ANTI–PLASMODIUM VIVAX DUFFY BINDING PROTEIN ANTIBODIES MEASURE EXPOSURE TO MALARIA IN THE BRAZILIAN AMAZON

ISABELA P. CERÁVOLO, OSCAR BRUÑA-ROMERO, ÉRIKA M. BRAGA, COR J. F. FONTES, CRISTIANA F. A. BRITO, JOSE M. SOUZA, ANTONIANA U. KRETTLI, JOHN H. ADAMS, AND LUZIA H. CARVALHO

Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil; Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; Instituto de Evandro Chagas, Belém, Pará, Brazil; Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana

Abstract. Plasmodium vivax Duffy binding protein (DBP) is functionally important in the erythrocyte invasion process and provides a logical target for vaccine-mediated immunity. In the current study, we demonstrated that DBP is naturally immunogenic in different populations of the Brazilian Amazon, and the proportions of DBP IgG positive subjects increased with exposure to malaria, reaching a peak in those subjects with long-term exposure (> 15 years) in the Amazon area. This profile of antibody response was significantly different from the one observed for the P. vivax merozoite surface protein 1 (MSP110), which was relatively uniform in areas with markedly different levels of malaria transmission. In a small sample of adults with symptomless P. vivax infection, we could not detect any significant correlation between antibodies against these P. vivax proteins and asymptomatic infection. Our study provided an additional insight by demonstrating cumulative exposure as a determinant that acts independently of host age in generation of anti-DBP IgG response.

INTRODUCTION

Of the four species of malaria parasites known to infect humans, Plasmodium vivax has achieved the widest global distribution and is responsible for > 50% of all malaria cases outside of Africa, including Asia, the Middle East, the Western Pacific, and Central and South America.1,2 Certain features of the transmission biology of P. vivax give this species greater resilience than Plasmodium falciparum; therefore, as control measures become more effective, the residual malaria burden is increasingly pointing to P. vivax.3

Plasmodium vivax merozoites initiate erythrocyte invasion by an active process mediated by parasite ligands that interact with erythrocyte receptors. A key step in the host cell invasion is the irreversible commitment of the merozoite to the selected host cell by the formation of a tight junction between parasite and erythrocyte.4 In the case of P. vivax, junction formation is mediated by the Duffy binding protein (DBP) and its receptor on erythrocytes, the Duffy blood group antigen,4,5 also known as Duffy receptor for chemokines (DARC).7 Individuals that lack the Duffy antigen on their erythrocytes are naturally resistant to P. vivax.8 Thus, DBP is an important vaccine candidate against the asexual stages of the parasite.

DBP is a protein of 140 kDa that belongs to a family of homologous Duffy binding-like erythrocyte binding proteins located within the micronemes of Plasmodium merozoites.9,10 DBP is likely to be exposed on the merozoite surface during invasion, enabling it to bind to its receptor but also making it accessible to serum antibodies. Because DBP is a molecule of very low abundance on the parasite, and because of limitations in culturing P. vivax parasites, the available data on immune responses to DBP in human population are still limited.11–14 In Latin America, a single study on the prevalence of anti-DBP antibodies has been carried out so far.15 However, this study cannot be extrapolated to other areas of Latin America because it was performed in an area on the Pacific coast of Colombia where most people are probably resistant to P. vivax infection; that is, 93% of them were black of African origin (Duffy negative trait).12

In Brazil, like in most regions where P. vivax is prevalent, malaria transmission rates are low, and infections affect people of all ages.13 The distribution of malaria in the Amazon region is not homogeneous, and it is concentrated in areas with uncontrolled establishment of rural and mining settlements, being associated with poorly maintained dwellings and favorable transmission conditions.16 During the past 8 years, our group has conducted a number of immuno-epidemiologic studies in different areas of the Brazilian Amazon.17–20 Benefiting from those studies, we now analyzed the immunologic response to DBP in the Amazon area as a contribution to current efforts on vaccine development against P. vivax. For this purpose, we have compared the profiles of antibody responses to DBP as well as to another vaccine candidate, the 19-kDa C-terminal fragment of P. vivax merozoite surface protein 1 (MSP110), among individuals from three well-characterized areas of the Brazilian Amazon where malaria transmission levels are markedly different.

MATERIALS AND METHODS

Study areas and subjects. We analyzed subjects from three previously well-characterized areas of the Brazilian Amazon19–21 who had been exposed to different levels of malaria transmission (Table 1). The first group consisted of 36 individuals living in Belém, the capital of the State of Pará. They had acquired a single episode of P. vivax malaria after short trips to islands located near the capital (1 to 6 hours by boat) where levels of malaria transmission are low and unstable. The second group was composed of 47 individuals who had lived for about 10 years in a small rural community of Mato Grosso (MT) State, Terra Nova do Norte (TNN), where malaria is endemic with intermittent transmission. These individuals reported a variable number of previous malaria episodes caused by P. falciparum and/or P. vivax. The third group, named Apiacás group, consisted of 37 migrant miners who had lived for approximately 17 years in several gold-mining areas of the Brazilian Amazon where malaria is endemic. At the time of blood collection, these subjects were living in the municipality of Apiacás, MT, which is considered mesoendemic for malaria and where transmission is continu-
These workers were constantly moving inside the Amazon area and have high exposure to Plasmodium-infected mosquitoes. As a consequence, those gold miners had experienced a high number of previous malaria episodes (Table 1). At the time of blood collection, the three groups studied (Belém, TNN, and Apiacás) consisted of aparasitemic individuals, as assessed by microscopic examination, who were specifically treated for malaria, 2 to 5 months prior to the time of blood sample collection.

A previous study on the prevalence of malaria in Apiacás has suggested that gold miners develop resistance to clinical malaria (Fontes CJF and others, unpublished data). In this area, among 527 gold miners characterized and followed-up by C. J. F. Fontes, asymptomatic malaria infection was identified in 38 (7.2%) individuals, 17 of them being infected with P. vivax, 19 with P. falciparum, and 2 with Plasmodium malariae. In the current study, we selected 15 out of 17 gold miners with asymptomatic P. vivax infection and 13 out of 15 with symptomatic P. vivax infection. Asymptomatic malaria infection was defined as absence of classic malaria symptoms, such as fever, headache, muscle and/or joint pain, for at least 72 hours after parasite detection. The mean age of these 28 enrollees was 32 ± 10 years, and most of them were males who had reported a high number of previous malaria episodes.

The ethical and methodological aspects of this study were approved by the Ethical Committee of Research on Human Beings from the Centro de Pesquisas René Rachou/ Fiocruz (Report 002/2002), according to the Resolution of the Brazilian Council on Health-CNS 196/96, and by the WHO Secretariat Committee for Research Involving Human Subjects (SCRIHS).

**Recombinant antigens.** Recombinant DBP (rDBP) was expressed in prokaryotic system and purified using only minor modifications of the protocol described previously. Briefly, a portion of DBP from amino acids 177 to 815 that includes regions II to IV (DBP11-IV) was inserted in frame with glutathione S-transferase (GST) in the expression vector pGEX-2T. The GST fusion protein was purified directly from bacterial lysates through affinity chromatography using matrix glutathione sepharose 4B, according to the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ). Even though the fusion protein was cleaved by thrombin treatment, one contaminant of 70 kDa, probably one bacterial chaperonin, was frequently co-purified with the rDBP (data not shown). Efficient removal of the chaperonin from the rDBP was achieved using a standard protocol for gel electrophoresion, as described. Briefly, after sodium dodecyl sulfate–polyacrylamide gel electrophoresion (SDS-PAGE) of the Sepharose column–eluted proteins, the band of interest was excised from the gel, and protein was electrophoretically eluted from the mincd gel. This procedure was very effective to obtain purified rDBP, as required for enzyme-linked immunosorbent assay (ELISA). A recombinant protein representing the 19-kDa C-terminal region of the merozoite surface protein-1 of P. vivax (rMSP119) was kindly provided by Dr. Irene Soares (Universidade de São Paulo, SP, Brazil). Detailed construction of this GST fusion protein, which represents the amino acids 1616–1794 of the MSP-1 (Belém strain) of P. vivax, has been described elsewhere.

**Antibody measurement.** The ELISA for total IgG antibodies was carried out according to the method described elsewhere, the optimal antigen concentration and the dilution of the primary and secondary antibodies determined empirically by cross-titration. Briefly, 96-well plates (Maxysorp, Nunc, Denmark) were coated overnight at 4°C with 5 μg of rDBP or 1 μg rMSP119 per milliliter in phosphate-buffered saline (PBS, pH 7.4), rinsed in wash buffer (0.05% Tween 20 in PBS), and incubated for 1 hour in blocking buffer (5% skim milk in wash buffer). After rinsing, the antigen-coated wells were incubated for 1 hour with duplicate serum samples diluted 1:100 (rDBP) or 1:80 (rMSP119) in 1.5% skim milk in wash buffer, rinsed again, and incubated for 1 hour at 37°C with peroxidase-conjugated anti-human IgG (Sigma-Aldrich, St. Louis, MO). The presence of bound IgG was detected using o-phenylenediamine dihydrochloride (OPD) as substrate (Sigma-Aldrich) and reading the absorbance at 492 nm using a microplate reader (Stat Fax-2100, Awareness Technology, Palm City, FL). For the recombinant antigens, rDBP and rMSP119, the final optical density (OD) was calculated by deduced OD obtained with purified GST (Sigma-Aldrich), used as antigen control. For both antigens, the threshold of positivity was an OD value of 0.1, which was based on the mean plus three standard deviations (SD) reactivity of sera from 30 nonexposed subjects. For all test samples, both positive and negative control samples were added to each plate, and the reaction was stopped such that ODs varied less than 10% between test plates.

**Statistical analysis.** Statistical analysis was performed using the Epi-Info 2002 software (Centers for Disease Control and Prevention, Atlanta, GA) or MiniTab statistical software (Minitab Inc., State College, PA). Differences in means were tested by Student’s t test or one-way analysis of variance (ANOVA) with Tukey’s post hoc test to identify the significant differences between groups. The log-transformed data were applied for situations where analysis of variance normality assumption was not applied. Differences in proportions were evaluated by the Yates’ χ² (χ²) or Fisher’s exact tests and analysis for linear trend in proportions by χ² for trends (χ² trend). P values < 0.05 were considered significant.

**RESULTS**

To investigate the effect of exposure to malaria transmission on levels of antibodies to the rDBP, we studied individu-

---

**Table 1**

Demographic and epidemiologic data of the subjects exposed to endemic malaria transmission in the Brazilian Amazon

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Belém (N = 36)</th>
<th>TNN (N = 47)</th>
<th>Apiacás (N = 37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)*</td>
<td>32 ± 19</td>
<td>40 ± 28</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>Time of malaria exposure (mean ± SD) &lt;1 month</td>
<td>10 ± 5 years†</td>
<td>17 ± 11 years†</td>
<td></td>
</tr>
<tr>
<td>Number of previous malaria episodes</td>
<td>1</td>
<td>1–10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Exposure to malaria transmission</td>
<td>Sporadic</td>
<td>Variable</td>
<td>Constant</td>
</tr>
<tr>
<td>Previous malaria episodes by</td>
<td>Pv</td>
<td>Pv and/or Pf</td>
<td>Pv and/or Pf</td>
</tr>
</tbody>
</table>

* Differences not significant (F = 1.47, P = 0.234)
† Significant differences between TNN and Apiacás (t = −3.16, P = 0.003)
als from three well-characterized areas of the Brazilian Amazon. The proportions of rDBP IgG positive subjects increased with exposure to malaria transmission (Table 2). The highest IgG seroreactivity to rDBP was found among gold miners from Apiacás (65%) who had long-term exposure to malaria. The group who had experienced a single P. vivax infection (Belém) had very low response to rDBP (14%). The levels of IgG antibodies against rDBP were also associated with malaria exposure, as the greatest levels were observed in gold miners from Apiacás (Figure 1A). In contrast, the proportions as well as the levels of IgG antibodies against rMSP119 were not correlated with exposure (Table 2, Figure 1B), the protein being immunogenic to a large proportion of individuals (66–73%).

Because individuals from TNN had experienced a variable number of previous episodes of clinical malaria, we reasoned that individuals who had been infected multiple times with malaria in TNN should exhibit higher frequency of antibodies against rPvDBP than those who had been single-infected. Eighteen (49%) of 37 individuals who had reported multiple infections developed anti-rDBP antibodies (Figure 2); on the other hand, none of the individuals who had reported a single malaria infection had antibodies that recognize the rDBP. The rMSP119 antibody response was similar in both groups (Figure 2).

In a further analysis, individuals from Apiacás and TNN who had been exposed to several previous malaria episodes were analyzed according to their last malaria episode, whether by P. vivax or P. falciparum. In both cases, the results show a non-significant tendency toward increased prevalence of anti-rDBP antibodies among individuals whose last infection was by P. vivax (P > 0.05 for all statistical comparisons) (Figure 3). The frequency of antibodies against the rMSP119 was similar between P. vivax and P. falciparum groups (data not shown).

We also analyzed individuals acutely infected with P. vivax in Apiacás who had (N = 13) or not (N = 15) developed clinical malaria after a follow-up of 72 hours. For both recombinant proteins, antibody response was higher in individuals with asymptomatic infection than in those suffering clinical symptoms of malaria, even though this difference was not of statistical significance (P > 0.05 for all comparisons) (Figure 4).

**DISCUSSION**

Direct evidence for naturally acquired antibodies to DBP was first demonstrated in residents of a highly endemic region in Papua New Guinea (PNG). This observation was subsequently confirmed in a low-endemic P. vivax malaria region in Colombia, where 40% of the individuals displayed specific antibodies to the PvDBP. Recently, data from a study in PNG have identified four dominant B-cell epitopes in the cystein-rich ligand domain (region II) of the protein.

In the current study, we have demonstrated that a recombinant protein containing region II-IV of the DBP (rDBP) is immunogenic in different populations of the Brazilian Amazon. In these areas, the prevalence and levels of antibodies to the rDBP showed marked differences according to the exposure to malaria transmission; higher antibody levels were associated with higher exposure to transmission. Thus, the frequency of responders to the rDBP was higher (65%) among those subjects with a long-term exposure to malaria in Apiacás when compared with subjects less exposed, including those sporadically exposed in Belém area (14%).

**TABLE 2**

Antibody response to the rDBP and rMSP119 in three groups of individuals exposed to different situations of malaria transmission (sporadic, variable, constant)

<table>
<thead>
<tr>
<th>Locality</th>
<th>Antibody response to (sporadic)</th>
<th>Belem (variable)</th>
<th>TNN (variable)</th>
<th>Apiacás (constant)</th>
<th>χ² linear trend, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rDBP</td>
<td>5/36 (14%)</td>
<td>18/47 (38%)</td>
<td>24/37 (65%)</td>
<td>19.75, P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>rMSP119</td>
<td>25/35 (71%)</td>
<td>31/47 (66%)</td>
<td>27/37 (73%)</td>
<td>0.024, P = 0.877</td>
<td></td>
</tr>
</tbody>
</table>

*Number of individuals with a positive antibody response, no./total (%).
*χ² linear trend, P

**FIGURE 1.** Effect of exposure to malaria transmission on levels of antibodies to the rDBP (A) or rMSP119 (B). IgG antibody responses are expressed as optical density (OD) detected by ELISA in sera from adults living in three areas of the Brazilian Amazon, which have different levels of malaria transmission (Apiacás > TNN > Belém). For both proteins, the threshold of positivity was an OD value of 0.1 (mean ± 3 standard deviations of unexposed controls subjects). Different letters on the top of the figure indicate significantly different means (P < 0.05) determined by analysis of variance with Tukey’s high significance degree post hoc test.
Previous studies demonstrate that anti-DBP antibodies increased significantly with age, suggesting a possible boosting of the DBP antibody response due to accumulated age-related exposure. Because host age affects both the quantitative and qualitative nature of the immune response to Plasmodium antigens, those studies carried out in highly endemic areas of PNG could not differentiate the effects of age from those of cumulative exposure. Because the groups studied in Belém, TNN, and Apiács were comparable with respect to their age and gender (most adults, males), our study allows a separation of the two effects, age and cumulative malaria exposure. We conclude that cumulative exposure, independent of host age, apparently represents a key determinant of the quantitative nature of the IgG response to P. vivax DBP. However, at this time, we cannot exclude an additional effect of the host age in this antibody response.

As expected, the serological responses to rDBP and rMSP119 were different in these samples from the Brazilian Amazon. Whereas rDBP fitted a pattern expected for a molecule that is less immunogenic or has restricted exposure to the host immune response, rMSP119 appeared to be highly immunogenic in the Amazon area. Also, there appeared to be little boosting effect to MSP119 from accumulated malaria exposure, a result that corroborates previous findings in PNG. Several reasons, not mutually exclusive, may account for the fact that a larger proportion of sera, including those from single-infected individuals, reacted with P. vivax MSP119. First, the P. vivax MSP119 encoding DNA displays

![Figure 2](image-url)  
**Figure 2.** Antibody response to the rDBP and rMSP119 detected in sera of individuals from Terra Nova do Norte (TNN) who had experienced a single malaria episode (single-infection) or a variable number of previous malaria episodes (multiple infections). IgG antibody responses are expressed as optical density (OD), as described in the legend of Figure 1. Values on the bottom of the figure represent the overall frequency of response for each group. For rDBP, there was a significant difference between single-infection and multiple-infections groups (*t* = 3.42, *P* < 0.001 for log-transformed OD values; **P** < 0.05 for proportions compared by Fisher’s exact test).

![Figure 3](image-url)  
**Figure 3.** Antibody response to the rDBP detected in sera of individuals from Apiács or TNN whose last malaria episode was by P. vivax (Pv) or P. falciparum (Pf). In TNN, only individuals who had reported a variable number of previous episodes of malaria were included for analysis; at the time of blood collection, all individuals were aparasitemic. IgG antibody responses are expressed as optical density (OD), as described in the legend of Figure 1. Values on the bottom of the figure represent the overall frequency of response for each group. Differences in mean OD values between Pv and Pf groups were not statistically significant (*t* = 0.12, *P* = 0.906 for Apiács; and *t* = 1.50, *P* = 0.142 for TNN). Also, there was no significant differences in proportions (Fisher’s exact test, *P* = 0.100 for Apiács; *χ²* value = 1.38, *P* = 0.240).
very limited allele polymorphism in different regions of the world. Second, DNA sequences encoding \( P. vivax \) MSP1\(_{19} \) are less variable compared with equivalent regions of other species of malaria parasites such as \( P. falciparum \). Consistent with this notion, our previous results in the Amazon area demonstrated that antibody response against a recombinant \( P. falciparum \) MSP1\(_{19} \) increased with the number of infections. Third, despite the existence of two allelic forms of MSP1\(_{19} \), antibodies are directed mainly to conserved epitopes present in both allelic forms. Consequently, a high frequency of responders to the C-terminal region of \( P. vivax \) MSP1 has also been described in other surveys performed in Brazil, Papua New Guinea, and Korea.

We detected a non-significant tendency toward an increased prevalence of anti-DBP antibodies among individuals whose last infection was by \( P. vivax \). In a follow-up of a population exposed to a \( P. vivax \) malaria outbreak, outside of the Amazon area, we have found that individuals serologically negative to the rDBP became positive after a \( P. vivax \) relapse (I. P. Cerávolo and L. H. Carvalho, unpublished results). These results suggest that boosting of the anti-DBP antibody response is achieved at the time of a new \( P. vivax \) episode. Because \( P. vivax \) relapses in Brazil are usually caused by the same parasite strain of the initial infection, at this point we cannot rule out the possibility that a booster with DBP needs to be strain-specific.

In the Brazilian Amazon area, instability of transmission is the dominant feature of malaria. The exposed populations consist of migrants mostly from malaria-free areas. In these individuals, the infection is generally accompanied by clinical symptoms of variable degrees of intensity. Nevertheless, during the past few years, epidemiologic studies carried out among individuals with long-term exposure to malaria in Brazil clearly shown the existence of symptomless infections by \( P. vivax \). In Apiacás area, asymptomatic malaria infections were identified among 7% of the gold miners followed for up to 2 months (Fontes CJF and others, unpublished results). Although the protective nature of the anti-rDBP antibody response was not the focus of the current work, the serum samples available from clinically and parasitologically defined subjects in Apiacás offered an excellent opportunity to correlate asymptomatic malaria and the IgG antibody response.

When we divided the \( P. vivax \) blood-smear positive individuals from Apiacás into asymptomatic (\( N = 15 \)) and symptomatic (\( N = 13 \)) groups, the former group had a higher IgG response against rDBP and rMSP1\(_{19} \) than did the symptomatic group. Although this observation failed to reach the level of statistical significance, it is in line with the results of a previous study in PNG, which shows an increase in the antibody response to DBP with age, concomitant with a decline in the prevalence and intensity of \( P. vivax \) infection. However, the low number of asymptomatic infections analyzed in our study precludes any strong conclusion about the existence of an association between antibodies to DBP and the development of clinical malaria. Unfortunately, the size of our sample could not be increased in Apiacás area; the difficulties for geographical access and the political conflicts, which often affect the gold-mining areas of the Brazilian Amazon, hampered the continuation of our study there. Furthermore, a failure to see a closer correlation between anti-DBP and protection against clinical malaria may have occurred because not all anti-DBP antibodies seem to have a functional role in inhibiting the interaction of DBP with erythrocytes; up to now, this anti-functional antibody response was demonstrated only in highly endemic regions of PNG, which is representative of only some tropical regions where \( P. vivax \) is prevalent.

In conclusion, we have demonstrated cumulative exposure as a determinant that acts independently of host age in generation of anti-DBP IgG responses among Brazilian migrants from the Amazon area. Studies are in progress to clarify further whether individuals in Brazil acquire antibodies that have the ability to block erythrocyte cytoadherence to DBP.

Figure 4. Antibody response to the rDBP and the rMSP1\(_{19} \) among 28 individuals infected with \( P. vivax \) in Apiacás who had (\( N = 13 \)) or not (\( N = 15 \)) developed symptomatic malaria. IgG antibody responses are expressed as optical density (OD), as described in the legend of Figure 1. Values on the figure represent the overall frequency of response for each group. Differences in mean OD values between asymptomatic and symptomatic groups were not statistically significant (\( \chi^2 = 1.29, P = 0.229 \) for rDBP; and \( \chi^2 = 2.0, P = 0.157 \) for rMSP1\(_{19} \)). Also, there were no significant differences in proportions (\( \chi^2 = 0.00, P = 0.950 \) for rDBP; and \( \chi^2 = 2.69, P = 0.101 \) for rMSP1\(_{19} \)).
Authors’ addresses: Isabel P. Cerávalo, Cristiana F. A. Brito, Antoniana U. Krettli, and Luzia H. Carvalho, Laboratório de Malária, Centro de Pesquisas René Rachou, FIOCRUZ, Av. Augusto de Lima 1715, CP 1743, 30190-002 Belo Horizonte, MG, Brazil. Oscar Bruiña-Romero and Érika M. Braga, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil. Cor J. F. Fontes, Hospital Júlio Muller, Universidade Federal de Mato Grosso, Rua L s/n, Jardim Alvorada, 78070-150 Cuiabá, MT, Brazil. José M. Souza, Instituto Evandro Chagas, Av. Almirante Barroso 492, 66900-000 Belém, PA, Brasil. John H. Adams, Department of Biological Sciences, University of Notre Dame, P.O. Box 369, Notre Dame, IN 46556-0369.

Reprint requests: Luzia H. Carvalho, Laboratório de Malária, Centro de Pesquisas René Rachou, FIOCRUZ, Av. Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil. Telephone: 55-31-32953566, Fax: 55-31-32953115, E-mail: lhcvalho@spqrr.fiocruz.br.

REFERENCES

32. Park JW, Moon SH, Yecos J, Lim KJ, Sohn MJ, Jung WC, Cho

