

## ANTIGENICITY, IMMUNOGENICITY, AND PROTECTIVE EFFICACY OF *PLASMODIUM VIVAX* MSP1 Pv200L: A POTENTIAL MALARIA VACCINE SUBUNIT

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**Abstract.** The merozoite surface protein 1 (MSP-1) is expressed in all *Plasmodium* species and is considered a major malaria vaccine candidate. We found that MSP-1 from *Plasmodium vivax* (PvMSP-1) contains a region of significant sequence homology with the 190L subunit vaccine derived from the *P. falciparum* MSP-1. The fragment, termed Pv200L, was expressed as a recombinant protein in *Escherichia coli* (rPv200L) and used to assess its immunologic relevance as a vaccine target. A cross-sectional, seroepidemiologic study conducted in Buenaventura, Colombia showed that 52.2% (95% confidence interval [CI] = 39.8–64.3) of individuals previously exposed to *P. vivax* and 72.8% (95% CI = 61.8–82.1) of *P. vivax*-infected patients had IgG antibodies to rPv200L. Immunization of BALB/c mice and *Aotus* monkeys induced IgG antibodies (titer > 10<sup>6</sup>) that cross-reacted with *P. vivax* parasites. Immunized monkeys displayed partial protection against a challenge with *P. vivax* blood stages. Our results suggest that Pv200L is a new malaria vaccine subunit and deserves further testing.

### INTRODUCTION

Malaria caused by *Plasmodium vivax* is seldom lethal, but it is widely distributed and characterized by periodic relapses that generate a significant socioeconomic burden in endemic disease-areas.<sup>1</sup> Several immunologic, biologic, and epidemiologic reasons support the development of a vaccine to evoke specific protective immunity to this *Plasmodium* species.<sup>1</sup> Although several *P. vivax* antigens have been proposed as potential vaccine candidates and have been tested in preclinical studies,<sup>2</sup> to date only two antigens, PvCS and Pvs25, have undergone clinical testing.

Merozoite surface protein 1 (MSP-1) is an antigen abundantly expressed on the surface of mature merozoites<sup>3</sup> that has been shown to participate in the parasite invasion of the erythrocyte.<sup>4</sup> Because antibodies specific to this protein, particularly to the C-terminal region (42-kD and 19-kD subfragments), have been shown to block parasite invasion *in vitro*<sup>5–7</sup> and to induce protective immunity in animal models,<sup>8–13</sup> this MSP-1 fragment has long been considered a major vaccine candidate.<sup>12,14,15</sup> Limited effort has been invested in the definition and testing of other MSP-1 fragments as potential vaccine candidates.

In previous studies, we tested the immunogenicity and protective efficacy of a recombinant protein fragment (Pf190L) derived from the *Plasmodium falciparum* MSP-1 (PfMSP-1) protein in *Aotus* monkeys. This fragment was highly immunogenic and induced partial protection against an otherwise lethal parasite challenge.<sup>9</sup> More recently, a clinical trial conducted with a combination of *P. falciparum* antigens including Pf190L, MSP-2, and RESA (combination B) induced a parasitemia reduction in the volunteers.<sup>16</sup> Pf190L was shown to be the most immunogenic molecule in that vaccine cocktail and is likely to be responsible for part of the protection induced.<sup>17</sup> Here we report the results of studies conducted with a *P. vivax* MSP-1 protein fragment termed Pv200L because of its sequence homology to Pf190L. This protein fragment was expressed as a recombinant protein in *Escherichia coli*

(rPv200L) and tested for its antigenicity and vaccine potential both in BALB/c mice and *Aotus* monkeys.

### MATERIALS AND METHODS

**Sequence analysis.** We constructed a consensus sequence of the Pf190L fragment (Pf190Lcons) using seven complete PfMSP-1 sequences reported in the Swissprot database, which correspond to the FC27, MAD20, RO-33, Camp, Palo Alto, K1, and Wellcome strains. We used the Pf190Lcons sequence to run a standard BLAST/p search at the National Center for Biotechnology Information (NCBI) (Bethesda, MD) server to identify homologous protein sequences in *P. vivax*.<sup>18</sup> To identify the exact homologous region, we aligned 42 complete PvMSP-1 sequences reported in the EntrezProtein (NCBI) database to Pf190Lcons. The N- and C-terminal ends were defined, and a consensus sequence of the homologous fragment (Pv200Lcons) was constructed. Sequences were aligned with Clustal X (MSA mode),<sup>19</sup> and the consensus sequences were created with the Emboss tool *Cons*, after evaluation of the alignments with T-coffee. Homology cutoff was designed as  $e < 0.01$  and identity > 30%. We used the *Dotmatcher* tool from Emboss to illustrate the homolog regions in a dot plot.

**Cloning, expression, and purification of rPv200L.** Genomic DNA obtained from a *P. vivax*-infected patient who had given informed consent was used to isolate the *pv200L* gene fragment using primers that were designed based on the available sequence of *pvmsp1* of the Salvador I strain (AF435593). The primers 200L-1 5'-GCG GAT CCA AAC AAT CAG GTT AGC GTT-3' and 200L-2 5'-GTT AGT GTG AGG TCT CTT TCA GGG TTG G-3' were designed to amplify the region from basepairs 254 to 1308. A polymerase chain reaction (PCR) was then performed under the following conditions: 30 seconds at 95°C, 60 seconds at 55°C, and 90 seconds at 72°C. The PCR products were cloned into pGEM-T Easy vectors (Promega, Madison, WI) and subcloned in the expression vector pRSET-B (Invitrogen, Carlsbad, CA) after digestion with *Eco* RI and *Pst* I. This vector provided a polyhistidine (6-His) tag to facilitate further purification. We confirmed the open reading frame by automated sequencing in

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an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA) using a BigDye Terminator version 3.1 sequencing kit. This construct was then used to transform *E. coli* BL21-CodonPlus (DE3)-RIL expression hosts (Stratagene, La Jolla, CA). Expression was determined by immunoblotting that was revealed with a monoclonal antibody specific to the 6-His tag.

Bench-quality recombinant protein batches were obtained in a small laboratory scale of two liters using standard procedures recommended in *The QIAexpressionist* (Qiagen, Valencia, CA).<sup>20</sup> Briefly, a single colony-forming unit was cultured in 20 mL of selective Luria-Bertani (LB) medium for 14–18 hours (37°C, pH 7.2, and 350 rpm) and added to two liters of nonselective LB medium. Optical density (OD) was monitored until the OD<sub>630</sub> was ~0.600 and 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was then added. Expression was induced for 4–5 hours and cells were harvested by centrifugation. The cell paste was weighed and stored at –80°C until use. To extract the protein, we used denaturing methods based on 6 M guanidine hydrochloride and 8 M urea plus disruption by sequential freezing with liquid nitrogen and thawing with water (42°C). This material was centrifuged and the supernatant was sequentially filtered through 0.4-μm and 0.2-μm pores. The His-tagged recombinant protein was purified by standard immobilized metal ion affinity chromatography (IMAC) with Ni-nitrilotriacetic acid matrix (Qiagen) and eluted with 150 mM imidazol. Purified batches were dialyzed for 34 hours against phosphate-buffered saline (PBS) at 4°C and reprocessed 2–3 additional times to obtain material 90% free of *E. coli* contaminants with an endotoxin content less than 1,000 endotoxin units (EU)/50 μg of protein. Final batches of rPv200L were monitored for homogeneity and purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels under reducing conditions and analytical reverse-phase high-performance liquid chromatography (HPLC) with a C4 column. Endotoxin content was measured using the QCL-1000<sup>®</sup> chromogenic *Limulus* amoebocyte lysate endpoint assay (Biowhittaker, Walkersville, MD).<sup>21</sup> All buffers used during purification were prepared with pyrogen-free ultrapure water.

**Antigenicity study in humans.** A cross-sectional seroepidemiologic study was conducted in Buenaventura, the main port on the Pacific coast of Colombia (population approximately 400,000) and a malaria-endemic area with low and unstable malaria transmission.<sup>22</sup> We collected plasma samples from patients infected with *P. vivax* who were attending the outpatient clinic of the Malaria Vaccine and Drug Development Center for malaria diagnosis. Additionally, we studied asymptomatic individuals who had been permanently exposed to malaria in La Delfina, a disease-endemic village in rural Buenaventura where *P. vivax* causes almost 90% of the malaria cases every year.<sup>23</sup> These volunteers reported that they had at least one previous *P. vivax* malaria episode. We also included a group of volunteers recruited in Cali, a city in a non-endemic area of Colombia, who had no previous malaria experience. These individuals were selected based on a negative *P. vivax* immunofluorescent antibody test (IFAT) result. All volunteers provided 5 mL of blood (EDTA was added to samples as an anticoagulate) from which we obtained plasma fractions that were stored at –80°C until use. Malaria diagnosis was performed in all plasma donors using Giemsa-stained thick blood smears.<sup>24</sup>

**Immunogenicity studies in BALB/c mice.** Sixteen BALB/c mice were randomly selected and distributed to immunized and control groups of eight animals each that were used to test for Pv200L immunogenicity. The immunized group was injected intraperitoneally three times at 20-day intervals with 50 μg of rPv200L emulsified in decreasing amounts of complete/incomplete Freund's adjuvants: 50%, 25%, and 12.5%. The control group was injected intraperitoneally at the same immunization schedule with material purified from nontransformed expression host cells processed using the same purification procedures as for rPv200L. Approximately 150 μL of blood was collected from the tip of the tail on days 0, 20, 40, and 60. Sera was obtained by centrifugation and stored at –80°C until use.

**Immunogenicity and protective efficacy studies in Aotus monkeys.** Twelve healthy adult *Aotus lemurinus griseimembra* monkeys were randomly selected from a larger group of animals from the Primate Center in Cali, Colombia and were assigned to immunized and control groups of six monkeys each. The immunized group received 100 μg/dose of rPv200L, and the control group received saline solution (0.9%). Both preparations were formulated in Freund's adjuvants as described for the mice. Animals were immunized subcutaneously on days 0, 20, and 40 with a total volume of 500 μL of vaccine. An additional boosting dose formulated solely in 50% incomplete Freund's adjuvant was administered on day 120. Sera was obtained from blood samples collected on the same immunization days as described elsewhere.<sup>25</sup> Peripheral blood mononuclear cells (PBMCs) were also collected and cryopreserved and are currently being studied. All monkeys were challenged on day 140 with an intravenous injection of  $1 \times 10^5$  *P. vivax* (Sal I strain)–parasitized red blood cells. Parasitemia was followed-up by staining of thick blood smears with Giemsa every two days from day 152 until day 160 and daily until day 180; final values corresponded to means of four independent readings. Hemoglobin (mg/dL) and hematocrit (%) were tested once a week during the whole trial. Hemoglobin levels < 7.0 mg/dL or hematocrit levels < 25% were indicative of severe anemia and were defined as parameters to curatively treat the animals.

**Electrophoresis and immunoblotting.** We used standard SDS-PAGE on 12.5% gels under reducing conditions and immunoblotting to analyze the recombinant protein and parasite crude extracts as described elsewhere.<sup>26</sup> Separated proteins were stained with Coomassie brilliant blue silver stains (Sigma, St. Louis, MO). After blotting onto polyvinylidene difluoride membranes, antigen preparations were incubated for one hour with a 1:1,000 dilution of mice or monkey sera or with human immune plasma diluted in Tween 20-PBS (TPBS). The blotted strips were washed with T-PBS incubated for one hour with a 1:2,500 dilution of γ chain-specific anti-mouse or anti-human IgG alkaline phosphatase conjugate (Sigma) in TPBS. Antibody reactions were developed with SigmaFast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma). Prestained SDS-PAGE or Kaleidoscope molecular weight standards (Bio-Rad, Hercules, CA) were included on each gel to estimate relative molecular weights.

**Reverse-phase high-performance liquid chromatography.** Purified protein batches were analyzed on an HPLC system (Watters, Milford, MA), using a protein C4 column (Capital C4/300-Nucleosil) at 35°C. Purified samples were di-

luted 1:2 in mobile phase A (water plus 0.1% [w/v] trifluoroacetic acid [TFA]) and applied to the column. Bound material was eluted with a linear gradient (0–80%) of mobile phase B (100% acetonitrile plus 0.083% [w/v] TFA) over 20 column volumes. Elution was monitored by absorption at 216 nm.

**Serologic studies.** Human plasma samples and animal sera samples were evaluated using an enzyme-linked immunosorbent assay (ELISA) as described elsewhere.<sup>27</sup> Briefly, Maxisorp flat-bottomed, 96-well microplates (Nunc, Rochester, NY) were coated with 100 mM of rPv200L per well and incubated overnight at 4°C. Microplates were blocked for two hours with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (Sigma). After one washing step with T-PBS human and monkey samples diluted 1:200 and mice sera diluted 1:1,000 were added and incubated for one hour at room temperature. Two-fold serial dilutions of samples collected from mice and monkeys 20 days after the last immunization were also tested. Plates were washed five times with PBS containing 0.05% Tween 20, and secondary anti-mouse or anti-human IgG antibodies labeled with alkaline phosphatase (Sigma) were added at a final dilution of 1:2,500. Plates were incubated for one hour at room temperature, washed with T-PBS and developed with *p*-nitrophenylphosphate (Sigma). The reaction was stopped after 15 or 45 minutes with 10  $\mu$ L of 1 N NaOH and read at 405 nm. Every sample was tested in duplicate. The ELISA IgG cutoff was defined as the average of the negative control plus three standard deviations.

To determine the recognition of the native MSP-1 protein by mice and monkey sera, an IFAT was performed as previously described<sup>28</sup> using *P. vivax* mature schizonts obtained after short-term cultures of parasites collected from *P. vivax*-infected patients as antigen. Briefly, serum from either control or immunized groups of mice and monkeys were tested in two-fold dilutions in PBS from 1:50 to 1:3,200 and incubated on antigen slides in a moisture chamber at room temperature for 30 minutes. The slides were then washed three times with PBS, and goat anti-mouse and goat anti-human IgG fluorescein isothiocyanate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) conjugates were used as secondary antibodies for mice and monkey samples, respectively. Slides were then incubated as described earlier in this report, mounted with 50% glycerol, and evaluated with a fluorescence microscope (Nikon, Avon, MA).

**Statistical analysis.** Antigenicity was evaluated in human volunteers in two ways. First, we computed the proportion of IgG responders by group and its exact 95% confidence interval based on the binomial distribution, and we compared the groups using a chi-square test. Also, we fitted a multiple linear regression of the OD values to study differences among the study groups adjusted by age as a potential confounding factor. For immunogenicity in BALB/c mice and *Aotus* monkeys, we described antibody titers in the study groups. To assess the protective efficacy in *Aotus* monkeys, we compared the following parameters between immunized and control groups: 1) prepatent period, defined as the number of days from challenge to the first *P. vivax*-positive thick blood smear in every monkey; 2) parasitemia peak, defined as the maximum parasites/300 white blood cells (WBCs) in each monkey during the whole trial; 3) cumulative parasitemia, defined as the sum of a monkey's daily parasites/300 WBCs; and 4) the area under the parasitemia curve (AUC), defined as a summary measure of the magnitude and duration of the parasit-

emia. These last two parameters were determined for two different periods: 1) the time from the first to last positive thick blood smear in all monkeys (parasitemia clearance period) and 2) the time from first positive thick blood smear to first curative treatment in the whole trial (pretreatment period). We also compared the hematologic parameters (hemoglobin and hematocrit) in terms of the slope (m) during these same periods. We used the Mann-Whitney U test to compare all parameters between control and immunized groups. All data were analyzed with Stata version 8.3 software (Stata Corporation, College Station, TX).

**Ethical guidelines.** The study protocol was reviewed and approved by the institutional review board of Universidad del Valle, and written informed consent was obtained from all human adult participants and from parents or legal guardians of minors donors before plasma samples were taken. Animal research was approved by the Animal Ethical Committee of Universidad del Valle, and animals were housed and handled following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## RESULTS

**Sequence homology of Pf190L and Pv200L.** Sequence analysis of Pf190L from seven complete PfMSP-1 sequences available at the Swissprot database confirmed that it is a highly conserved fragment located near the N-terminal region, in the 83-kD fragment, which comprises amino acids 174–350 of the *P. falciparum* Palo Alto strain sequence. Standard BLASTp results for a 190-residue consensus sequence of Pf190L (Pf190Lcons) showed the existence of a region with significant homology scores ( $e < 0.01$  and identity/similarity > 30%) in most PvMSP-1 sequences reported at the NCBI database Entrez Nucleotide (Figure 1). The same analysis, using a Blosum 80 matrix, showed two major homologous fragments corresponding to amino acids 1–125 and 140–190 with similarity scores ranging from 66% to 72% (Figure 1). We also found that Pf190Lcons aligned with part of the high-binding region I (HBRI), which is a region with high-binding activity for reticulocytes.<sup>29</sup>

Clustal X analyses of 42 PvMSP-1 complete sequences available in the Entrez protein database indicated the alignment of the Pf190Lcons between amino acids 75/77 and 330/340 of the different PvMSP-1 sequences. A BLASTp analysis displayed a central stretch of high size and sequence polymorphism (Figure 1). A consensus sequence of the homologous region (281 characters) with a central string below 50% of conservation was obtained. Figure 1 shows a dot plot alignment of the two consensus sequences using the Emboss tool Dotmatcher. The defined homologous sequence was termed Pv200L by analogy to Pf190L.

**Cloning, expression, and purification of rPv200L.** We expressed Pv200L as a recombinant protein in *E. coli*. The PCR primers were designed based on the *pvmsp1* sequence of Salvador I strain to obtain the Pf190L homologous region plus a major section part of the HBR-I region (Figure 2).<sup>29</sup> The *pv200L* gene fragment ( $\pm 1$  kB) was amplified by PCR using genomic DNA obtained from a *P. vivax*-infected patient, cloned in pGEM, subcloned in pRSET, and expressed in *E. coli* BL21(DE3)-RIL Codon-Plus. Differential expression patterns and immunoblots using an anti-His monoclonal antibody showed that the B open reading frame of pRSET al-



Query	Pf190Lcons
Captured sequences of PvMSP-1	81
<b>e ranges:</b>	
Global (1-190)	2e-41 – 4e-44
Fragment A (1-125)	4e-38 – 8e-39
Fragment B (140-190)	8e-06 – 3e-09
<b>Identity ranges:</b>	
Global (1-190)	37 – 40%
Fragment A (1-125)	52 – 54 %
Fragment B (140-190)	43 – 50 %
<b>Similarity ranges:</b>	
Global (1-190)	54 – 56%
Fragment A (1-125)	72 – 76%
Fragment B (140-190)	66 – 71%

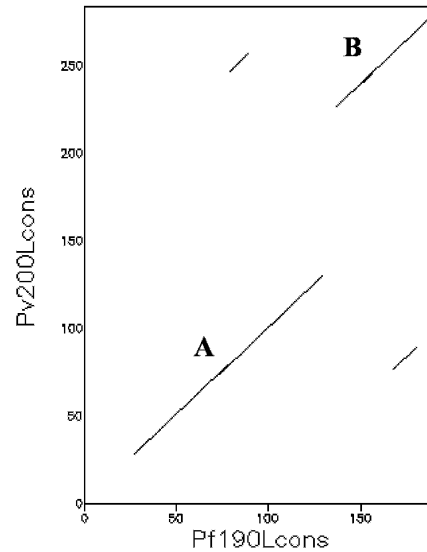


FIGURE 1. Summary (left) of BLAST/p results using a *Plasmodium falciparum* 190L consensus sequence (Pf190Lcons) as a query at the Entrez protein database (December 2004). The dot plot (right) shows the similarity of fragments A and B between Pf190Lcons and *P. vivax* 200Lcons. PvMSP-1 = *P. vivax* merozoite surface protein 1.

lowed the expression of a His-tagged protein with a molecular mass of ± 50 kD. Sequence analysis showed 99% identity with the already annotated PvMSP-1 sequence of the isolate BR44 from Brazil, with only two mutations: S/F at amino acid 84 and H/N at amino acid 106 (Figure 2).<sup>30</sup>

Optimal expression conditions were 2 mM IPTG for six

hours after bacteria reached an OD<sub>600 nm</sub> ~0.600. Since protein minipreps showed that rPv200L was forming inclusion bodies, combined cryofracture and urea-denaturing methods were required to prepare bacteria lysates, with optimal elution obtained using 100 mM imidazol. With these conditions, we established a scale production (two liters) system to pro-

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Sal1      ASANNNNNQVSVLTSKIRNFLSKFLELQIPGHTDLLHLIRELAVEPNGIKYLVSSEYEEF 118
Belem    --ANNNNNNQVSVLTSKIRNFVVGKFLLELQIPGHTDLLHLIRELAFEPNGIKYLVSSEYEEF 120
rPv200L  -----ITIFPSVLTSKIRNFVVGKSLLELQIPGHTDLLHLIRELAFEPHGIKYLVSSEYEEF
HBRIcons -----
Sal1      NQLMHVINFHVDLLRAKLHDMCAHDYCKIPEHLKISDKELDMLKKVVLGYRKPDLNPKDD 178
Belem    NQLMHVINFHVDLLRAKLHDMCAHDYCKIPEHLKISDKELDMLKKVVLGYRKPDLNPKDD 180
rPv200L  NQLMHVINFHVDLLRAKLHDMCAHDYCKIPEHLKISDKELDMLKKVVLGYRKPDLNPKDD
HBRIcons -----
Sal1      IGKLETFITKNKITIKNISDLIIAENKKRSGHPTTTTNGAGTQPANGSIAAASSETTQIS 238
Belem    IGKLETFITKNKETISNINKLISDENAKRGGQSTNTTNGTGAQNN----AAQGST----- 231
rPv200L  IGKLEAFITKNKETISNINKLISDENAKRGSQSTNTTNGTGAQNN----AAQGST-----
HBRIcons -----
Sal1      GSSNSGSSSTGSSNSGSSSTGSSSGTGTGTGQSPPAADASSTNANYEAKKIIYQAVYNT 298
Belem    -----GNTETGTTRSSASNTLSGGDGTTVVGTSSPAPAAPSSTNEDYDEKKKIYQAMYNG 286
rPv200L  -----GNTETGTQSSASNTLSGGAGTTVVGTSSPAPAAPSSTNANYEAKKIIYQAIYNG
HBRIcons -----
Sal1      IFYTNQLQEAQKLIQVLEKRVKVLKEHKDIKVLLEQVAKEKEKLPDYDPTNTLNTN-VHK 357
Belem    IFYTSQLEEAQKLIQVLEKRVKVLKEHKGIKALLEQVEAEKPKDNTTNTPLTDEQQK 346
rPv200L  IFYTNQLQEAQKLIQVLEKRVKVLKEHKGIKALLEQVEAEKPKDNTTNTPLTDEQQK
HBRIcons IFYTSQLEEAQKLIQVLEKRVKVLKEHKKIKXLLQVXXEKEKLPXDNXXXTXLTDXXXX
Sal1      EAESKIAELEKKEIAIAKTVNFDLDGLFTDAEELEYLREKAKMAGTLIIPESTKSAGTP 417
Belem    AAQKKIADLESQIVANAKTVNFDLDGLFTDAEELEYLREKAKMAGTLIIPESTKSAGTP 406
rPv200L  AAQKKIADLESQIVIAIAKTVNFDLDGLFTDAEELEYLREKAKMAGTLIIPESTKSAGTP
HBRIcons XAEXKIADLEKXIXIAIAKTVNFDLDGLFTDAEELEYLREKAKMAGTLIIPESTKSAGTP
Sal1      GKTVP TLKETYPHGISYALAENS IYELIEKIGSDETFGDL 457
Belem    GKTVP TLKETYPHGISYALAENS IYELIEKIGSDETFGDL 446
rPv200L  GKTVP TLKETYPH-----
HBRIcons GKTVP TLKETYPHGISYALAENS IYELIEKIGSDETFGDL
    
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FIGURE 2. Amino acid sequence alignment showing the *Plasmodium vivax* 200L subunit (rPv200L) expressed as a recombinant protein (bold) and its corresponding locations in Pv merozoite surface protein 1 from Belem (amino acids 69–430) and Sal-I (amino acids 71–419). Sequence homologous to *P. falciparum* 190L is indicated in **italic bold** type. The high-binding region I (HBRI) is indicated by underlining. HBRIcons is the consensus of the five HBRI regions annotated in the Entrez nucleotide database (AAC32532, AAC3253, AAC32534, AAC32535, and AAC32536).

duce enough protein for antigenicity and immunogenicity studies. Dialysis and IMAC cycles were repeated as needed until we obtained 200 mg of rPv200L that was 90% homogeneous and with an endotoxin content < 1,000 EU per 50  $\mu$ g of recombinant protein (Figure 3A). Silver staining of SDS-polyacrylamide gels showed additional protein populations in the rPv200L preparations that did not account for more than 10% of the total protein content, as shown by analytical reverse phase HPLC (Figure 3B).

**Antigenicity of rPv200L.** As shown in Figure 4A, rPv200L was specifically recognized at levels as low as 50 ng of protein by human IgG antibodies that displayed a positive IFAT reaction to *P. vivax* blood stages. Negative human control sera did not recognize the protein (Figure 4A). Of the 194 human plasma samples studied, 81 corresponded to infected patients and 69 to previously exposed individuals. The control group, which was obtained from malaria-naive volunteers in Cali, included 44 sera samples.

We found a significantly higher number of IgG responders (59) to rPv200L in the *P. vivax*-infected group than in the exposed and control groups ( $P < 0.001$ ) (Table 1). Similarly, the number of IgG responders in the exposed group (36) was significantly higher than that in the control group (3) ( $P < 0.001$ ). Using a multiple linear regression analysis, we found that the infected individuals had a significantly higher OD mean than did those in the exposed and control groups ( $P < 0.001$ ). However, the OD mean of the exposed group was not significantly higher than the OD mean in the control group ( $P = 0.837$ ). Figure 4B shows OD values for the infected and exposed groups according to age; the highest values were found in individuals approximately 20 and 50 years old.

**Immunogenicity in mice.** Mice immunized with the protein developed specific IgG antibodies to titers  $>10^6$  as deter-

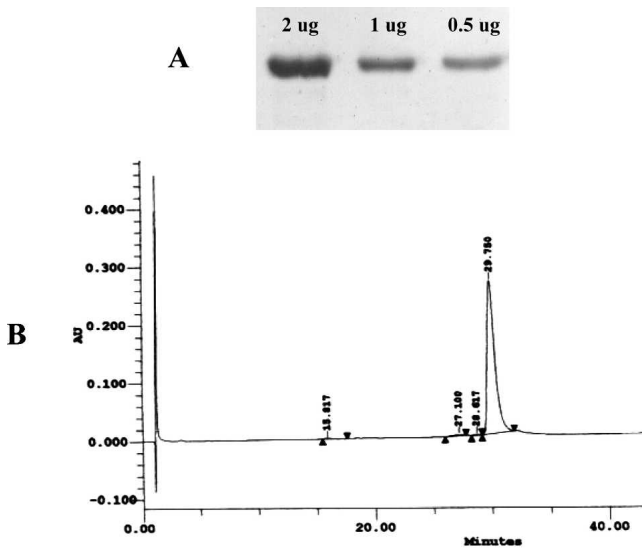


FIGURE 3. Recombinant *P. vivax* 200L (rPv200L) corresponding to a homogeneous product of approximately 50 kD with other protein bands that are less than 10% of the total protein content. **A**, Analysis of bench-quality batches of rPv200L analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue. **B**, Analysis by high-performance liquid chromatography showing the homogeneity of rPv200L preparations. AU = absorbance units.

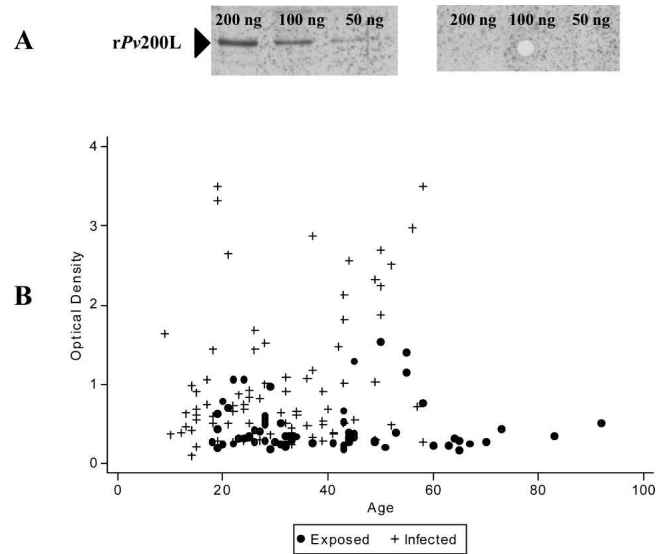


FIGURE 4. Humoral immune response to recombinant *P. vivax* 200L (rPv200L) in individuals from the malaria-endemic area. **A**, Immunoblot of rPv200L using sera from infected individuals (left) or sera from malaria-naive individuals (right). **B**, Distribution of optical density values against rPv200L according to age.

mined by ELISA (Figure 5A). These antibodies were able to recognize the recombinant protein rPv200L (Figure 5B) and a recombinant *Pf190L* (Figure 5C) by immunoblot. Importantly, sera of immunized mice were able to recognize *P. vivax* mature schizonts by IFAT with antibodies titers ranged from 1:200 to 1:3,200 dilution serum (Figure 6A–C). Although *Pf190L* was recognized by immunoblot, we could not detect cross-reactivity with mature *P. falciparum* blood schizonts. Sera of mice in the control group did not recognize the recombinant protein either by ELISA or immunoblot.

**Immunogenicity and protective efficacy in Aotus monkeys.** Monkeys immunized with rPv200L formulated in Freund's adjuvant developed specific IgG antibodies to the recombinant protein in titers  $> 10^6$  as determined by ELISA (Table 2). These antibodies cross-reacted with mature *P. vivax* schizonts by IFAT with titers up to a serum dilution of 1:2,000 (Figure 6D–I). Preliminary assays demonstrated that PBMCs from immunized monkeys produce interferon- $\gamma$  (IFN- $\gamma$ ) *in vitro* upon stimulation with the recombinant Pv200L protein. Two animals from the experimental group and two from the control group died during the immunization period of causes unrelated to the immunization procedure or the immunogen preparation. Three died of aseptic pneumonia and one of a cardiovascular accident.

TABLE 1  
IgG antibody response to rPv200L\*

Group	No. of samples	% IgG responders (exact 95% CI)†	OD mean (95% CI)	Antibody titers
Infected	81	72.8 (61.8–82.1)	1.015 (0.828–1.203)	$1 \times 10^3 - 5 \times 10^5$
Exposed	69	52.2 (39.8–64.3)	0.464 (0.363–0.506)	$1 \times 10^2 - 1 \times 10^4$
Control	44	6.8 (1.40–18.1)	0.264 (0.246–0.368)	–

\* OD = optical density.

† Exact 95% confidence interval (CI) based on a binomial distribution.

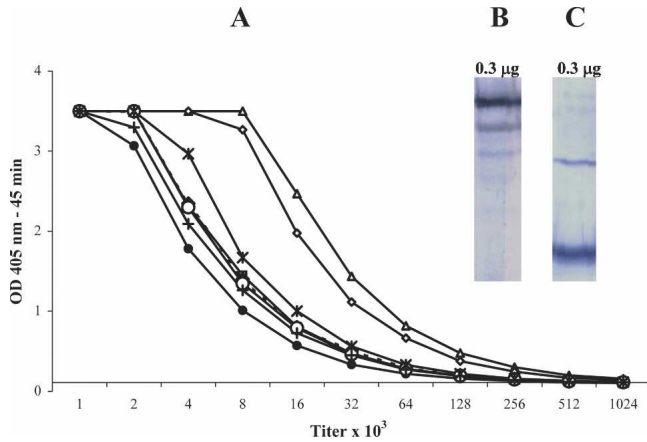


FIGURE 5. Immunogenicity of recombinant *P. vivax* 200L (rPv200L) in BALB/c mice. **A**, Titration of the IgG antibody response to rPv200L in immunized mice. OD = optical density. **B**, Immunoblot of rPv200L with sera from immunized mice. **C**, Immunoblot of a recombinant version of *P. falciparum* 190L with sera from rPv200L-immunized mice. min = minutes.

All monkeys from the experimental and control groups developed patent parasitemia after challenge (Figure 7), with similar ranges of prepatent periods: 14–22 days versus 14–20 days, respectively. However, the parasitemia peak was lower in the experimental group, with a median of 39.5 parasites/300 WBCs (range = 2–47) than in the control group, which had a median of 68.5 parasites/300 WBCs (range = 13–132). In general, immunized animals had lower cumulative parasitemia and AUC medians, either for the parasitemia clearance

TABLE 2  
Parameters determined in the protective efficacy trial\*

Group	Monkey code	Antibody titer		CP		AUC	
		ELISA	IFAT	Pcl	Ptx	Pcl	Ptx
C	292*788	< 1,000	Negative	575	562	840	800
C	875*319	< 1,000	Negative	201	179	280	232
C	886*541	< 1,000	Negative	90	36	121	40
C	884*081	< 1,000	Negative	425	400	586	537
I	894*582	$2 \times 10^7$	2,200	19	11	25	13
I	882*849	$2 \times 10^7$	2,000	235	95	339	93
I	292*308	$2 \times 10^7$	2,400	259	240	353	292
I	382*876	$2 \times 10^7$	2,600	231	210	312	266

\* ELISA = enzyme-linked immunosorbent assay (titer before challenge); IFAT = immunofluorescent antibody test; CP = cumulative parasitemia; Pcl = parasitemia clearance period; Ptx = pretreatment period; AUC = area under the curve; C = control; I = immunized.

period or for the pretreatment period (Table 2). However, none of the parasitic parameters showed statistically significant differences between the immunized and control groups. We also observed a tendency in the monkeys to have lower levels of parasitemia expressed AUC at higher OD values in the ELISA.

Three of four monkeys in the control group had to be treated with curative doses of sulfadoxine-pyrimethamine (on days 169, 175, and 176) because of severe anemia (hemoglobin level < 7.0 mg/dL and/or hematocrit < 20%), whereas all animals from the immunized group recovered spontaneously (Table 2).

DISCUSSION

In the search for *P. vivax* malaria parasite subunits with vaccine potential, we focused efforts toward the N-terminal region of *P. vivax* MSP-1. There we identified a fragment that had significant sequence homology to the *P. falciparum* Pf190L subunit vaccine candidate and also contained part of the PvMSP-1 HBRI domain. We called this fragment Pv200L and collected evidence of its immunologic relevance as a vaccine candidate.

First, Pv200L has shown a high antigenicity as determined by the level of naturally induced IgG to rPv200L in individuals from malaria endemic areas. Both patients carrying active *P. vivax* infections and asymptomatic individuals previously exposed to *P. vivax* infection frequently had antibodies to the recombinant protein. These results are in agreement with those from previous seroepidemiologic studies conducted in Brazil using longer fragments from the N-terminal region of PvMSP-1.<sup>31,32</sup> It was striking that actively infected individuals had circulating antibodies more frequently (> 70%) than did individuals without infection at the time of the anti-rPv200L ELISA (> 50%). This differs from the normal finding in *P. falciparum* where the parasite blood infection appears to induce a transient suppression of the immune responses.<sup>33–35</sup> The higher antibody responses we found in infected patients may correspond to an early immunologic boosting by parasites after natural infection and may provide advantages for boosting of antibody responses induced by vaccination.

Second, Pv200L displayed a high immunogenicity in mice and monkeys. The rPv200L formulated in Freund's adjuvant was highly immunogenic in BALB/c mice, inducing unexpectedly high titers of IgG antibodies (> 10<sup>6</sup>) in the immunized mice, which not only recognized the rPv200L but also cross-

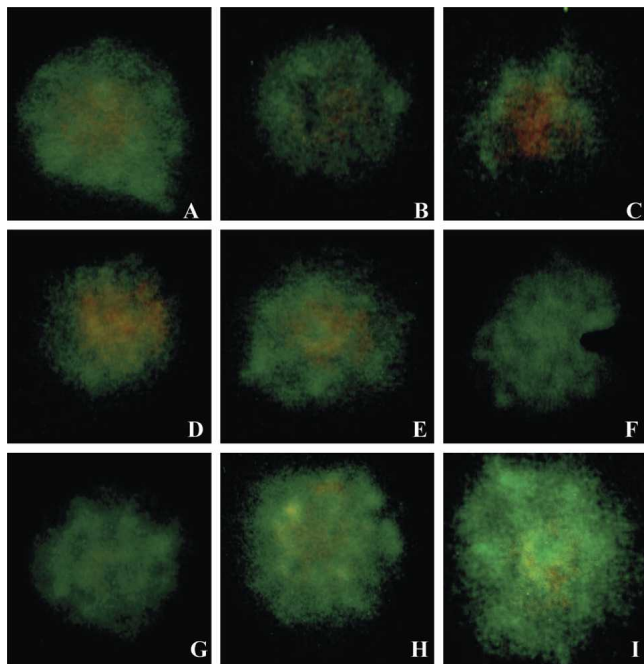


FIGURE 6. Immunofluorescent antibody test results of *Plasmodium vivax* schizonts exposed to IgG antibodies to recombinant *P. vivax* 200L raised in BALB/c mice (**A–C**) and *Aotus* monkeys (**D–I**). Slides were obtained after short-term *in vitro* cultures and the results correspond to a 1:200 dilution of a pool of serum from mice and monkey experimental groups, respectively.

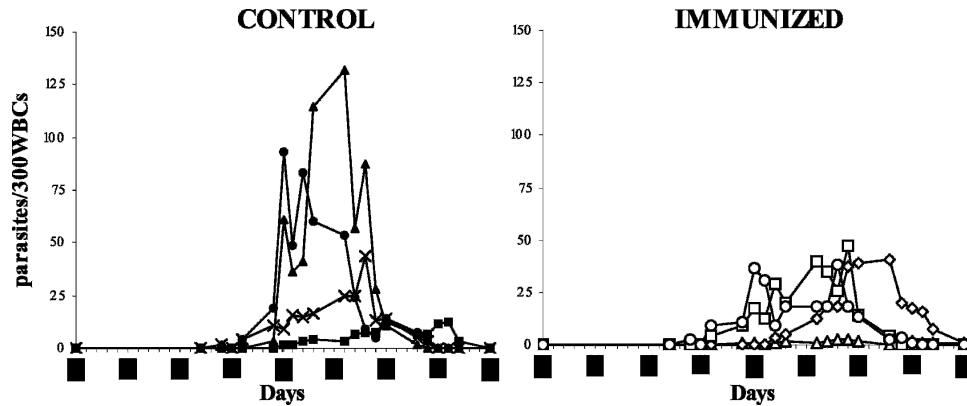


FIGURE 7. Parasitemia (parasites/300 white blood cells [WBCs]) in control monkeys (left) and monkeys immunized with recombinant *P. vivax* 200L (right) after challenge with *P. vivax* (Sal 1). The follow-up period was 40 days.

reacted with mature *P. vivax* blood schizonts. Similarly, the protein was highly immunogenic in *Aotus* monkeys, where it induced antibody titers  $>10^6$ , which are not usual in this experimental model. As in the mice, these antibodies cross-reacted with *P. vivax* schizonts.

Third, immunization induced partial protection of *Aotus* monkeys against experimental heterologous challenge with *P. vivax* (Salvador I) blood stages. Although the number of animals was limited (four per group), we observed protective tendencies in the experimental group in terms of parasitemia peak, cumulative parasitemia, and AUC. Additionally, the fact that none of the monkeys in the immunized group had to be curatively treated, whereas three of four animals among the controls had to be treated because severe anemia had developed, supports the induction of anti-disease immunity. Additionally, the strength of the antibody response in monkeys seems to be associated with protective efficacy. However, we believe that this protection is the result of both antibody and T cell responses, e.g., the production of protective cytokines such as IFN- $\gamma$ . Preliminary analyses indicated that PBMCs from immunized monkeys release IFN- $\gamma$  upon stimulation with the recombinant protein. We have previously shown that better protection is achieved in *Aotus* monkeys immunized with *Pf190L*, which simultaneously produce better antibody and IFN- $\gamma$  responses.<sup>36</sup>

We consider the heterologous challenge a more similar scenario for a real vaccine in the field where the circulating parasites will contain a certain degree of heterogeneity to the immunization variant. Our interpretation is that if it is possible to confer partial protection with a divergent variant of Pv200L, it is likely that a better protective efficacy could be achieved with more related variants. Relevant protective B and T cell epitopes may be spread through both polymorphic and conserved regions and the overall protection might be different depending on the degree of homology between the parasite 200L sequence and that of the vaccine. Additionally, the protein expression in *E. coli* may have failed to completely reproduce the native conformation and this would induce the production of immune responses without protective effect.

We propose that this protein fragment has similar features to those of *Pf190L*, which has long been considered a major subunit vaccine candidate for *P. falciparum* malaria.<sup>9,16,17</sup> *Pf190L* has been shown to contain a domain that binds to the

human erythrocyte spectrin.<sup>37</sup> The sequence homology found between the *Pf190L* subunit and *Pv200L* ( $> 66\%$  for fragments A and B) and the existence of the HRBI region, which is considered a *P. vivax* merozoite ligand for reticulocyte binding and invasion,<sup>29</sup> suggest that these are homologous regions with similar functions in both parasites (Figures 1 and 2). In addition to the sequence homology between the two protein fragments, the cross-recognition of the recombinant *Pf190L* fragment by antibodies to *Pv200L* elicited in BALB/c mice indicate sharing of B epitopes of the two MSP-1 proteins (Figure 5).

In conclusion, the results presented here provide evidence that rPv200L is a novel potential *P. vivax* subunit vaccine target, with similar features to the leading vaccine candidates to *P. vivax* malaria (i.e., MSP1-19 or Duffy binding protein [DBP]). We could not claim significantly better protection with this protein fragment compared with that achieved with these other *P. vivax* proteins. However, we have conducted preclinical studies using DBP-RII and the level of protection achieved by vaccination with this protein was moderate compared with that reported herein. (Arevalo-Herrera and others, in this supplement). Moreover, we are certain that no solid protection can be achieved with a single parasite protein. We are now optimizing our protein expression and purification systems and planning studies to immunize larger numbers of animals with rPv200L formulated in adjuvants eligible for human use that would be challenged with both homologous and heterologous parasite strains.

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