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Expression and Serologic Activity of a Soluble Recombinant *Plasmodium vivax* Duffy Binding Protein

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***Plasmodium vivax* Duffy binding protein (DBP) is a conserved functionally important protein. *P. vivax* DBP is an asexual blood-stage malaria vaccine candidate because adhesion of *P. vivax* DBP to its erythrocyte receptor is essential for the parasite to continue development in human blood. We developed a soluble recombinant protein of *P. vivax* DBP (rDBP) and examined serologic activity to it in residents of a region of high endemicity. This soluble rDBP product contained the cysteine-rich ligand domain and most of the contiguous proline-rich hydrophilic region. rDBP was expressed as a glutathione *S*-transferase (GST) fusion protein and was isolated from GST by thrombin treatment of the purified fusion protein bound on glutathione agarose beads. *P. vivax* rDBP was immunogenic in rabbits and induced antibodies that reacted with *P. vivax* and *Plasmodium knowlesi* merozoites. Human sera from adult residents of a region of Papua New Guinea where malaria is highly endemic or *P. vivax*-infected North American residents reacted with rDBP in an immunoblot and an enzyme-linked immunosorbent assay. The reactivity to reduced, denatured *P. vivax* rDBP and the cross-reactivity with *P. knowlesi* indicated the presence of immunogenic conserved linear B-cell epitopes. A more extensive serologic survey of Papua New Guinea residents showed that antibody response to *P. vivax* DBP is common and increases with age, suggesting a possible boosting of the antibody response in some by repeated exposure to *P. vivax*. A positive humoral response to *P. vivax* DBP correlated with a significantly higher response to *P. vivax* MSP-1_{19}. The natural immunogenicity of this DBP should strengthen its usefulness as a vaccine.**

The development of malaria in an individual infected with *Plasmodium* is dependent on the repeated cycles of asexual replication of the parasites in the blood. Each generation of merozoites must rapidly invade new blood cells to maintain the infection. Each merozoite must interact with a series of specific erythrocyte surface receptors to complete the invasion process (see reference 11). Blocking these receptor-ligand interactions offers a potentially effective mechanism to reduce or eliminate the blood-stage malaria parasites and hence prevent clinical malaria.

Plasmodium vivax Duffy binding protein (DBP) is a parasite-produced adhesion molecule that has an essential role in the invasion of human erythrocytes (4, 19, 31). *P. vivax* DBP is the prototype of a superfamily of adhesion proteins expressed in the blood stages of malaria parasites (24), including other erythrocyte-binding proteins (EBP) (e.g., *Plasmodium knowlesi* DBP and EBA-175) (2) and the *Plasmodium falciparum* variant surface antigens (e.g., *P. falciparum* EMP1) involved in cytoadherence of parasite-infected erythrocytes (5, 24, 28, 29). Conservation of a functionally important cysteine-rich domain (region II) is the principal characteristic of members of the superfamily. The EBP have the additional characteristics of (i) a similar gene structure, (ii) localization in the micronemes, and (iii) a second conserved carboxyl cysteine-rich domain (2). Because of their important biological and pathological roles in malaria, the EBP adhesion molecules are important candidates for asexual blood-stage malaria vaccines (22).

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P. vivax DBP is likely to be exposed on the merozoite surface during invasion, enabling it to bind its receptor but also making it accessible to serum antibodies. Evidence that DBP is the target of an effective immune response was suggested by hypervariability of amino acids in the critical ligand domain of the amino cysteine-rich erythrocyte-binding domain (3, 30). However, direct evidence for naturally occurring antibodies reactive to DBP from a malaria-exposed population has not yet been demonstrated. Availability of a purified form of DBP has been one of the major difficulties in assessing the immunological activity against the cysteine-rich erythrocyte-binding domain of this and other EBP. The purpose of this project was to express *P. vivax* DBP as a recombinant protein containing the cysteine-rich receptor binding domain. In this work, we have expressed this functionally important conserved merozoite protein as an immunogenic soluble recombinant protein and demonstrated its serologic reactivity with naturally occurring human serum antibodies from residents of a region where *P. vivax* malaria is highly endemic.

MATIERALS AND METHODS

Expression constructs *P. vivax* DBP. The putative full-length ectodomain of *P. vivax* DBP Sal-1 (M37514) (10), from amino acids 177 to 1050 (regions II to VI; $DBP_{II–VI}$), was inserted in frame with glutathione *S*-transferase (GST) in the plasmid expression vector pGEX-2T (27). This fragment is thought to correspond to the soluble form of the parasite-derived erythrocyte-binding protein (31). The DNA encoding the DBP ectodomain was PCR amplified from the original cloned genomic fragment (5′ primer, AAGAGCTCGGATCCGGAGA ACATAAAACTGATAGT; 3′ primer, ACCCAAGCTTCACTTTAAAGCCCC CTTTCGTAAAGC). The polylinker of the expression plasmid pGEX-2T was modified to include a *Hin*dIII site, and the PCR-amplified product was cleaved with the appropriate restriction enzymes and cloned into the *Bam*HI site at the 5′ end and the *Hin*dIII site at the 3′ end. This full-length ectodomain was

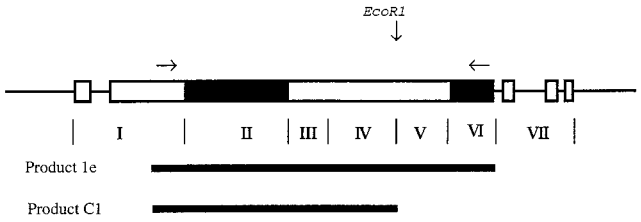

FIG. 1. Expression of the *P. vivax* DBP Sal-1 (10) as a soluble recombinant protein. The exons of the DBP gene are boxed, and the black areas identify the cysteine-rich regions. The structurally distinct regions of the EBP family products are shown under the diagram in Roman numerals (2). Region II, the amino cysteine-rich region, is the principal ligand or erythrocyte-binding domain (7). The putative ectodomain of the DBP, regions II to VI, was PCR amplified and cloned in frame with the gene for the GST protein of *S. japonicum* to generate the clone 1e; the protein expressed from this fragment was thought to correspond to the soluble form of the parasite-derived erythrocyte-binding protein, but the 1e product was poorly soluble and could not be readily purified. A second construct, C1, was derived from this full-length version by excision of the *Eco*RI fragment encoding regions V to VI. The C1 product, $rDBP_{II–IV}$, was purified as a soluble protein and used for subsequent immunologic assays.

designated product 1e (Fig. 1) and expressed fusion protein GST-*P. vivax* $DBP_{II–VI}$ (or GST-$DBP_{II–VI}$).

A truncated version of the DBP expression plasmid, from amino acids 177 to 815 (regions II to IV; $DBP_{II–IV}$), was derived directly from the expression plasmid containing the full-length ectodomain. The 1e pGEX plasmid was treated with *Eco*RI to remove the 3′ *Eco*RI fragment of the DBP by use of an internal restriction site and one in the plasmid cloning site, followed by self ligation of the plasmid. This truncated form of the DBP ectodomain was designated product C1 (Fig. 1) and expressed fusion protein GST-*P. vivax* $DBP_{II–IV}$ (or GST-$DBP_{II–IV}$). Both the 1e and C1 plasmids were used to transform *Escherichia coli* DH5α (Bethesda Research Laboratories). Clones for each were isolated and selected based upon their ability to express a recombinant fusion protein of approximately the correct molecular weight. The open reading frame of each expression construct was sequenced to verify that the DBP gene segment was correctly inserted into the GST open reading frame to express DBP as a fusion protein.

Recombinant protein expression and purification. The GST-DBP recombinant proteins were expressed by use of the recommended protocol with only minor modifications (27). A single colony was grown overnight in LB medium with 100 μg of ampicillin per ml (LB-A) at 37°C, diluted 1:10 in LB-A, and incubated for 2 to 3 h at 37°C. Expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 0.1 mM, the medium was incubated for 2 to 3 h, and the cells were harvested by centrifugation. The bacteria were resuspended in 1/20 volume of phosphate-buffered saline (PBS) and sonicated three times for 1.5 to 2 min each time. Triton X-100 was added to 1%, the lysate was incubated for 30 min with agitation and centrifuged for 10 min at 12,000 × *g*, and the supernatant was incubated in a 50:1 dilution with reduced glutathione agarose CL-4B beads (Fluka). The standard method to isolate GST fusion proteins was followed. The beads with adsorbed fusion protein were washed three times in ≥10 volumes of PBS, removing all liquid after the final wash, and the recombinant proteins were eluted into the supernatant with three sequential treatments with 10 mM reduced glutathione–50 mM Tris (pH 8.0). To isolate purified DBP separate from the GST component, the beads were washed as described above and the recombinant protein was released into the supernatant by treatment with 10 cleavage units of thrombin (Sigma) for 3 to 5 h at room temperature. The standard method to purify GST fusion proteins and isolate the product by thrombin cleavage worked well for the truncated form, GST-$DBP_{II–IV}$, but not for the full-length ectodomain, GST-$DBP_{II–VI}$. The full-length ectodomain product remained bound to the beads even after thrombin cleavage or with the addition of chaotropic agents or high-ionic-strength solutions.

Expression and purification of recombinant *P. vivax* MSP-1_{19} (rMSP-1_{19}) was described previously (18). Briefly, The 19-kDa carboxyl terminus of *P. vivax* MSP-1 containing the two epidermal growth factor-like domains (residues Leu_{1639} to Ser_{1729}) was expressed in the yeast *Saccharomyces cerevisiae*. The rMSP-1_{19} was secreted into the culture supernatant and purified on nickel-nitriloacetic acid agarose.

Antisera. Rabbits (New Zealand White) were immunized with purified recombinant proteins by use of approved protocols. The C1 protein, GST–$DBP_{II–IV}$, 100 μg in solution, was emulsified with Freund's complete adjuvant for the first injection and vaccinated subcutaneously. To boost the antibody response, the antigen was emulsified in incomplete Freund's adjuvant and rabbits were vaccinated subcutaneously at 2-week intervals, five times for the C1 fusion protein and six times for the 1e fusion protein. Ten days after the last immunization, serum was collected from clotted blood, decomplemented, aliquoted, and stored at −20 or −80°C. Because the full-length ectodomain 1e protein remained adsorbed to the glutathione beads, the immunization protocol was modified so that the animal was injected with an emulsion of a suspension containing antigen-adsorbed beads. Anti-GST serum was prepared against a nonfusion GST protein produced by expression of nonrecombinant pGEX-2T.

Human plasma samples from *P. vivax*-exposed patients were collected among residents of Madang, Papua New Guinea (PNG). A preliminary sample collected from 13 healthy adults in the Madang area were used for an initial immunoblot assay and to establish the conditions for the enzyme-linked immunosorbent assay (ELISA). A second set of 100 previously age-grouped serum samples (subject age range, 0 to 70 years) from the Madang area were then screened only by ELISA. These samples were collected from July to September 1982 (dry season) (6) and were subsequently kept in long-term storage (−70°C) at the Queensland Institute of Medical Research; the samples were age grouped and kindly made available for our purposes by Allan Saul.

A sample of positive pooled sera was obtained from North American residents that were diagnosed as having only *P. vivax* infections; this serum sample reacted (by immunofluorescence assay [IFA]) with *P. vivax* only. It was kindly provided by W. Collins, Malaria Branch, Centers for Disease Control and Prevention, Chamblee, Ga. Presumably, most of these patients were infected with *P. vivax* malaria while traveling in regions outside of the United States where malaria is endemic and did not have chronic exposure. Fifteen serum samples from North American residents with no history of malaria and that had never traveled to areas where malaria is endemic were used as negative controls (NA-Neg).

Serum from a rhesus monkey (no. 626) immune to *P. knowlesi* and reactive with the *P. knowlesi* DBP was also used (1). This serum was a collection of samples pooled over several months from an animal that had been infected several times with different strains of *P. knowlesi* and treated by chemotherapy (20).

Electrophoresis and immunoblotting. Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with reducing sample buffer was used to analyze the recombinant proteins. Proteins were separated on running gels with 8% acrylamide; prestained molecular weight standards (Bethesda Research Laboratories) were included on each gel to estimate relative molecular weights. For immunoblotting, proteins separated by SDS-PAGE were electroblotted onto nitrocellulose membranes in the presence of 15% methanol. For antigen detection, membranes were blocked with 1% gelatin (Sigma) in 0.05% Tween 20–PBS (TPBS), washed in TPBS, incubated for 1 h with a 1:100 dilution of primary serum in TPBS, washed in TPBS, incubated for 1 h with a 1:7,500 dilution of anti-immunoglobulin G (IgG) alkaline phosphatase conjugate (Promega) in TPBS, washed in TPBS, and developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate.

Immunofluorescence microscopy. Thin blood films of cultured *P. vivax* and *P. knowlesi* schizonts were prepared as described previously (1, 12) and stored desiccated at −80°C. Unless noted otherwise, all incubations were done at 23°C. The *P. vivax* IFA was done on air-dried unfixed thin smears of infected blood that had wells formed from fingernail polish lines. The primary antibody was incubated with recombinant GST (100 μg of antiserum diluted 1:50 per ml) and *E. coli* extract for 30 min prior to use, diluted 1:200 in PBS, and incubated in a humidified box at room temperature for 1.5 h. The slide was then washed three times by standing in a Coplin jar filled with PBS–0.2% bovine serum albumin for 5 min for each wash. The secondary antibody was affinity-purified goat anti-rabbit IgG(FC) conjugated to fluorescein isothiocyanate (Kirkegaard & Perry Laboratories), incubated for 1.5 h as described above, washed three times as before, and then air dried. A coverslip was then mounted with antifade in 50% glycerol and viewed by epifluorescence. The *P. knowlesi* IFA was done on air-dried thin films fixed for 5 min in 1% formaldehyde diluted in PBS, rinsed in PBS, and incubated for 5 min in block buffer (0.1% Triton X-100 and 2.5 mg of normal goat serum per ml, in PBS) and then for 60 min in a humidified chamber at 37°C with primary antibody, diluted 1:200 in block buffer. The slides were then washed three times, for 5 min each time, in block buffer and incubated for 60 min, in the dark, with fluorochrome-labeled secondary antibodies (fluoroscein-conjugated goat anti-rabbit IgG; Kirkegaard & Perry Laboratories) diluted 1:10 in PBS. The slides were washed three times, for 5 min each time, in block buffer, mounted in 80% glycerol–0.1% *p*-phenylenediamine–10 mM Tris (pH 7.4), and then viewed on a MRC-1024 laser scanning confocal imaging system (Bio-Rad) under a 10% laser with a Kalman filter.

ELISA to *P. vivax* DBP and MSP_{19}. The optimal antigen concentration to coat the wells and the dilution of the primary and secondary antibodies were determined empirically by cross-titration; at the concentrations used, all of the initial test sera generated a negligible optical density (OD) when reacted with microtiter plates coated with GST (negative control antigen) in place of the purified *P. vivax* $rDBP_{II–IV}$. Purified thrombin-cleaved $rDBP_{II–IV}$ diluted to 2 μg/ml in PBS was adsorbed overnight at 4°C onto microtiter ELISA plates (Immulon I; Dynatech), rinsed in wash buffer (0.2% Tween 20 in PBS), incubated for 15 min with block buffer (1% BSA in PBS), rinsed in wash buffer, and used immediately or stored sealed at 4°C until used. After rinsing, the antigen-coated wells were incubated for 90 min with triplicate serum samples diluted 1:400 in block buffer, the plates were rinsed in wash buffer, incubated for 90 min with secondary antibody (goat anti-IgG [heavy plus light chains] human alkaline phosphatase conjugate) diluted to 0.5 μg/ml in block buffer, and rinsed in wash buffer, and to each well, *p*-nitrophenolphosphate was added. The anti-*P. vivax* DBP antibody activity was detected by recording the absorbance (OD) at 405 nm; the OD of blank wells with substrate alone was subtracted from all values. The average absorbance and standard deviation were calculated for each serum sample. The

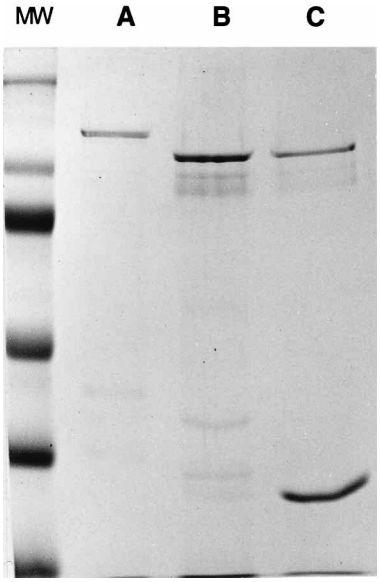

FIG. 2. $rDBP_{II–IV}$ was expressed as a soluble GST fusion protein of 140 kDa. This product was purified on and eluted from glutathione agarose beads (lane A). Thrombin treatment of the purified fusion protein on the glutathione beads released most of the $rDBP_{II–IV}$ as a relatively pure soluble protein into the thrombin treatment buffer (lane B). However, some cleaved DBP remained adsorbed on the glutathione beads along with the GST after thrombin treatment and was released only in the SDS-PAGE sample buffer (lane C). Sizes of the recombinant proteins were determined in reference to known molecular weight markers (MW; in descending order, 220,000 to 205,000, 105,000, 70,000, 43,000, 29,000, and 18,400).

average plus 2 standard deviations of 15 negative control serum samples was used as the negative cutoff. For the 100 age-grouped PNG samples, the ELISA was done as described above, with each sample tested in triplicate. Each ELISA plate contained serial dilutions of two separate pooled immune PNG plasma samples (16 and 10 individuals) for use as a standard curve reference.

Serological reactivities to *P. vivax* $rMSP_{19}$ were assayed for comparison. The ELISA was done under the same conditions and concentrations as those described above, but substituting $rMSP_{19}$ expressed in yeast as the antigen.

Data analysis for age-grouped samples. An antigen-specific antibody unit (AU) value was determined for each of the 100 test samples by plotting its average OD against that plate's standard curve of titrated pooled immune sera. A sample was considered positive when its AU value was greater than the mean AU value of the pooled NA-Neg plus 2 standard deviations. Statistical analysis was performed with the Systat 5 program for the Macintosh by using log-transformed values. The Spearman correlation was used for pairwise comparisons of the response to the two antigens and parasitemia. Mean antigen-specific antibody responses were compared between age groups (0 to <5, 5 to <10, 10 to <19, 20 to <40, ≥40) by analysis of variance with a Post Hoc to test for significance between age groups (Fisher's least significant difference and Tukey's test with age as a covariant within the age groups). One-way analysis of variance (Kruskal-Wallis) was used to test for the mean response to one antigen based upon the category of responsiveness to the other antigen (e.g., mean anti-MSP response of the anti-DBP positive responders). Statistical significance was defined as a P of <0.05.

RESULTS

rDBP expression. Regions II to IV of the *P. vivax* DBP (i.e., $DBP_{II–IV}$) were expressed as a soluble GST fusion protein of 150 kDa. This product was an abundant soluble protein in the cytosol 2 h after induction and was readily purified from lysed bacteria by use of glutathione agarose beads (Fig. 2, lane A). Thrombin treatment of the purified fusion protein on the beads released most of the $rDBP_{II–IV}$ as a relatively pure soluble protein of approximately 110 kDa (Fig. 2, lane B). Some $rDBP_{II–IV}$ remained associated with the GST on the beads (Fig. 2, lane C), even though the fusion protein was cleaved by the thrombin treatment. The purified protein remained stable when stored at −20°C.

The $rDBP_{II–IV}$ was derived from a GST-$DBP_{II–VI}$ construct by removal of an *Eco*RI fragment encoding the second (carboxyl) cysteine-rich domain of DBP regions V to VI. Removing this cysteine-rich region from the expressed rDBP converted a relatively insoluble fusion protein of 170 kDa (data not shown) into a soluble 150-kDa fusion protein. The toxicity of the full-length rDBP in bacteria appeared related to its insolubility. Neither rDBP product had specific erythrocyte binding activity (data not shown).

Generation of antibodies specific to *P. vivax* DBP. To assess the immunologic activity of the rDBP, rabbits were immunized with the recombinant fusion proteins emulsified with adjuvant. Both forms of the rDBP fusion protein were immunogenic and induced antibodies that reacted with rDBP and GST, but the anti-$rDBP_{II–VI}$ serum was considerably less reactive (data not shown). To determine whether either of these antibodies also reacted with epitopes of native *P. vivax* DBP, the sera from the immunized rabbits were screened for reactivity to blood-stage parasites of *P. vivax* and *P. knowlesi.*

The rabbit anti-$rDBP_{II–IV}$ serum reacted to merozoites in both *P. vivax* and *P. knowlesi* schizonts (Fig. 3A and B, respec-

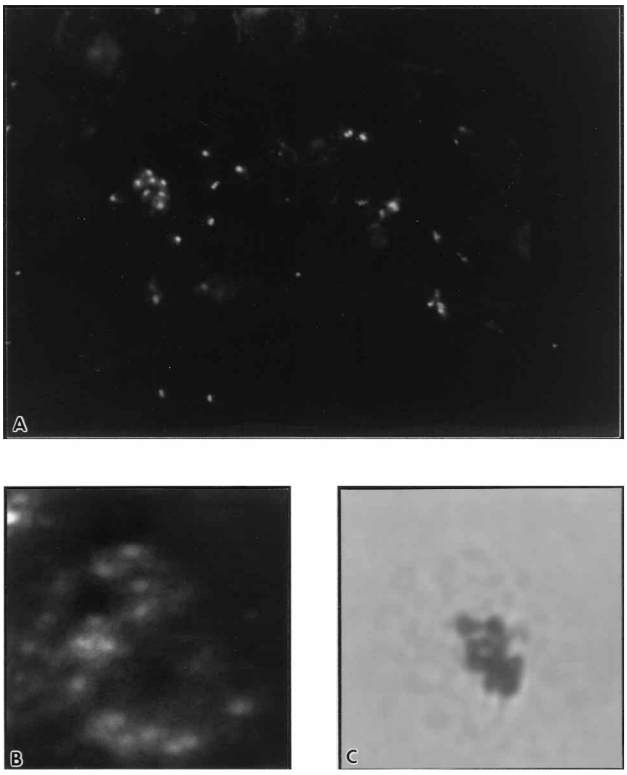

FIG. 3. Indirect immunofluorescence microscopy with anti-*P. vivax* $rDBP_{II–IV}$ sera reacted with smears of *P. vivax* and *P. knowlesi*. (A and B) Rabbit antiserum to $rDBP_{II–IV}$ was incubated with smears of blood-stage parasites matured in short-term cultures. In both *P. vivax* (A) and *P. knowlesi* (B), the anti-rDBP serum reactivity pattern produced an apical fluorescence pattern in mature schizonts and free merozoites. Immature schizonts and trophozoites showed a diffuse pattern of no immunofluorescence. This immunofluorescence pattern is similar to that observed with antiserum to the *P. knowlesi* DBP and *P. falciparum* EBA-175 reacted with *P. knowlesi* and *P. falciparum*, respectively (1, 25). (C) A nonconfocal transmission image of the mature *P. knowlesi* schizont demonstrates that the fluorescence pattern seen in panel B is located in the apical end of the merozoites pointed away from the residual pigment body. The *P. vivax* smears were viewed by epifluorescence microscopy and have an approximate final magnification of ×600. The *P. knowlesi* fluorescence was viewed by scanning confocal laser microscopy in 0.2-μm optical sections and has a final magnification of ca. ×3,000.

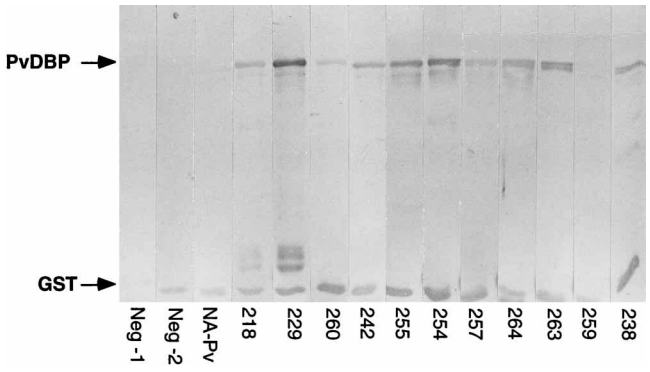

FIG. 4. Immunoblot of *P. vivax* DBP (PvDBP) reacted with human sera. Purified, thrombin-cleaved *P. vivax* $rDBP_{II–IV}$ and GST were separated by SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose. The blot was blocked and reacted with human serum samples from PNG adults (numbered samples), pooled sera from North American residents exposed to *P. vivax* malaria (NA-Pv), or North American residents not exposed to *P. vivax* malaria (Neg-1 and Neg-2). The size of the $rDBP_{II–IV}$ was 110 kDa and was estimated by comparison with known molecular weight standards as described in the legend to Fig. 2. The NA-Neg produced a result similar to that of the individual serum samples Neg-1 and Neg-2.

tively). There was a discrete punctate, apical fluorescence pattern in both parasites that was consistent with the microneme localization of the mature native protein. The rabbit anti-$rDBP_{II–VI}$ serum reacted more weakly to merozoites in *P. vivax* schizonts than the rabbit anti-$rDBP_{II–IV}$ did and did not react with *P. knowlesi* schizonts (data not shown). Preimmune rabbit sera did not react with either parasite.

Reactivity of human sera to rDBP and MSP-1_{19}. Sera and plasma from individuals exposed to *P. vivax* were used to determine whether the rDBP had epitopes reactive with antibodies induced from a naturally occurring infection. For the initial analysis, plasma was collected at random from a small sample of adults living in Madang, PNG, a region with year-round mosquito transmission where *P. vivax* malaria is highly endemic. Additional sera used for these assays were from North American residents and included pooled sera from patients diagnosed with *P. vivax* and serum from individuals with no exposure to malaria. Each serum sample was incubated separately against purified, thrombin-cleaved $rDBP_{II–IV}$ that was size fractionated by SDS-PAGE and blotted onto nitrocellulose. *P. vivax*-exposed individuals had antibodies that reacted with $rDBP_{II–IV}$, but sera from the unexposed donors did not (Fig. 4). The patients with the strongest positive reactivity also reacted with several of the smaller degraded products of $rDBP_{II–IV}$.

A quantitative assay (ELISA) of antibody reactivity to the $rDBP_{II–IV}$ as well as to the C-terminal fragment of *P. vivax* MSP-1 ($rMSP\text{-}1_{19}$) was developed by using these initial sera to establish the assay conditions (Fig. 5). We found that nearly all individuals in a small sample of PNG adults had a positive response to *P. vivax* DBP and most had a positive response to the $rMSP\text{-}1_{19}$. Generally, the reactivity of each serum sample in the ELISA was similar in relative intensity to that of the immunoblot. The exception was the pooled anti-*P. vivax* immune sera from the exposed North American residents that reacted relatively more strongly to $DBP_{II–IV}$ in the ELISA. None of the sera tested reacted significantly with GST at an equivalent concentration.

Serologic reactivity of human responses to DBP and MSP-1_{19}. To better determine the serological responses to *P. vivax* DBP and MSP-1, we examined the serum antibody responses of a collection of 100 plasma samples obtained from Madang residents during the dry season of 1982 (6). When the subjects were divided into age groups, significant differences were seen in serologic responses to DBP but not to MSP-1 (Table 1). The mean anti-DBP response increased significantly from group 1 (ages 0 to 4 years) to group 2 (ages 5 to 9 years), including a doubling in the percent positive individuals; but, in group 3 (ages 10 to 19 years), the mean response fell back to near that of the youngest group. The mean response increased progressively in groups 3 to 5, becoming significantly higher in the oldest group (age, $\geq$40 years). For all groups but group 2, there was a progressive increase in the antibody response, as shown by the group median, the range, and the percent positive individuals; this was not true for the anti-MSP-1 responses, which were relatively uniform for all age groups. With all of the patients, antibody responses to the two antigens were not correlated ($r_s = 0.329$). However, there was a positive correlation ($P < 0.012$) when the antibody response to MSP-1 was investigated in relation to whether a patient had a positive or negative response to DBP; persons with a positive response to DBP had on average a significantly higher response to MSP than DBP nonresponders did. The reverse was not true, and persons with a positive response to MSP-1 did not have a higher average response to DBP. There was no correlation between antigen responsiveness and parasitemia or village of residence.

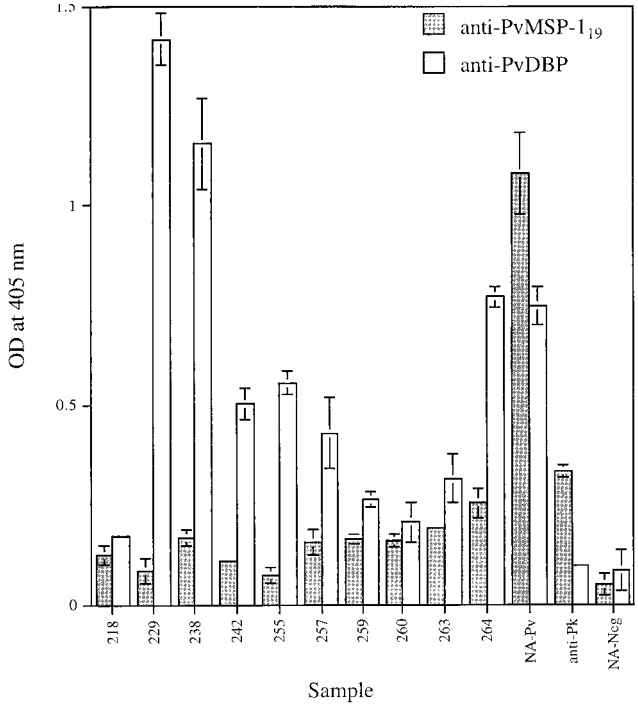

FIG. 5. ELISA. The relative reactivity of human antibodies to *P. vivax* antigens assayed by ELISA. Purified thrombin-cleaved *P. vivax* $rDBP_{II–IV}$ or purified *P. vivax* $rMSP\text{-}1_{19}$ was used to coat microtiter plates and reacted in triplicate with a 1:400 dilution of each serum sample. The presence of human antibody reactive with the $rDBP_{II–IV}$ was assayed with anti-IgG human alkaline phosphatase conjugate and a colorimetric substrate. Bars represent the average plus the standard deviation of the three OD readings of each serum for its anti-*P. vivax* DBP (anti-PvDBP) and anti-*P. vivax* MSP-1_{19} (anti-$PvMSP_{19}$) activity. The samples were from the PNG adults (numbered samples), pooled sera from North American residents exposed to *P. vivax* malaria (NA-Pv), or North American residents not exposed to *P. vivax* malaria (NA-Neg). The NA-Neg value is the group average and standard deviation for 15 samples; the cutoff for positive anti-DBP activity was 0.184 OD, and that for anti-MSP-1 activity was 0.105 OD. Sample 254 was not tested by ELISA. Anti-PK, anti-*P. knowlesi.*

TABLE 1. Serum antibody responses of Madang residents

Group	Age range (yr)	*n*	Serological responses to *P. vivax* antigens							
			Anti-DBP				Anti-MSP			
			% Positive	AU			% Positive	AU		
				Mean[a]	Median	Range		Mean	Median	Range
1	0–4	19	42	2.935A	2.887	2.46–3.58	84	2.908	2.932	2.39–3.92
2	5–9	21	86	3.280B	3.214	2.34–3.98	81	2.911	2.895	2.24–3.80
3	10–19	26	46	2.931A	2.929	2.36–3.55	81	2.878	2.899	2.29–3.55
4	20–39	25	56	3.068A	2.972	2.38–4.12	88	3.010	2.941	1.99–3.90
5	≥40	9	89	3.664C	3.681	2.91–4.49	78	3.022	2.932	2.24–4.01
Total	0–70	100	60	3.110	3.097	2.34–4.49	83	2.946	2.916	1.99–4.01

[a] Values followed by different letters identify significantly different means.

DISCUSSION

The *P. vivax* DBP is a well-conserved molecule that has an essential role in the parasite's development during asexual blood-stage infection. *P. vivax* merozoites require the Duffy blood group antigen as a surface receptor to invade human erythrocytes (19). Unlike other malaria parasites (8, 9, 16, 21, 23, 26), *P. vivax* does not have an alternate pathway of invasion in humans and must use the Duffy receptor-ligand interaction (19). Because of these factors, *P. vivax* DBP is a promising asexual blood-stage malaria vaccine candidate (22).

In the logical development of a vaccine, it is important to study the natural immunogenicity and antigenicity of the candidate immunogen. The use of a purified material is preferable for these immunological analyses. Because *P. vivax* DBP is a molecule of very low abundance in the parasite and because of limitations in culturing *P. vivax* parasites, it was necessary to express the DBP as a recombinant protein. Unfortunately, the functionally important part of the DBP is a cysteine-rich domain with many aromatic residues, and this has hampered its expression in recombinant systems. In this study, we have expressed the cysteine-rich functional domain of the DBP as a soluble recombinant protein by including most of the contiguous hydrophilic cysteine-free region following the receptor-binding domain. This soluble rDBP was useful for subsequent immunological analysis.

The rDBP products were immunogenic in laboratory animals. Antibodies induced to these recombinant proteins reacted with native DBP from *P. vivax* asexual blood-stage parasites. In addition, the anti-*P. vivax* $rDBP_{II–IV}$ sera reacted to the closely related simian malaria parasite, *P. knowlesi*, and immune serum from a rhesus immune to *P. knowlesi* reacted with the *P. vivax* rDBP. The anti-*P. vivax* $rDBP_{II–IV}$ immune serum gave similar fluorescence patterns in both *P. vivax* and *P. knowlesi* that were consistent with the apical fluorescence expected for a microneme or another apical organelle protein (1, 11). The *dbp* genes of *P. knowlesi* and *P. vivax* have 70% amino sequence identity in their cysteine-rich erythrocyte-binding domains (2) but less than 10% similarity in the hydrophilic regions III to IV included on the recombinant protein. This indicates, therefore, that some of the reactive antibody epitopes were present in the conserved sequence motifs of the functional cysteine-rich domain.

The immunogenicity of the *P. vivax* DBP in humans exposed to *P. vivax* malaria was demonstrated by immunoblotting and ELISA, reacting plasma samples from residents of Madang, PNG, with purified rDBP. The Madang population from which the 100 plasma samples were collected lived in a region where both *P. vivax* malaria and *P. falciparum* malaria were highly endemic (6, 14). The total malaria prevalence (i.e., diagnosed positive by blood smear) for all *Plasmodium* species was approximately 40%, and that for *P. vivax* was close to 10%, with slight seasonal and annual variations; the prevalence of *P. vivax* extended to over 16% in subjects aged 1 to 9 years, followed by an initially sharp and then progressive decline with age (6). The high exposure level is also evident in a high prevalence (>90%) of antibody to total (*P. falciparum*) parasite antigen (6).

The PNG serum antibodies reacted equally well to both reduced and nonreduced rDBP antigen. The strong reactivity of the human antibodies to the reduced, denatured rDBP established the presence and immunogenicity of linear epitopes on the native protein. In addition, the reactivity of the pooled immune sera from *P. vivax*-infected North Americans gave a relatively greater response in the ELISA compared to its very weak reactivity by immunoblot, suggesting that there were additional epitopes on the nondenatured form of the rDBP. Together, these data indicate that the rDBP contained some conformational immunodominant epitopes of the native protein and that the humoral response after a single or limited exposure was relatively stronger to these conformational epitopes than to the linear epitopes. Response to the linear cryptic epitopes may require repeated exposure to *P. vivax*, as would be the case with PNG residents.

Previous studies of the EBP from *P. falciparum* have shown that linear epitopes present on EBA-175 in the hydrophilic region (III to V) may be important in blocking parasite invasion (17, 25). Rabbit sera to peptides in this hydrophilic region of EBA-175 inhibited growth of *P. falciparum* in culture (25), and residents of a country in Africa where malaria is endemic responded immunologically to peptides from this hydrophilic region (17). However, we expect that antibodies to conformational epitopes in the cysteine-rich ligand domain (region II) will be most effective in inhibiting parasite invasion because this is the ligand domain that mediates binding of *P. vivax* DBP and EBA-175 to their respective receptors (7, 26). Demonstrated hypervariability in the coding sequence for the *P. vivax* DBP ligand domain from PNG isolates is consistent with this domain as a principal target of an inhibitory immune response (30).

The serological responses to *P. vivax* DBP and MSP-1 were distinctly different, as observed in this sample of the Madang population. MSP-1 appeared as a highly immunogenic molecule for most individuals in all age groups and has a prevalence expected based on existing data (13, 18). There appeared to be little boosting effect to MSP-1 from accumulated age-related exposure, since neither the mean response nor the maximum responses increased significantly with age. *P. vivax* DBP proved to be a less-immunogenic molecule and fits a pattern of that expected for a conserved malarial antigen (14, 15); the increase in the mean as well as the maximum response with age is

consistent with boosting by repeated exposure. Although the significant jump in serological response in subjects between 5 and 9 years of age does not fit this pattern, it may be a result of the high rate of parasitemia and exposure in this age group. It is difficult to know the significance of this phenomenon with our limited sample size and single time point samples. More extensive analysis of the serologic responses together with correlation with clinical illness will help to determine whether these patterns are consistent and whether a positive serologic response is associated with protection from *P. vivax* malaria.

In summary, we have shown that *P. vivax* DBP is naturally immunogenic and induces a positive humoral response in residents of a region where *P. vivax* malaria is endemic. Natural immunogenicity may be useful in the development of *P. vivax* DBP as an asexual blood-stage malaria vaccine to provide boosting against the vaccine immunogen. Future studies will need to investigate the protective nature of the antibody response and factors associated with the weak immune response in some individuals despite repeated exposure to *P. vivax.*

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