poor B-cell immunogenicity of the interspecies conserved blocks of the PvMSP-1 molecule most likely reflects important and unknown structural or functional features of these regions. Whether or not the exclusive presentation of ICBs from the N-terminal region of the PvMSP-1 molecule will elicit protective immune responses in a *P. vivax* monkey vaccine trial, is thus now amenable to experimental verification.

In conclusion, studies on the MSP-1 gene and its protein of *Plasmodium vivax* have been facilitated trough the use of recombinant DNA technology. Thus, a variant segment of the PvMSP-1 gene has been amplified from parasite genomic DNA and used as a genetic marker to determine the extent of allele polymorphism among natural parasite populations. Moreover, fusion proteins expressing the N-terminal region of the PvMSP-1 molecule have been produced and used to characterize the naturally acquired humoral immune responses of malarious patients in endemic regions. Studies on T-cellular immune responses against the PvMSP-1 protein are yet to be reported and other portions of the molecule need to be analyzed. Together, this information should contribute in the near future to the design of rationale MSP-1 vaccine candidates against the asexual blood-stages of *P. vivax*.

NOTE

This paper was written exclusively for the proceedings of the meeting and consequently detailed data and extensive references were omitted in order to avoid complications with publication of the results as original articles. Details are however available on request.

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