Naturally Acquired Antibodies to *Plasmodium vivax* Duffy Binding Protein (DBP) in Rural Brazilian Amazon

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Abstract. Duffy binding protein (DBP), a leading malaria vaccine candidate, plays a critical role in *Plasmodium vivax* erythrocyte invasion. Sixty-eight of 366 (18.6%) subjects had IgG anti-DBP antibodies by enzyme-linked immunosorbent assay (ELISA) in a community-based cross-sectional survey in the Brazilian Amazon Basin. Despite continuous exposure to low-level malaria transmission, the overall seroprevalence decreased to 9.0% when the population was reexamined 12 months later. Antibodies from 16 of 50 (36.0%) subjects who were ELISA-positive at the baseline were able to inhibit erythrocyte binding to at least one of two DBP variants tested. Most (13 of 16) of these subjects still had inhibitory antibodies when reevaluated 12 months later. Cumulative exposure to malaria was the strongest predictor of DBP seropositivity identified by multiple logistic regression models in this population. The poor antibody recognition of DBP elicited by natural exposure to *P. vivax* in Amazonian populations represents a challenge to be addressed by vaccine development strategies.

INTRODUCTION

Almost 40% of the world's population is currently exposed to *Plasmodium vivax*, with 130–435 million clinical episodes recorded each year.^{1–3} The emergence of multi-resistant *P. vivax* isolates associated with severe and fatal malaria^{4,5} highlights the need to consider both *P. vivax* and *Plasmodium falciparum* when implementing measures designed to reduce the malaria burden in regions where both species coexist.

The Duffy binding protein (DBP) stands out as the most promising *P. vivax* vaccine candidate antigen.^{6,7} The DBP plays a major role in red blood cell invasion by *P. vivax*; blocking DBP binding to the Duffy antigen/receptor for chemokines (DARC) reduces the parasite's ability to invade new erythrocytes.^{8–10} Binding domains of DBP is located in the N-terminal cysteine-rich region II (DBP_{II}), which contains 330 amino acids. The critical residues map to the central, 170-amino-acid stretch of DBP_{II}, which includes cysteines 5–8.^{11–13}

Naturally acquired antibodies to DBP_{II} may block DBP_{II}-DARC interaction^{14–16} and inhibit erythrocyte invasion *in vitro*.¹⁰ Antibody recognition of DBP has been described in individuals exposed to hyperendemic malaria,^{14,16–18} but little is known about naturally acquired antibodies in areas where substantially lower levels of malaria transmission prevail, such as the frontier settlements across the Amazon basin.^{15,19} Here, we measure levels of naturally acquired antibodies to DBP in a well-characterized population exposed to frontier malaria in Brazil,²⁰ and investigate whether these antibodies block DBP_{II}-DARC interaction *in vitro*. We also investigate levels of sequence diversity in DBP_{II} among local parasites and examine factors that might impair antibody recognition of DBP_{II} by populations exposed to low-level *P. vivax* transmission.

SUBJECTS, MATERIALS, AND METHODS

Study area and population. The State of Acre is located in the Western Amazon Basin of Brazil, bordering with Peru, Bolivia, and the Brazilian states of Amazonas and Rondônia (supplementary Figure 1, available at www.ajtmh.org). The study site, Granada (9°41'S–9°49'S, 67°05'W–67°07'W), was a sparsely peopled rubber tapper settlement that became part of the Pedro Peixoto Agricultural Settlement Project in 1982. The study site and local malaria transmission patterns have been described in detail elsewhere.²⁰ Malaria morbidity in Granada has been shown to be associated with 1) forest-related activities such as land clearing; 2) time of residence in the settlement, with the probability of having malaria decreasing with years of residence in the settlement, but it is not affected by the subject's age; and 3) place of residence in the study area, with a significant spatial clustering of malaria risk in the areas of most recent settlement.

Blood samples for laboratory diagnosis of malaria and serum separation were collected between March 2004 and May 2005. Both *P. falciparum* and *P. vivax* are transmitted year-round. Recruitment strategies have been described elsewhere, with 466 dwellers < 1 to 90 years of age (98.5% of the 473 permanent residents in the study area) enrolled at baseline and 43 individuals (mostly newcomers to the area) enrolled between September and October 2004.²⁰ A questionnaire was applied to all study participants to obtain demographic and clinical information and assess their cumulative exposure to malaria. Because most (60.1%) study subjects were migrants from malaria-free areas, their ages do not necessarily correlate with exposure to malaria or risk of malaria during the follow-up.²⁰ Cumulative exposure to malaria was therefore estimated as the length of residence in malaria-endemic areas (either in Acre or elsewhere in the Amazon area) and the self-reported number of lifetime malaria episodes. Recent exposure to *P. vivax* was estimated as the number of slide-confirmed *P. vivax* malaria episodes recorded in the three local malaria diagnosis outposts between January and December 2003.

The 425 study participants ≥ 5 years of age were invited to contribute a 5-mL venous blood sample for serum separation; 366 subjects (86.1% of the eligible; age range, 5–90 yr)

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FIGURE 1. Inhibition of DBP_{II}-DARC binding in sequential samples from 50 individuals who had conventional anti-DBP antibodies by enzyme-linked immunosorbent assay (ELISA) at the time of enrollment. Each sample was assayed at baseline and $\geq$ 12-month latter. Conventional anti-DBP antibodies were detected by ELISA (at 1:100 plasma dilution), and inhibitory antibodies by erythrocyte-binding assays (at 1:40 plasma dilution) with COS cells expressing the most common DBP_{II} variant identified in the study population (Acre-1) or Sal-1 DBP_{II} (*P. vivax* laboratory reference), as described in Material and Methods. Numbers on the left refer to the individual code and values at the bottom of the figure represent the overall frequency of responders for each assay.

had their baseline serum samples tested for IgG antibodies to *Plasmodium vivax* DBP. All households were revisited in February–March 2005, when 323 venous blood samples were collected from their inhabitants 5 years of age or older and examined for IgG antibodies to DBP. Of 366 subjects enrolled at baseline, 287 (78.4%) still lived in the area and had a paired serum sample tested for anti-DBP antibodies.

The ethical and methodological aspects of this study was approved by the Ethical Committee of Research on Human Beings from the Institute of Biomedical Sciences, USP, São Paulo, SP, Brazil (Reports 318/CEP, July 19, 2002 and 538/CEP, January 7, 2004), according to the Resolution of the Brazilian Council on Health-CNS 196/96.

Malaria surveillance and acute-phase serum samples. Malaria episodes were diagnosed during 15 months of follow-up (March 2004 through May 2005) through both active and passive case detection. For active case detection, all households in the study area were visited 5 days/week by our field team and blood samples were collected from all subjects having fever or other symptoms suggestive of malaria since the last visit. Additional malaria episodes were found by passive case detection when symptomatic study participants had a malaria diagnosis confirmed at one of the three government-run malaria outposts in the study area. Asymptomatic malaria infections were detected during three cross-sectional surveys of the whole study population carried out in March–April

2004, September–October 2004, and February–March 2005. The combined active and passive case detection strategy identified 244 laboratory-confirmed *P. vivax* infections (183 symptomatic, 61 asymptomatic) among 138 subjects 5 years of age or more (mean, 1.7 episode per subject; range, 1–6). Ninety-four infections (38.5%) were missed by thick-smears microscopy, being only diagnosed by nested polymerase chain reaction (PCR); of them, 57 (60.6%) were asymptomatic. Molecular diagnosis detected both *P. falciparum* and *P. vivax* in 70 (28.7%) infections, but only three of these mixed-species infections had been diagnosed by conventional microscopy, which usually detected only the predominant species.^{21,22} Acute-phase serum samples collected during 143 laboratory-confirmed *P. vivax* infections were tested for IgG antibodies to DBP.

Laboratory diagnosis of malaria. Two methods were used to diagnose malarial infections: examination of Giemsa-stained thick smears under 1,000× magnification (minimum of 100 microscopic fields examined) and nested PCR amplification of a species-specific segment of the *18S rRNA* gene of human malaria parasites.^{23,24} Two sets of slides were sent for review by an expert microscopist at the National Reference Laboratory of the Ministry of Health of Brazil, in Brasília: 1) all positive slides and 2) negative slides from patients with acute febrile illness. Samples with either positive microscopy (confirmed by expert review) or positive nested PCR were considered positive for malaria parasites.

Clinical assessment. The prevalence and intensity of the symptoms associated with 174 *P. vivax* episodes diagnosed in our cohort subjects 5 years of age or more, during 15 months of follow-up, were assessed essentially as described elsewhere.^{25,26} Only single-species episodes were considered. Briefly, a semiquantitative questionnaire addressing nine common symptoms (fever, chills, sweating, headache, myalgia, arthralgia, abdominal pain, nausea, and vomiting) was applied to all patients. The same medical doctor (MdSN) assessed all infections, to minimize inter-observer variation. According to the patient's perception, each clinical manifestation (except for fever) was considered to be absent, mild, moderate, or severe; fever was classified as absent, mild, or severe. Numerical scores of 0, 1, 2, or 3 were assigned to symptoms reported to be absent, mild, moderate, or severe, respectively. Asymptomatic subjects were given scores of 0 for each symptom. To minimize recall bias, patients were interviewed during the acute malaria episode or up to 1 week after treatment. Severe malaria episodes were not diagnosed in our study population during the follow-up.

Recombinant proteins and serological assay. The recombinant Duffy binding protein, which includes amino acids 132 to 771 (regions II to IV), was expressed as a soluble glutathione S-transferase (GST) fusion protein of 140 kDa.^{17,19} To assess IgG antibodies against DBP an enzyme-linked immunosorbent assay (ELISA) was carried out as previously described.¹⁹ Serum samples were assayed at 1:100 and the recombinant proteins used in the final concentrations of 5 µg/mL (DBP). Specific optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST alone (antigen control). The threshold of positivity was an OD value of 0.2 for DBP, which was based on the mean plus two standard deviations reactivity of sera from 20 non-exposed subjects.

DBP-pEGFP constructs. Region II of DBP (DBP_{II}) from a *P. vivax* laboratory reference clone (Sal-1)²⁷ has previously

been subcloned into the pEGFP-N1 plasmid (Clontech), with a flanking signal sequence from the herpes simplex virus glycoprotein D1 (HSVgD1).¹⁴ This targets expression to the surface of the transfected COS cells as a green fluorescent protein (GFP) fusion protein. The original Sal-1 DBP-pEGFP plasmid was kindly provided by Dr. J. H. Adams, University of South Florida, FL. An additional GFP construct with the DBP_{II} sequence from a common DBP_{II} variant circulating in the study area was made by subcloning a fragment corresponding to aa 198–522 of region II into pEGFP-HSVgD1 plasmid, using primers described previously.²⁸ Recombinant plasmids were purified by use of an endotoxin free plasmid DNA purification system (Qiagen, Valencia, CA).

COS cell transfection and erythrocyte-binding assays.

Recombinant plasmids were transfected into COS-7 cells (American Type Culture Collection, Manassas, VA) by use of lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocols. Briefly, COS-7 cells in six-well culture plate (1.5×10^5 cells/well) were transfected with plasmids (0.5 µg/well)-liposome complexes (5% Plus-reagent and 3% lipofectamine) in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) without serum. After 6 hr of cell exposure to DNA-liposome complexes (37°C, 5% CO₂), transfection medium was replaced by DMEM with 10% of fetal bovine serum (Gibco-BRL, Gaithersburg, MD). At 24 hr after transfection, culture medium was replaced again and efficiency of transfection was assessed by fluorescence. Forty-eight hours after transfection, the erythrocyte-binding assays were performed as previously described.¹⁵ Briefly, antiserum was added at 1:40, and plates were incubated for 1 hr at 37°C in 5% CO₂. The 1:40 dilution was chosen because in previous experiments this dilution provided a wide range of inhibitory activity among different plasmas. Human O⁺ erythrocytes in a 10% suspension were added to each well (200 µL/well), and plates were incubated for 2 hr at room temperature. Unbound erythrocytes were then removed by washing the wells three times with phosphate buffered saline (PBS). Binding was quantified by counting rosettes observed over 10–20 fields of view (200×). Positive rosettes were defined as adherent erythrocytes covering more than 50% of the COS cell surface. For each assay, pooled plasma samples from Acre residents, characterized as non-responders by ELISA, were used as a negative control (100% binding). For this purpose, only plasma that does not inhibit erythrocyte binding (as compared with sample from unexposed Brazilian donors) has been pooled as a negative control (usually, 10 plasma samples/pool). An additional control included a pool of plasma from individuals with long-term exposure to malaria in the Amazon area (positive control). The percent inhibition was calculated as $100 \times (R_c - R_t)/R_c$, where R_c is the average of the number of rosettes in the control wells and R_t is the average of the number of rosettes in the test wells.

Plasmodium vivax DBP_{II} amplification and sequencing.

Extracted DNA was used as a template in the PCR to amplify the fragment corresponding to nucleotide positions 870 to 1,545 (amino acids 290–515) of the DBP_{II} encoding gene.²⁹ Platinum high fidelity *Taq* DNA polymerase (Invitrogen Life Technologies) was used in PCR to reduce possible nucleotide misincorporation. Amplicons were purified using the GFX-96 PCR kit (Amersham Biosciences, Little Chalfont, UK) and directly sequenced using DYEnamic ET dye terminator kit (Amersham Biosciences) and MegaBace 500 automated

DNA sequencer (Amersham Biosciences). The sequences were analyzed using Bioedit sequence alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify DBP_{II} polymorphisms relative to the SAL-1 sequence.²⁷

Statistical analyses. A database was created with SPSS 13.0 software (SPSS Inc., Chicago, IL). Proportions were compared in 2 × 2 tables with χ^2 tests with Yates correction for continuity or Fisher's exact tests, as appropriate. Pairwise correlations were evaluated with the Spearman's correlation coefficient ρ . Multiple logistic regression models with stepwise backward deletion were built to describe independent associations between covariates and the presence of antibodies to DBP during the cross-sectional surveys. Age, gender, time of residence in Amazonia, recent or current laboratory-confirmed episode of *P. vivax* malaria, and sector of residence within the study area were included into logistic regression models. Because of the nested structure of the data (there may be two observations per individual), we used two-level logistic models with robust standard errors, with level-1 variables corresponding to each observation (one or more per individual) and level-2 variables corresponding to each individual. Malaria transmission is heterogeneously distributed across Granada because of different patterns of land use and deforestation rates.²⁰ To adjust for these differences in logistic models, we divided the study area into four relatively homogeneous sectors with increasing malaria incidence: 1) sector A (92 subjects at baseline, 0.46 *P. vivax* episodes/100 person-months at risk between March 2004 and May 2005); 2) sector B (97 subjects, 0.79 *P. vivax* episodes/100 person-months at risk); 3) sector C (130 subjects, 3.44 *P. vivax* episodes/100 person-months at risk); and 4) sector D (47 subjects, 9.71 *P. vivax* episodes/100 person-months at risk). The HML software package (version 6.03, Scientific Software International, Lincolnwood, IL) was used for multilevel analysis. Only variables associated with statistical significance at the 5% level were maintained in the final models.

RESULTS

Naturally acquired IgG antibodies to DBP in cross-sectional surveys. We have studied baseline antibody responses to DBP in 366 subjects 5 to 90 years of age (median, 24.5 yr), with a male:female rate of 1.13:1 (Table 1). These subjects had between 1 month and 72 years of residence in the Brazilian Amazon area (median, 14 yr), where they are continuously exposed to *P. vivax* infections; 39 (10.7%) subjects had at least one recent symptomatic *P. vivax* malaria episode, diagnosed by passive case detection, between March 2003 and March 2004. At enrollment, 18.6% (68 of 366) study subjects had antibodies to DBP. Table 2 shows the proportions of study subjects who had IgG antibodies to DBP detected by ELISA at the cohort baseline and in February–March 2005. The overall proportion of responders differed significantly in the surveys (18.6% versus 9.0%, $P = 0.0004$, χ^2 test with Yates correction). *Plasmodium vivax*-infected subjects examined during the second cross-sectional survey had a greater prevalence of antibodies to DBP than those free of *P. vivax* infection ($P = 0.007$, χ^2 test with Yates correction), but no similar association between current *P. vivax* infection and seropositivity was found at the baseline survey. No significant association was found between recent exposure to the parasite (in the absence of current infection) and positive serology at the cohort baseline or in the

second cross-sectional survey (Table 2). Similar proportions of subjects with baseline anti-DBP antibodies detected by ELISA (14 of 68, 20.6%) and those without detectable anti-DBP antibodies (50 of 298, 16.8%) had one or more *P. vivax* infections diagnosed by either passive or active case detection during the first 15 months of cohort follow-up ($P = 0.480$, Fisher's exact test).

Baseline plasma samples from 50 subjects with IgG antibodies to DBP detected by ELISA (age range, 8–75 yr) were further tested for their ability to inhibit *in vitro* the erythrocyte-binding function of DBP ligand domain. The Sal-1 DBP_{II} variant was included in these assays because it was being developed as a *P. vivax* vaccine candidate, and, of importance, Sal-1 DBP_{II} variant was present in Acre *P. vivax* isolates with a frequency greater than 10% (Table 3). We also tested the inhibitory ability of sera against the most common DBP_{II} variant identified in the study population, haplotype-1 (Table 3; renamed here Acre-1), which is quite common across the Amazon region of Brazil (Sousa TN and others, unpublished data). Overall, a plasma sample from 16 subjects (32.0% of those with anti-DBP antibodies that were tested) displayed significant (> 50%) inhibitory activity with one or both variants (13 of them had inhibitory antibodies against both DBP_{II} variants) (Figure 1). The subjects with inhibitory antibodies were slightly older (median age, 30.5 versus 26.5 years) and had a longer length of residence in the Amazon area (median length, 17 versus 14 years) than those who had ELISA-detected antibodies with no inhibitory activity ($P = 0.160$ and 0.327, respectively, Mann-Whitney test). Five baseline samples tested for inhibitory antibodies were collected from subjects with current *P. vivax* infection; two of them yielded significant erythrocyte-binding inhibition. A smaller proportion of subjects with inhibitory antibodies at the cohort baseline (3 of 16, 18.7%), when compared with those with anti-DBP antibodies that were unable to inhibit erythrocyte binding (7 of 34, 20.6%), had one or more *P. vivax* infections diagnosed during the first 15 months of follow-up, but the sample size is too small for meaningful statistical analysis. The baseline levels of IgG antibodies detected by ELISA (as estimated with corrected absorbance values) were positively correlated with the inhibitory activity of these antibodies against both Sal-1 ($\rho = 0.426$, $P = 0.003$, Spearman's correlation test) and Acre-1 ($\rho = 0.453$, $P = 0.001$, Spearman's correlation test) DBP variants.

We used multiple logistic regression models to determine whether cumulative, recent, or current exposure to *P. vivax* infection predicted the presence of anti-DBP antibodies during the cross-sectional surveys, after controlling for several covariates putatively associated with malaria risk in our population, such as age, gender, and sector of residence in the study

TABLE 1

Demographic, epidemiologic, and immunologic data of the 366 subjects who had baseline serum samples tested for IgG antibodies to Duffy binding protein (DBP)

Characteristics	
Median age, years (range)	24.5 (5–90)
Gender, male:female	1.13:1
Acute <i>Plasmodium vivax</i> infection, n (%) [*]	30 (8.2)
Years of malaria exposure, median (range)	14 (0–72)
Anti-DBP antibodies, n (%) [†]	68 (18.6)

^{*} Fifteen out of 30 were mixed infections by *P. vivax* and *Plasmodium falciparum*.

[†] Positive antibody response, as detected by enzyme-linked immunosorbent assay (ELISA).

TABLE 2

Prevalence of IgG antibodies to Duffy binding protein (PvDBP) in relation to current or recent *Plasmodium vivax* infections, Acre, Brazil, 2004–2005

<i>P. vivax</i> infection	Baseline (March–April 2004)			<i>P</i> (yes vs. no)	February–March 2005			<i>P</i> (yes vs. no)
	No.	Subjects with IgG antibodies			N°	Subjects with IgG antibodies		
Current		No.	(%)			No.	(%)	
Yes	30*	6	(20.0%)	0.971	28†	7	(25.0%)	0.007
No	336	62	(18.5%)		295	22	(7.4%)	
Total	366	68	(18.6%)		323	29	(9.0%)	
Recent								
Yes	34‡	9	(26.5%)	0.299	40§	6	(15.0%)	0.103
No	302	53	(17.5%)		255	17	(6.7%)	
Total	336¶	62	(18.8%)		295	22	(7.4%)	

* Fifteen *P. vivax* infections and 15 mixed *P. vivax*–*Plasmodium falciparum* infections.† Nineteen *P. vivax* infections and 9 mixed *P. vivax*–*P. falciparum* infections.‡ Occurrence of one or more laboratory-confirmed *P. vivax* infections between March 2003 and March 2004.§ Occurrence of one or more laboratory-confirmed *P. vivax* infections during the follow-up (March 2004 to March 2005).¶ Thirty subjects with current *P. vivax* infections excluded.|| Twenty-eight subjects with current *P. vivax* infections excluded.

site. Because of the significant difference in seropositivity rates between surveys, the time of survey was included as one of the covariates to be controlled. The number of years of residence in the Brazilian Amazon, a surrogate measure of cumulative exposure to malaria, was a strong predictor of the presence of IgG antibodies (adjusted odds ratio [aOR], 1.02; 95% confidence interval [CI], 1.00–1.04, $P = 0.044$). In other words, each additional year of exposure to malaria increased the probability of having anti-DBP IgG antibodies by 2%. Neither age nor current or recent exposure to *P. vivax* (as defined in Table 2) were significant predictors of DBP seropositivity. As expected, however, the sector of residence was significantly associated with the presence of IgG antibodies. We conclude that the cumulative exposure to malaria, but not the recent or current exposure to *P. vivax*, was a significant independent predictor of the presence of anti-DBP IgG antibodies during the cross-sectional surveys.

Malaria surveillance and anti-DBP antibodies in sequential serum samples. We next compared the prevalence of conventional anti-DBP antibodies detected by ELISA and of inhibitory antibodies detected by erythrocyte-binding assays in paired samples (baseline versus second cross-sectional survey) obtained from subjects who experienced or did not experience one or more clinical episodes of *P. vivax* malaria between the surveys (Table 4). Subjects who had asymptomatic *P. vivax* parasitemia detected during one of the population-wide cross-sectional surveys but had no disease ($N = 11$) were not included in this analysis. No significant association was found between the presence of antibodies (either conventional or inhibitory) in the second cross-sectional survey and the occurrence of *P. vivax* malaria between the blood draws. A significant correlation between the levels of IgG antibodies detected by

ELISA and the inhibitory activity of these antibodies against both Sal-1 ($\rho = 0.513$, $P < 0.0001$, Spearman's correlation test) and Acre-1 ($\rho = 0.471$, $P < 0.0001$, Spearman's correlation test) variants. Four subjects had no anti-DBP antibodies detected by ELISA during the second cross-sectional survey but maintained high-level rosette-inhibitory activity against both DBP_{II} variants (Figure 1). Although overall levels of anti-DBP antibodies tended to decrease between the first and the second surveys, serum inhibitory activity remained relatively stable in the majority of the responders (14 of 16). Of relevance, both the frequency and levels of inhibitory antibodies to Sal-1 and Acre-1 variants were quite similar (Figure 1, supplementary Figure 2, available at www.ajtmh.org).

The putative antibody boosting effect of current exposure to *P. vivax* was further assessed by analyzing acute-phase serum samples from 80 cohort participants who experienced laboratory-confirmed *P. vivax* infections diagnosed during the follow-up. Of 143 acute-phase sera tested for IgG antibodies to DBP, 34 (23.8%) were positive. The prevalence of anti-DBP antibodies increased linearly with increasing parasitemias (χ^2 for trend = 5.325, 1 degree of freedom, $P = 0.0021$), and only 10 of 67 (14.9%) acute-phase sera collected during subpatent infections had detectable antibodies. These data suggest that exposure to very low parasitemias may be poorly effective in inducing anti-DBP antibody boosting.

Finally, we compared the prevalence of anti-DBP IgG antibodies in consecutive samples collected from 53 cohort subjects who experienced one or more laboratory-confirmed *P. vivax* infections. Individual results are shown in Figure 2, with subjects categorized as 1) seroconverters (initially seronegative subjects who acquired anti-DBP antibodies at any time-point of the study; $N = 13$, Figure 2A); 2) subjects who

TABLE 3

Common *Plasmodium vivax* DBP_{II} haplotypes identified among 25 *P. vivax* isolates from the study area, State of Acre, Brazil*

AA residue	333	371	375	384	385	386	390	417	419	424	437	503	Frequency (%)
Sal-1†	L	K	N	D	E	K	R	N	I	L	W	I	12
1	—	.	.	G	.	.	H	.	.	.	.	K	20
2	.	.	.	G	K	N	H	.	.	.	R	K	12
3	.	.	.	.	.	.	.	K	.	I	R	K	12
4	F	.	D	G	K	N	H	K	.	I	R	.	16

* Eleven DBP_{II} haplotypes were identified in Acre population, and those present at a frequency greater than 10% were listed in Table 3. Grey areas highlight the trio of polymorphisms in DBP_{II} that form part of a cluster surrounding the Duffy antigen/receptor for chemokines (DARC)-binding site, and which are under positive selection (Souza TN and others, unpublished data).† Sal-1 sequence, accession no.: M61095.²⁷

TABLE 4

Prevalence and inhibitory activity of naturally acquired antibodies to Duffy binding protein (PvDBP) in consecutive cross-sectional surveys in relation to *Plasmodium vivax* malaria episodes during the follow-up; Acre, Brazil, 2004–2005*

Antibodies in consecutive surveys	<i>Plasmodium vivax</i> malaria during the follow-up			All subjects
	Yes	No.	<i>P</i> †	
Conventional ELISA				
antibodies				
−/−	29	194	0.358	223
+/−	8	30		38
−/+	3	8		11
+/+	2	13		15
Total	42	245		287
Inhibitory antibodies				
(Sal-I or Acre-1)				
−/−	6	27	1.00	33
+/−	1	2		3
−/+	1	0		1
+/+	2	11		13
Total	10	40		50

* ELISA = enzyme-linked immunosorbent assay.

† P for a comparison between (–/– or +/-) vs. (–/+ or +/+) with Fisher's exact test.

lost their anti-DBP antibodies ($N = 10$, Figure 2B); and 3) subjects who failed to develop antibody responses to DBP despite documented exposure to the parasite ($N = 25$, Figure 2C). Five out of 53 subjects who could not be classified following these group criteria were not included in the analysis (data not shown). DBP antibody acquisition was likely to be related with cumulative exposure to malaria, as defined by the years of residence in the Amazon area (median, 19 yr versus 12 to 13 yr) or by the number of previous malaria episodes (median, 11 episodes versus 3 to 5 episodes), but these differences were not statistically significant.

Clinical expression of *P. vivax* malaria and anti-DBP antibodies. The prevalence and severity of symptoms associated with uncomplicated *P. vivax* malaria were analyzed in 174 laboratory-confirmed single-species infections diagnosed in our cohort. Fever (67.8%), headache (71.3%), chills (58.6%), and myalgia (57.5%) were the most prevalent symptoms; 34 (19.5%) infections were symptomless and 60 (48.6%) infections, 33 of them asymptomatic, were diagnosed by PCR only.

We next examined whether the levels of anti-DBP IgG antibodies measured during *P. vivax* infections correlated to the perceived severity of symptoms. For that, each symptom was assessed separately for its correlation with levels of anti-DBP antibodies. Because of the confounding effect of parasitemias, which affect both anti-DBP responses and the clinical expression of malaria, we restricted this analysis to 57 patent infections. No significant correlation was found between ELISA absorbance values and the severity of any symptom (P value range, 0.161–0.943, Spearman's correlation test). Quite similar results were obtained when only the first infection experienced by each subject was analyzed ($N = 31$, P value range, 0.172–0.929, Spearman's correlation test).

DISCUSSION

Here, we show that a relatively small proportion of rural Amazonians exposed to low-level malaria transmission have detectable antibodies to DBP; in contrast, the MSP1₁₉, a much more abundant surface antigen, was previously recognized by

≈50% of these individuals.³⁰ Given the fact that DBP is localized in an apical secretory organelle (micronemes), and it is probably not released until erythrocyte attachment,³¹ the host immune system seems to have little opportunity to mount an efficiently antibody response, particularly as the invasion process may take less than a minute to be completed.³² It may partially explain why in Acre population a long-term exposure to malaria and, apparently, a certain level of parasitemia have to be reached until subjects acquire anti-DBP antibodies. Consistent with this hypothesis, our previous study in the Amazon area showed that high-levels of anti-DBP antibodies could be reached only among gold miners¹⁹ whose behavioral patterns place them at a higher risk of exposure to infected mosquitoes.^{33,34} In fact, malaria prevalence in Granada (3.6%)²⁶ is far lower than that measured in mining areas (13.8–35.0%).^{34–36} However, the “just-in-time” hypothesis of DBP exposure³⁷ does not completely explain the large proportion of individuals who remain unresponsive to DBP after prolonged exposure to malaria, especially because a few dominant DBP_{II} haplotypes seem to account for the majority of *P. vivax* infections in areas of high malaria transmission.³⁸ The reasons for this are not clear, but may relate to the complexity of immune responses in terms of genetic diversity of the human and parasite populations, and stochastic events associated with the induction of a specific antibody response.^{39–41} Such complexity may provide a plausible explanation for the difficulties that have been encountered in *Plasmodium* vaccine development.

The goal in developing DBP as a vaccine against blood-stages of *P. vivax* is to elicit an antibody response that inhibits the adhesion of this parasite ligand to its cognate erythrocyte receptor and thereby abrogate merozoite invasion. In the Acre population, we further analyzed whether the conventional DBP immune response, as detected by ELISA, includes antibody activity that blocks the DBP_{II}-DARC interaction. A significant inhibitory activity was detected in about one-third of those subjects, and the presence of these inhibitory antibodies was related with a long-term residence in the Amazon area (median, 19 yr). In effect, by using multiple logistic regression models, it was possible to identify cumulative exposure to *P. vivax*—estimated by the time of residence in the Brazilian Amazon—as a strong predictor of the presence of anti-DBP antibodies during the cross-sectional surveys. Nevertheless, subjects' age was not associated with the presence of anti-DBP antibodies. It was not unexpected because in this area the pattern of malaria transmission is typically from those of frontier malaria, where exposed populations consist of migrants mostly from malaria-free areas, and malaria infection affects people of all ages.⁴² In conclusion, cumulative exposure, independent of host age, apparently represents a key determinant of the quantitative and qualitative nature of the IgG responses to DBP.

Recently, it has been suggested that DBP_{II} polymorphic residues surrounding the DARC binding domain might elude binding of inhibitory antibody.^{6,43} In fact, individuals briefly exposed to *P. vivax* developed anti-DBP inhibitory antibodies that are biased toward a specific DBP_{II} variant.⁴⁴ Although we cannot rule out the possibility that polymorphisms at DBP_{II} could contributed to the relatively low frequency (≈30%) of inhibitory antibodies among long-term residents in Acre, it seems unlikely because 1) to reduce the potential effects of DBP polymorphism on antibody recognition, we used two

different DBP_{II} variants that are commonly found in the study site (Sal-I and Acre-1) in erythrocyte binding assays; 2) Sal-1, Acre-1, and a number of Acre DBP_{II} haplotypes (5 out of 11) share the trio of polymorphisms (at codons 417, 419, and 424) that is suggested to play a role in DBP_{II} inhibitory binding (Sousa TN and others, unpublished data). Furthermore, a similar proportion of responders (39%) were found among immune children residing in a *P. vivax* hyperendemic area of Papua New Guinea (PNG), with only 18 of 208 (9%) presenting high-levels of inhibitory antibodies.¹⁶ Consequently, the low levels of DBP inhibitory antibodies should be expected in naturally malaria-exposed populations.

Significantly, the inhibitory ability of antibodies was relatively stable over time in Granada; 14 of 16 subjects retained their inhibitory DBP antibody response to at least one DBP_{II} variant (Acre-1) when studied 12 months after the baseline survey. To the best of our knowledge, a single study previously investigated the longevity of anti-DBP_{II} inhibitory antibodies in the endemic area.¹⁶ King and colleagues¹⁶ found that asymptomatic children residing in PNG developed inhibitory anti-DBP antibodies, which were remarkably stable over the 12-month follow-up period, a result corroborated by our study in Acre population. Together, these evidences imply that although the majority of people naturally exposed to *P. vivax* do not develop antibodies that inhibit the DBP_{II}-DARC interaction, once they are acquired; these inhibitory antibodies seem to be stable under continuous exposure to malaria transmission. Of note, a number of subjects with high rosette-inhibitory activity lost their conventional (ELISA-detected) anti-DBP antibodies during the follow-up. This is not completely unexpected, because standard serological assays with recombinant proteins do not account for fine epitope specificity and affinity, which are likely to be essential for inhibitory activity. However, levels of ELISA-detected antibodies correlated with levels of erythrocyte-binding inhibition, consistent with previous findings in areas with unstable or stable malaria transmission.^{14,15}

Although the size of our sample was not small, the low frequency of DBP responders had precluded a number of statistical comparisons. Consequently, we were unable to test whether the presence of DBP inhibitory antibodies are associated with protection from blood-stage *P. vivax* infection, as recently suggested among children from PNG.¹⁶

In conclusion, our results show low immunogenicity of DBP among individuals continuously exposed to malaria in a well-consolidated settlement of the Brazilian Amazon area. Future challenges include understanding why only a few malaria exposed-individuals develop an immune response able to inhibit DBP_{II}-DARC interaction, and to establish whether DBP inhibitory immune response predicts partial protection from infection and/or disease in semi-immune populations. Although essential, those studies will be a difficult task because protection against malaria in an outbred human population may be a higher-order phenomenon related to patterns of response and not attributable to any single antigenic target.⁴⁰

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