

**Ministério da Saúde
Fundação Oswaldo Cruz
Centro de Pesquisas René Rachou
Programa de Pós-graduação em Ciências da Saúde**

**Estudo de anticorpos inibitórios da interação
ligante-receptor na infecção pelo *Plasmodium vivax* em
populações expostas à malária na Amazônia brasileira**

por

Flávia Alessandra de Souza Silva

Belo Horizonte
Fevereiro/2010

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Foi avaliada pela banca examinadora composta pelos seguintes membros:

Prof. Dra. Luzia Helena Carvalho (Presidente)

Prof. Dra. Fabiana Maria de Souza Leoratti

Prof. Dra. Joseli de Oliveira Ferreira

Prof. Dr. Bernardo Simões Franklin (Suplente)

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“O que é necessário não é a vontade de acreditar, mas o desejo de descobrir, o que é exatamente o oposto”.

Bertrand Russell

Aos meus pais, irmã e ao Danilo pelo
carinho e amor em todos os
momentos.

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Lista de Abreviaturas e Símbolos

Acre-1 - Variante de DBPII de *P. vivax* isolado no Acre

CPqRR - Centro de Pesquisa René Rachou

COS-7 - Células do epitélio renal de símios transformadas pelo vírus SV-40

DARC - Antígeno do grupo sanguíneo *Duffy/DARC*; receptor para quimiocinas (*Duffy Antigen Receptor for chemokines*).

DBL - Domínio de ligação semelhante ao que se liga ao antígeno *Duffy/DARC* (*Duffy binding like domain*)

DBL-EBP's - Família de proteínas que se ligam aos eritrócitos e apresentam um domínio de ligação semelhante ao que se liga ao antígeno *Duffy/DARC* (*Duffy binding like domain Erythrocyte binding protein*)

DBP - Proteína de ligação ao eritrócito (*Duffy binding protein*)

DBP_{II} - Região II da *Duffy binding protein* de *P. vivax*.

DMEM - Meio essencial mínimo de Eagle modificado por Dulbeccos' (*Dulbeccos' Modified Eagle Medium*)

DNA - Ácido desoxirribonucleico

EDTA - Ácido etilenodiaminotetracético (*Ethylenediaminetetraacetic acid*)

EGFP - *Enhanced Green Fluorescent Protein*; proteína de fluorescência verde potencializada.

ELISA - Ensaio imunoenzimático (*Enzyme-linked immunosorbent assay*)

FIOCRUZ - Fundação Oswaldo Cruz

GST - glutationa S-transferase de *Schistosoma japonicum*

IgG - Imunoglobulina da classe G

kDa - Kilodalton

µg - Micrograma

µL - Microlitro

mL - mililitro

mM - Milimolar

nm - Nanômetro

OD - *Optical density*; densidade ótica

OPD - Cromógeno ortofenilenodiamino diidrocloreto (*o-phelylenediamine dihydrochloride*)

(ρ) - coeficiente de correlação de *Spearman*

pEGFP - Plasmídeo que codifica a proteína de fluorescência verde potencializada (*Enhanced Green Fluorescent Protein*)

PBS - Salina tamponada com fosfato (*Phosphate-buffered saline*)

PBS-T - Salina tamponada com fosfato acrescentada de 0,05% de Tween 20

PCR - Reação em Cadeia da Polimerase (*Polymerase Chain Reaction*)

PNCM - Programa Nacional de Controle da Malária

PNG - Papua Nova Guiné, Oceania

PNG 7.18 - Variante de DBP_{II} de *P. vivax* isolado na Papua Nova Guiné (VanBuskirk *et al.*, 2004a)

PNG 27.16 - Variante de DBP_{II} de *P. vivax* isolado na Papua Nova Guiné (VanBuskirk *et al.*, 2004a)

PvDBP - *Duffy binding protein* de *Plasmodium vivax* - proteína do *P. vivax* de ligação ao antígeno do grupo sanguíneo *Duffy/DARC*.

PvRBP - Proteína de ligação do *P. vivax* a reticulócitos (*P. vivax Reticulocyte Binding Protein*)

PvRBP-1 - Proteína 1 de *P. vivax* que se liga a reticulócito (*P. vivax Reticulocyte binding protein 1*)

PvRBP-2 - Proteína 2 de *P. vivax* que se liga a reticulócito (*P. vivax Reticulocyte binding protein 2*)

Sal-1 - Variante de DBP_{II} de *P. vivax* de um clone de referência de laboratório isolado em El Salvador (Fang *et al.*, 1991)

SBF - Soro Bovino Fetal (*Fetal bovine serum*)

TNN - Município de Terra Nova do Norte, Estado do Mato Grosso, Brasil

Tween 20 - Polioxietileno-sorbitano-monolaurato (*Polyoxyethylenesorbitan monolaurate*)

UI - Unidade Internacional

USP - Universidade de São Paulo

X² - teste estatístico qui-quadrado

Resumo

A *Duffy binding protein* do *Plasmodium vivax* (PvDBP) é uma proteína essencial para o processo de invasão do *Plasmodium vivax* em eritrócitos humanos Duffy/DARC positivos, sendo consequentemente uma forte candidata à vacina antimalárica. Contudo estudos sobre a resposta imune à PvDBP tem sido conduzidos principalmente em áreas hiperendêmicas da Papua Nova Guiné. Nos últimos anos, nosso grupo de pesquisa vem demonstrando que indivíduos expostos à malária na Amazônia brasileira desenvolvem uma resposta imune humoral contra a PvDBP (Cerávolo *et al.*, 2005). No presente trabalho, para investigar se os anticorpos detectados pela sorologia convencional (ELISA) incluíam aqueles capazes de bloquear a interação ligante-receptor, foram utilizados ensaios funcionais *in vitro*, onde células de mamíferos (COS-7) expressando a região do ligante (região II da PvDBP) formam rosetas na presença de eritrócitos *Duffy* positivos (receptor). Os resultados obtidos permitiram demonstrar pela primeira vez que indivíduos com história de longa exposição à malária em áreas de transmissão instável, como a Amazônia brasileira, desenvolvem anticorpos inibitórios, isto é, capazes de bloquear a interação entre o ligante do parasito e seu receptor presente na superfície da célula hospedeira. Para caracterizar esta resposta imune anti-PvDBP e verificar se esta resposta está associada à imunidade clínica, foi realizado um estudo de coorte entre 366 indivíduos de um assentamento agrícola no Estado do Acre. Na linha de base, amostras de soro de apenas cerca de 20% dos indivíduos apresentaram anticorpos anti-PvDBP detectados pelo ELISA, sendo observado que, entre outros fatores, o tempo de exposição ao *P. vivax* estava mais associado à presença desses anticorpos. Nesta população, foi possível demonstrar que apenas um terço dos indivíduos com anticorpos no ELISA apresentaram anticorpos inibitórios. Neste grupo, a resposta de anticorpos inibitórios não se correlacionou com a cepa do parasito, já que os níveis de inibição foram semelhantes com células COS-7 expressando a região II de diferentes variantes da PvDBP. De interesse, embora a maioria dos indivíduos estudados não apresentaram anticorpos inibitórios, quando estes foram adquiridos, se mantiveram estáveis ao longo do período estudado (\cong 12 meses). Por outro lado, a associação entre presença de anticorpos funcionais anti-PvDBP e a imunidade clínica não pôde ser avaliada devido ao pequeno número de indivíduos que apresentaram anticorpos inibitórios. Diante disso, faz-se necessária a realização de novos estudos em populações semi-imunes para entender porque apenas poucos indivíduos expostos à infecção pela malária desenvolvem anticorpos inibitórios e quais são os fatores envolvidos na aquisição da proteção contra a infecção e/ou a doença clínica.

Abstract

Duffy binding protein (DBP), a leading malaria vaccine candidate, plays a critical role in *Plasmodium vivax* erythrocyte invasion. Antibody recognition of DBP has been described in individuals exposed to hyperendemic malaria, 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, also naturally acquired antibodies to DBP II may block DBP II - DARC interaction and inhibit erythrocyte invasion in vitro (Cerávolo *et al.*, 2008). In the present study, we examined the ability of sera from different populations of the Brazilian Amazon – an area of markedly unstable malaria transmission – to inhibit the erythrocyte-binding function of the DBP ligand domain (region II, DBPII). We found that long-term exposure to malaria in the Amazon area elicits DBP-specific antibodies that inhibit the binding of different DBPII variants to erythrocytes. Further to test whether the presence of DBP inhibitory antibodies are associated with protection from blood-stage *P. vivax* infection we performed a prospective cohort study of 366 subjects of agricultural frontier settlements across the Amazon basin. Sixty-eight of 366 (18.6%) subjects had IgG anti-DBP antibodies by enzyme-linked immunosorbent assay (ELISA). Cumulative exposure to malaria was the strongest predictor of DBP seropositivity identified by multiple logistic regression models in this population. 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). Of relevance, both the frequency and levels of inhibitory antibodies to different DBP_{II} variants were quite similar. 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). 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. 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.

1 Introdução

A malária é uma das doenças infecciosas mais prevalentes no mundo, atingindo 105 países, infectando cerca de 500 milhões de pessoas e causando mais de um milhão de mortes anuais, principalmente entre crianças jovens, gestantes e adultos não-imunes (Greenwood *et al.*, 2008; WHO, 2008).

Esta parasitose tem como agente etiológico um protozoário do filo Apicomplexa pertencente ao gênero *Plasmodium*. Sabe-se que a doença humana é causada por quatro espécies: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, sendo esta última espécie limitada principalmente ao continente Africano e parte do sudeste asiático (Mueller *et al.*, 2008). Entretanto, estudos recentes trazem forte evidência de que o *P. Knowlesi*, comumente tido como parasito de primatas não humanos, infecta populações humanas, principalmente na Ásia (White, 2008; Oon *et al.*, 2008; Peter *et al.*, 2009).

Das quatro espécies principais de protozoários causadores da malária humana, o *P. vivax* é o mais difundido sendo responsável por significativa morbidade no sul e sudeste da Ásia, Oceania e América Latina (Baird *et al.*, 2007). Embora a malária causada por *P. vivax* seja conhecida como benigna e raramente fatal, vários estudos clínicos recentes mostram que esta espécie pode causar anemia severa, edema pulmonar e disfunção hepática (Tjitra *et al.*, 2008; Genton *et al.*, 2008; Fernandez-Becerra *et al.*, 2009; Barcus *et al.*, 2007; Kochar *et al.*, 2009).

O Brasil é responsável por cerca de 70% de um milhão de casos clínicos de malária registrados anualmente nas Américas (Breman & Holloway, 2007). Atualmente, a incidência de casos de malária no Brasil é de cerca de 300 mil casos anuais, sendo que mais de 99% das infecções adquiridas ocorrem essencialmente na Amazônia Legal (Estados do Acre, Amapá, Amazonas, Pará, Rondônia, Roraima, Tocantins, Mato Grosso e Maranhão), onde o *P. vivax* é responsável por aproximadamente 80% dos casos (SVS, 2008).

Na Amazônia Legal, as condições socioeconômicas e ambientais têm contribuído para a alta transmissão de malária. Além disso, durante décadas, a ocupação intensa e desordenada dos espaços peri-urbanos, desmatamento para extração de madeira, criação de gado, agricultura e assentamentos não-oficiais, tem agravado a transmissão de malária nessa região (Marques *et al.*, 1986; SVS, 2007; SVS, 2008). Outro fator colaborador é o aumento dos criadouros do mosquito vetor em função da atividade de piscicultura, com a construção de tanques artificiais (SVS, 2007). Por outro lado, a região extra Amazônica, apesar de não ser considerada área endêmica para malária, apresenta

uma média anual de cerca de 200 casos autóctones da doença (SVS, 2008). Nessa região, a transmissão da infecção se deve, em grande parte, ao fluxo constante de pessoas infectadas provenientes de áreas endêmicas dentro e fora do país, associado à existência dos vetores anofelinos (SVS, 2008).

Atualmente, está em vigência o Programa Nacional de Controle da Malária (PNCM) no Brasil, cujos objetivos são a redução da incidência e mortalidade, a eliminação da transmissão em áreas urbanas e manutenção da ausência da transmissão da doença nos locais onde ela foi interrompida. Para alcançar esses objetivos tem-se como prioridade o diagnóstico precoce, o tratamento oportuno, o fortalecimento da vigilância em malária e a capacitação de recursos humanos (SVS, 2007). Essas medidas de controle, associadas a uma série de outros fatores, como maior investimento de recursos e mudança no esquema de tratamento de primeira escolha da malária por *P. falciparum*, têm contribuído para uma redução do número de casos de malária nos últimos anos no país (SVS, 2008).

Recentemente foi publicado um estudo detalhado da distribuição do risco de malária por *P. falciparum* no mundo (Hay *et al.*, 2009). Iniciativas como essa são importantes para permitir a alocação eficiente de recursos visando uma intervenção mais eficaz no controle da malária. Faz-se necessário um estudo similar para o *P. vivax*, o que poderá contribuir para os esforços atuais no controle desta espécie, particularmente na América Latina e continente asiático.

1.1 Ciclo biológico dos parasitos da malária humana

Em diferentes momentos do seu ciclo de vida, o plasmódio sofre várias transformações que o capacitam a transpor diversas barreiras e se desenvolver em ambientes variados. Entre as mudanças estão o desenvolvimento do parasito em diferentes estágios, tais como móvel, invasivo, encistado, intracelular, sexuado e dormente (Greenwood *et al.*, 2008).

Em geral, o ciclo biológico dos parasitos da malária humana pode ser dividido em duas fases: uma fase assexuada ou esquizogônica, que se passa no hospedeiro vertebrado e outra sexuada ou esporogônica, que se passa em fêmeas do mosquito do gênero *Anopheles*. A fase assexuada ou esquizogônica se inicia durante o repasto sanguíneo das fêmeas de anofelinos, onde as formas infectantes (esporozoítos) são depositadas na pele do hospedeiro, podendo permanecer neste local por várias horas antes de encontrar um vaso sanguíneo (Amino *et al.*, 2006; Prudêncio *et al.*, 2006;

Yamauchi *et al.*, 2007). Recentemente, alguns autores descreveram uma nova via de migração dos esporozoítos, em que os mesmos, após atravessarem o epitélio do hospedeiro vertebrado, podem também atingir o sistema linfático. Entretanto, os parasitos não parecem atingir o fígado por esta via (Amino *et al.* 2006). Uma vez dentro do sistema circulatório, os esporozoítos atingem o fígado, onde infectam os hepatócitos.

O processo de invasão dos hepatócitos é complexo e depende de várias interações do tipo ligante-receptor. Recentemente foi demonstrado que os esporozoítos migram através de vários hepatócitos, antes de se desenvolverem dentro de um hepatócito específico, onde ocorre a formação de um vacúolo parasitóforo bem delimitado (Mota *et al.*, 2001; Frevert *et al.*, 2005). Uma vez dentro dos hepatócitos, os esporozoítos se diferenciam em trofozoítos que, após sofrerem várias divisões por esquizogonia, formam os esquizontes. Nas infecções por *P. vivax* e *P. ovale* algumas populações de parasitos se desenvolvem rapidamente nos hepatócitos, enquanto outras, as responsáveis pelos casos de recaídas (hipnozoítas), permanecem em estado de latência no fígado, meses ou até mesmo anos após a infecção inicial (Krotoski, 1985). Os esquizontes maduros liberam os merozoítos teciduais através de um processo de brotamento de vesículas (merosomas), que após atingirem a corrente sanguínea, repletos de parasitas, liberam os merozoítos (Sturm *et al.*, 2006). Os merozoítos teciduais invadem as hemácias iniciando assim a fase eritrocítica, que é responsável pelos sintomas clínicos da malária. Para que o merozoíto invada o eritrócito é necessário que haja também o reconhecimento inicial de receptores específicos (ver item 1.1.1). Após várias gerações de merozoítos sanguíneos, alguns se diferenciam dando origem a formas sexuadas, os gametócitos masculinos e femininos, os quais amadurecem sem sofrer divisão celular.

A fase sexuada ou esporogônica inicia-se quando os gametócitos são ingeridos pelo mosquito susceptível, dando início à jornada do parasita no hospedeiro invertebrado. Dentro do estômago do mosquito, os gametócitos masculinos e femininos se diferenciam transformando-se em gametas (Revisto por Vlachou *et al.*, 2006). Aproximadamente um dia após a fecundação, o zigoto se desloca com movimentos amebóides, passando a se denominar oocineto. O oocineto por sua vez atravessa a parede intestinal do mosquito por um mecanismo trans-celular (Barillas-Mury & Kumar, 2005) alojando-se na membrana basal onde se diferencia em oocisto. No interior do oocisto ocorrem diferenciação e divisão nuclear que culmina com a produção de esporozoítos (esporogonia) e em aproximadamente duas semanas, dependendo da espécie do plasmódio, a parede do mesmo se rompe liberando esporozoítos que invadem a

hemolinfa do inseto. Assim, muitos parasitas migrarão até atingir as glândulas salivares, completando o ciclo evolutivo dos plasmódios no hospedeiro invertebrado.

1.1.1 Processo de invasão do eritrócito pelo *Plasmodium vivax*

O processo de invasão do eritrócito pelos plasmódios é complexo e composto por uma série de interações específicas entre o parasito e a célula hospedeira. Resumidamente, este processo pode ser dividido em quatro fases: (1) reconhecimento e adesão reversível do merozoíto à membrana do eritrócito após colisão aleatória; (2) reorientação do parasito para que o seu complexo apical entre em contato com a membrana eritrocítica, formando uma junção irreversível entre as membranas do parasito e do eritrócito; (3) deslocamento da junção em direção ao pólo posterior do parasito com a concomitante liberação do conteúdo das organelas apicais, principalmente das roptrias e dos micronemas; (4) entrada do parasito no interior do eritrócito através da formação do vacúolo parasitóforo, no interior do qual o merozoíto se desenvolve (Cowman & Crabb, 2006; Oh & Chishti, 2005). Apesar do evento inicial que envolve o reconhecimento e adesão do parasito seja formado por interações de baixa afinidade, os eventos seguintes envolvem interações do tipo ligante-receptor de alta afinidade, e a partir desse ponto, o processo de invasão passa a ser irreversível. Embora todos os plasmódios utilizem esse mecanismo geral de entrada na célula hospedeira, que inclui a participação de organelas apicais do parasito, algumas espécies têm características únicas. No caso das espécies mais importantes do ponto de vista da saúde pública, *P. falciparum* e *P. vivax*, o processo de invasão se encontra melhor estudado. Numerosos estudos têm demonstrado que merozoítos de *P. falciparum* têm a habilidade de invadir os eritrócitos através de, pelo menos, cinco vias de invasão, sendo estas classificadas de acordo com a natureza do receptor no eritrócito (Gaur *et al.*, 2004). Algumas vias são dependentes de sialoglicoproteínas (glicoforinas A, B, C/D), e outras de receptores de eritrócitos ainda não conhecidos, denominados X, Y e Z (Chung *et al.*, 2008). A via mais bem caracterizada é aquela que envolve uma proteína da família EBAs (*erythrocyte-binding antigens*) denominada EBA-175, que se encontra localizada nos micronemas do parasito (Camus & Hadley, 1985), e que se liga a glicoforina A, (Sim *et al.*, 1994). Além dessa via, existem outras também importantes envolvidas no processo de invasão, que inclui proteínas da mesma família da EBA-175, tais como: BAEBL (EBA-140), JESEBL (EBA-181), e de outra família de proteínas PfRh (*reticulocyte-binding homolog*) tais como PfRh (1, 2a, 2b e 4) (Camus and Hadley, 1985; Duraisingh *et al.*, 2003; Gilberger *et al.*, 2003; Maier *et al.*, 2003; Mayer *et al.*, 2001; Rayner *et al.*, 2001; Stubbs *et al.*, 2005;

Thompson *et al.*, 2001; Triglia *et al.*, 2001, 2005). Além destas proteínas, existem outras envolvidas no processo de reconhecimento inicial do eritrócito, sendo melhor caracterizadas a AMA-1 (*apical membrane antigen*) e MSP1 (*merozoite specific protein 1*) (Cowman & Crabb, 2006). Entretanto, no caso do *P. vivax*, uma via de invasão parece ser a mais importante (Adams *et al.*, 1992).

Para que o merozoíto do *P. vivax* invada o eritrócito é necessária a interação da *Duffy binding protein* (PvDBP) e seu receptor presente na superfície dos eritrócitos – o antígeno do grupo sanguíneo Duffy, receptor para quimiocinas (DARC, *Duffy Antigen Receptor for chemokines*) (Fang *et al.*, 1991; Adams *et al.*, 1992; Horuk *et al.*, 1993). Dessa forma, indivíduos que não apresentam esse receptor em seus eritrócitos são altamente resistentes à infecção pelo *P. vivax* (Miller *et al.*, 1976). Entretanto, evidências recentes demonstraram que alguns indivíduos DARC negativos do oeste africano (Quênia) e do Brasil podem se infectar com esse parasito (Ryan *et al.*, 2006; Cavasini *et al.*, 2006; 2007), o que sugere uma via de invasão alternativa.

O *P. vivax* invade preferencialmente, se não exclusivamente, os reticulócitos (eritrócitos jovens), que compreendem uma minoria ($\cong 1\%$) da população de eritrócitos circulantes (Galinski *et al.*, 1992; Barnwell & Galinski, 1998). Isto significa que os merozoítos de *P. vivax* interagem com inúmeros eritrócitos maduros na circulação antes de invadirem um reticulócito. Tem sido proposto que interações prematuras entre a PvDBP e o antígeno do grupo sanguíneo DARC em eritrócitos maduros seriam prejudiciais para a sobrevivência do parasito (Barnwell *et al.*, 1989). Desta forma, acredita-se que antes da formação da junção merozoíto-eritrócito haveria uma seleção da célula hospedeira, impedindo, assim, a interação da PvDBP com uma célula não-alvo (Galinski *et al.*, 1992; Barnwell & Galinski, 1998). Se essa suposição estiver correta, ou seja, se existe a seleção dos reticulócitos antes da formação da junção, a especificidade de invasão de *P. vivax* poderia ser atribuída a pelo menos duas proteínas de ligação aos reticulócitos: PvRBP-1 e PvRBP-2 (*reticulocyte binding protein*, RBP) (Galinski *et al.*, 1992). Entretanto, até o momento, as funções dessas proteínas ainda necessitam ser esclarecidas já que proteínas homólogas das PvRBP também já foram encontradas em *P. falciparum* e *P. yoelli*, duas espécies de *Plasmodium* que infectam tanto reticulócitos quanto eritrócitos (Galinski *et al.*, 1992).

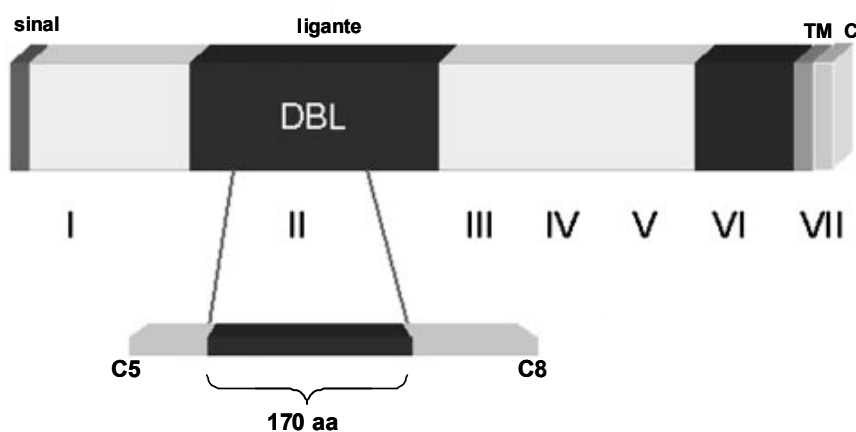
Diante do exposto, o fato da PvDBP ser importante para garantir o parasitismo humano, bem como as evidências de que a maioria dos indivíduos DARC negativos são resistentes à infecção por esse parasito, fazem desta proteína um dos antígenos

mais promissoras para o desenvolvimento de uma vacina contra a infecção causada pelo *P. vivax*.

1.2 Duffy Binding Protein de *Plasmodium vivax* (PvDBP)

A PvDBP é uma proteína de invasão que possui 140KDa sendo pertencente a uma família de proteínas homólogas que se ligam a eritrócitos – *Duffy binding like domain Erythrocyte binding protein* (DBL-EBP's) (Barnwell & Wertheimer, 1989; Adams *et al.*, 1992).

A PvDBP pode ser dividida em sete regiões, definidas a partir da similaridade da sua estrutura gênica e seqüência de aminoácidos com as outras DBL-EBPs (**Figura 1**). As regiões que constituem a PvDBP são: uma região que contém o peptídeo sinal (região I), duas regiões ricas em cisteínas amino e carboxiterminal (região II e VI, respectivamente) – sendo conservadas em todos os membros da superfamília DBL-EBP's - , três regiões hidrofílicas (região III, IV e V), um domínio transmembrana e um curto segmento citoplasmático (região VII) (Adams *et al.*, 1992; Fang *et al.*, 1991). O ligante funcional da PvDBP encontra-se localizado na região II da PvDBP e apresenta cerca de 300 aminoácidos, sendo seu sítio de ligação ao eritrócito situado em um fragmento de 170 aminoácidos que contém as cisteínas 5 a 8 (Ranjan and Chitnis, 1999; Singh *et al.* 2003).



(Adaptado de Vanbuskirk *et al.*, 2004b)

Figura 1: Representação esquemática dos domínios estruturais importantes da *Duffy binding protein* de *P. vivax*. Na figura, estão representadas as sete regiões, sendo seis extracelulares e uma transmembrana (indicadas em algarismos romanos). Esta proteína é caracterizada por um domínio N-terminal rico em cisteína, o domínio de ligação DBL (região II), e um segundo

domínio conservado rico em cisteína (região VI). O principal sítio de ligação para o reconhecimento do receptor (170 aa), é a porção central da PvDBP entre as cisteínas 5 (C5) e 8 (C8), sendo a porção mais polimórfica da proteína.

Embora a posição dos resíduos de cisteína seja conservada na região II, já foi demonstrado que outros aminoácidos são altamente polimórficos (Ampudia *et al.*, 1996). Este polimorfismo parece variar de acordo com a área geográfica. Estudos da variabilidade genética da região II foram realizados em isolados de *P.vivax* da Papua Nova Guiné (Tsuboi *et al.*, 1994; Xainli *et al.*, 2000), Colômbia (Ampudia *et al.*, 1996), Coreia do Sul (Kho *et al.*, 2001; Suh *et al.*, 2001), Tailândia (Gosi *et al.*, 2008) e, recentemente, no Brasil pelo nosso grupo (Sousa *et al.*, 2006; Sousa, 2009). Nestes estudos foi observado um alto padrão de polimorfismos não-sinônimos o que sugere a existência de uma pressão seletiva do sistema imune do hospedeiro como mecanismo de evasão pelo parasito.

Como a invasão dos eritrócitos por merozoítos é um processo complexo e dependente de interações entre ligantes e receptores, a caracterização desses componentes é essencial para o entendimento da dinâmica molecular do mecanismo de invasão. Sendo assim, conhecer os mecanismos imunes que permitem bloquear a invasão do merozoíto na célula hospedeira, poderia auxiliar na descoberta de um mecanismo que permitisse a redução, ou mesmo a eliminação dos parasitos sanguíneos da malária, e, conseqüentemente, impedir o estabelecimento da doença clínica.

1.3 Padrão de transmissão de malária e aquisição de imunidade clínica

Historicamente, as áreas de transmissão de malária têm sido classificadas como de transmissão estável ou instável (MacDonald, 1957). As áreas de transmissão estável são aquelas nas quais os indivíduos estão constantemente expostos à doença sendo acometidos ao longo do ano, por centenas de picadas infectantes dos vetores anofelinos. Por outro lado, em áreas de transmissão instável, os indivíduos estão expostos à infecção pelo plasmódio de forma menos intensa e irregular, sendo a transmissão focal, sazonal e, geralmente, relacionada com a atividade ocupacional (Atanaka-Santos *et al.*, 2006; SVS, 2008). Nestas áreas, os indivíduos estão sujeitos a poucas picadas infectantes ao longo do ano (Carter & Mendis, 2002).

Em áreas de transmissão estável, onde o *P. falciparum* é a espécie predominante, os recém-nascidos estão protegidos da malária grave, durante os primeiros seis meses de vida, devido, principalmente, à transferência passiva de anticorpos da mãe para o filho (Collins *et al.*, 1977; Chizzolini *et al.*, 1991). Após esse período, as crianças se tornam altamente susceptíveis à malária grave, sendo frequentes as infecções fatais nos primeiros 3 anos de vida. Com a idade, as crianças que vivem em condições de alta transmissão sofrem progressivamente menos episódios de malária aguda, embora possam apresentar ainda altas parasitemias (Mshana *et al.*, 1993). A razão desta aparente “tolerância” contra altas parasitemias ainda é desconhecida, e tem sido sugerido que isto pode refletir um estado de imunidade “anti-tóxica”, isto é, imunidade contra componentes solúveis do parasito que causam a doença (Playfair *et al.*, 1990). À medida que os indivíduos vão se tornando adultos, passam a apresentar sintomas menos pronunciados e baixa parasitemia, gerando uma imunidade “anti-parasito”. Entretanto, mesmo após muitos anos de exposição, os indivíduos residentes em áreas de transmissão estável são ainda susceptíveis à infecção, mostrando que tal imunidade não é completamente efetiva, podendo ser perdida com o afastamento da área endêmica e de novas infecções pelo parasito. Não estão bem esclarecidos os mecanismos imunes responsáveis pelo desenvolvimento e manutenção desta imunidade naturalmente adquirida, e, provavelmente, muitos tipos celulares, anticorpos de diferentes especificidades e uma cascata de citocinas estão envolvidos (Stevenson & Zavala, 2006; Langorne *et al.*, 2008). A caracterização dos mecanismos responsáveis por esta imunidade efetiva é dificultada devido, entre outros fatores, ao complexo ciclo de vida do plasmódio, ao fato de que a imunidade parece ser espécie e estágio-específica, considerável diversidade do parasito e a localização intracelular do parasito em algumas fases do seu ciclo (David *et al.*, 1988; Day & Marsh, 1991; Carter & Mendis, 1992).

Em áreas de transmissão instável, indivíduos de todas as idades são potencialmente susceptíveis à infecção pelo plasmódio. Em geral, considera-se que na Amazônia brasileira a transmissão da malária é de média e baixa intensidade sendo considerada área de transmissão instável (Camargo *et al.*, 1994, 1996). Nestas áreas, a população é constituída principalmente por adultos não-ímmunes migrantes de áreas livres de transmissão e sintomas clínicos de diferentes intensidades ocorrem quando os indivíduos se infectam por *P.falciparum* e/ou *P. vivax* (Tauil, 2006). Entretanto, tem sido relatado recentemente que indivíduos expostos à malária no Brasil podem apresentar o parasito circulante no sangue, e não manifestar sintomas clínicos. No Brasil, esta

infecção assintomática tem ocorrido entre indivíduos que apresentam história de longa exposição à transmissão de malária (Fontes, 2001; Alves *et al.*, 2002; Camargo *et al.*, 1999; Ladeia-Andrade, 2005). A definição de caso assintomático ainda não é bem determinada, pois o período de acompanhamento da ausência de sintomas tem variado de 72h (Andrade *et al.*, 1995; Fontes, 2001) até 30-60 dias (Alves *et al.*, 2002). Caracterizar os mecanismos imunes protetores que são induzidos pelo plasmódio em humanos, pode nos ajudar a desenvolver medidas de controle da doença nestas áreas de transmissão instável.

2 Justificativa

A malária causada pelo *P. vivax* atinge milhões de pessoas no mundo, resultando em importantes perdas sociais e econômicas, sendo que no Brasil, esta é a espécie mais prevalente (SVS, 2008). Aqui, a maior parte dos casos de malária é encontrada na região da Amazônia Legal, onde existe uma população migrante e dispersa, ainda com pouco acesso ao diagnóstico e tratamento imediato. Estes fatores, associados às condições favoráveis de transmissão de malária na região, constituem as maiores barreiras para o controle da doença no país. Dessa forma, estudos visando entender a resposta imune aos vários antígenos do parasito são de fundamental importância para o desenvolvimento de uma vacina antimalárica, considerada como uma ferramenta adicional para a luta contra a malária.

A PvDBP pode ser considerada uma importante candidata à vacina antimalárica, pois é o ligante que permite o parasito invadir os eritrócitos, garantindo o parasitismo humano. Entretanto, o fato desta proteína ser pouco abundante no parasito e a existência de limitações no cultivo do *P. vivax in vitro*, fazem com que estudos referentes a esta proteína e a resposta imune que ela é capaz de induzir ainda sejam limitados, tendo sido realizados, principalmente, em áreas de alta transmissão de malária (Fraser *et al.*, 1997; Michon *et al.*, 2000; Xainli *et al.*, 2002, 2003).

Estudos realizados em áreas de alta transmissão de malária, particularmente na Papua Nova Guiné, indicam que anticorpos anti-PvDBP_{II} são capazes de bloquear a interação entre o ligante e seu receptor DARC presente nos eritrócitos (Michon *et al.*, 2000), e conseqüentemente, impedir a invasão (Grinberg *et al.*, 2007). Entretanto, apesar desta resposta de anticorpos anti-PvDBP estar melhor caracterizada em indivíduos residentes em áreas de transmissão estável, nosso conhecimento a respeito da resposta imune anti-PvDBP em indivíduos residentes em áreas de baixa transmissão permanecem escassos (Cerávolo *et al.*, 2005; Tran *et al.*, 2005). Desta forma, é de fundamental importância estudar a resposta de anticorpos bloqueadores em áreas de transmissão instável, como é o caso da Amazônia brasileira. Além disso, a maioria dos estudos sobre resposta imune contra a malária tem avaliado anticorpos contra antígenos recombinantes, utilizando-se a sorologia convencional, sem avaliar a importância funcional destes anticorpos (Marsh & Kinyanjui, 2006; Persson *et al.*, 2006).

Em um estudo recente, realizado com crianças na Papua Nova Guiné, foi demonstrado que crianças que apresentavam altos títulos de anticorpos anti-PvDBP funcionais se infectavam com menor freqüência pelo *P. vivax*, sugerindo desta forma a

aquisição de proteção (King *et al.*, 2008). Assim, é de grande relevância verificar se a presença de anticorpos bloqueadores da interação PvDBP-DARC está relacionada com a imunidade clínica em indivíduos residentes na Amazônia brasileira, o que poderia ser feito através de um estudo de base populacional com acompanhamento longitudinal.

3 Objetivos

3.1 Objetivo Geral

Caracterizar a resposta imune anti-PvDBP, com ênfase em anticorpos bloqueadores da interação ligante-receptor, em populações expostas à malária na Amazônia brasileira e bem caracterizadas do ponto de vista epidemiológico.

3.2 Objetivos Específicos

Avaliar a presença de anticorpos IgG anti-PvDBP bloqueadores da invasão em soros de indivíduos expostos a diferentes condições de transmissão de malária na Amazônia brasileira;

Realizar um estudo de base populacional, do tipo prospectivo, para identificar as variáveis associadas ao desenvolvimento de uma resposta imune anti-PvDBP;

Verificar se existe relação entre os níveis de anticorpos anti-PvDBP detectados no ELISA, e a presença de anticorpos bloqueadores da interação ligante-receptor;

Relacionar a ação bloqueadora de anticorpos anti-PvDBP com a presença de infecção assintomática em indivíduos com história de longa exposição à malária.

4 Materiais e Métodos

4.1 Estudo transversal e coorte

O presente estudo envolveu duas abordagens epidemiológicas diferentes: um estudo do tipo transversal e um estudo de coorte. Inicialmente, para verificar a presença de anticorpos funcionais contra a PvDBP na Amazônia brasileira fez-se necessário um estudo de corte transversal. Em uma segunda etapa, visando caracterizar melhor a resposta imune identificada, foi realizado uma coorte aberta, na qual incluiu-se dois cortes transversais da população (linha de base e \cong 12 meses depois).

4.1.1 Estudo transversal, voluntários e coleta de sangue

O estudo de corte transversal foi realizado com amostras de soros/plasmas que se encontravam armazenados em um banco de soros do Laboratório de Malária do Centro de Pesquisa René Rachou, Fundação Oswaldo Cruz (CPqRR, FIOCRUZ), originadas de três áreas previamente bem caracterizadas da Amazônia brasileira e cujos indivíduos foram expostos a diferentes níveis de transmissão de malária (Carvalho *et al.*, 1997, 1999; Fontes 2001; Braga *et al.*, 2002). O primeiro grupo era composto por indivíduos residentes em Belém (n = 36), capital do estado do Pará, e que tinham apresentado uma única infecção de malária por *P. vivax* após uma curta viagem a uma ilha localizada próxima à capital, Cotijuba, onde os níveis de transmissão de malária são baixos e instáveis. O segundo grupo foi composto por indivíduos que residiram por aproximadamente 10 anos em uma comunidade rural de Mato Grosso, Terra Nova do Norte (TNN) (n = 47), onde a malária é endêmica e a transmissão intermitente, tendo os indivíduos relatado um número variável de episódios clínicos de malária causada pelo *P. vivax* e/ou *P. falciparum*. O terceiro grupo foi formado por indivíduos que se encontravam por aproximadamente 20 anos em diferentes regiões de garimpo de ouro em áreas da Amazônia brasileira, onde a malária é endêmica e a transmissão é constante, e que, no momento da coleta de sangue, residiam em garimpos de Apiacás, MT (n = 37).

Dos voluntários selecionados para o estudo foi coletado sangue total (\cong 10mL) obtido após assinatura de um termo de consentimento livre e esclarecido. Para a coleta dessas amostras, os critérios gerais de inclusão no estudo foram: (1) história de exposição prévia à malária; (2) ausência de sinais ou sintomas relacionados à malária

grave; (3) idade > 15 anos e, em caso do sexo feminino, (4) um indicativo de ausência de gravidez. Por ocasião da coleta de sangue, de todos os indivíduos foram confeccionadas gotas espessas e esfregaços sanguíneos sendo que aqueles que apresentavam parasitos circulantes foram encaminhados para tratamento imediato nos serviços de saúde locais, onde a supervisão era feita por médicos credenciados e que colaboram há vários anos com a equipe do Laboratório de Malária do CPqRR (Dr. C.J.F. Fontes, Universidade Federal de Mato Grosso, Cuiabá, MT e Dr. J.M. Souza, Instituto Evandro Chagas, Belém, PA). De grande relevância, em Apiacás foi observado que alguns indivíduos que apresentaram diagnóstico microscópico positivo para o plasmódio, ou seja, estavam na fase aguda da infecção, eram assintomáticos (Fontes, 2001). Assim, foram incluídos ainda no estudo 15 indivíduos com infecção assintomática e 10 indivíduos com infecção sintomática, diagnosticados por gota espessa.

Os aspectos éticos e metodológicos deste estudo foram aprovados pelo Comitê de Ética de Pesquisas em Seres Humanos do Centro de Pesquisa René Rachou (Protocolo N. 002/2002, N. 01/2006 e N. 07/2006) de acordo com a resolução do Conselho Nacional de Saúde 196/96, e pelo Secretariat Committee for *Research Involving Human Subjects* (SCRIHS) da Organização Mundial da Saúde.

4. 1. 2 Estudo de coorte

Para realização de um estudo de base populacional do tipo prospectivo foi escolhida uma comunidade rural do estado do Acre, denominada Ramal do Granada, localizada no assentamento Pedro Peixoto, município de Acrelândia (**Figura 2**). O perfil de transmissão de malária nesta área, caracterizada como de baixa transmissão de malária, foi recentemente descrita pelo grupo de pesquisa do Dr. Marcelo Urbano Ferreira (USP), que vem trabalhando na área há cerca de 8 anos (Silva-Nunes *et al.*, 2006, 2008). A maioria dos indivíduos era migrante de área livre de malária, principalmente do Sul e Sudeste do Brasil, se infectado em locais desmatados utilizados para a agricultura; principal atividade desenvolvida por esta população.

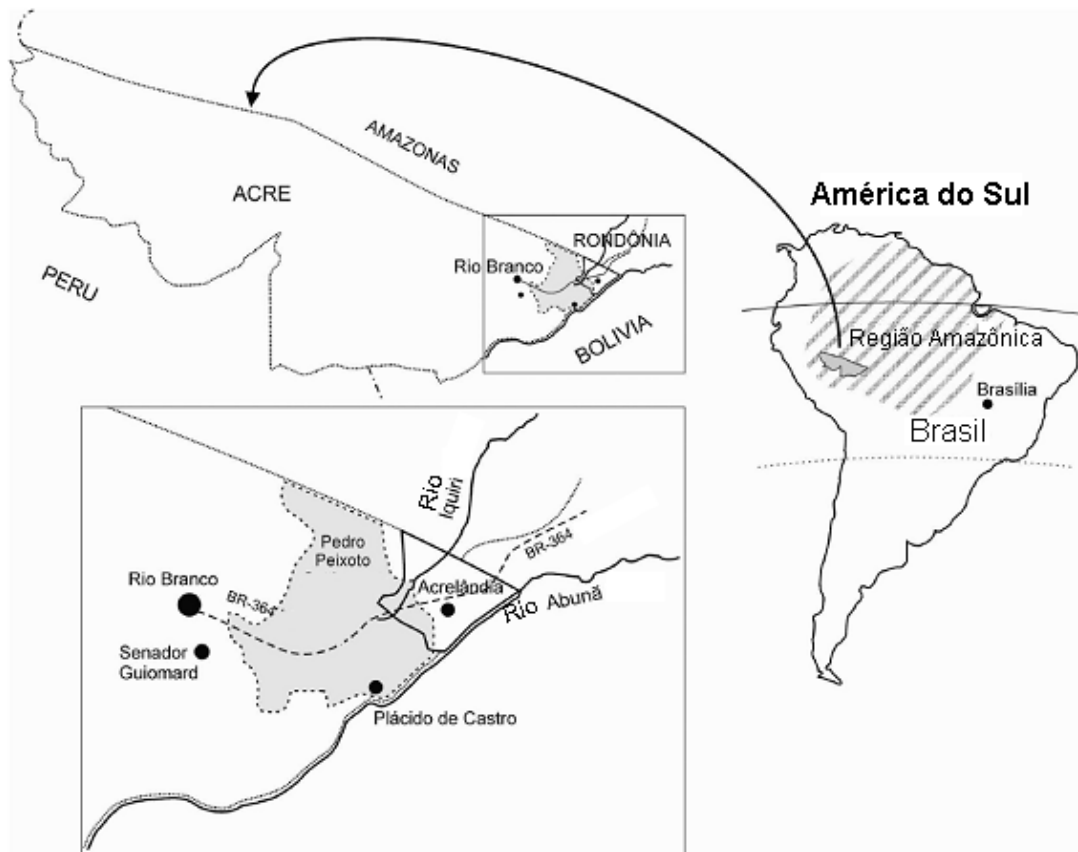


Figura 2: Mapa da América do Sul, mostrando a região Amazônica, o Estado do Acre, a localização do município de Acrelândia e o assentamento Pedro Peixoto. O ramal do Granada, onde foram coletadas as amostras de soro, localiza-se dentro de Pedro Peixoto.

4. 1.2.1 Voluntários e coleta de sangue

Durante a coorte foram realizados dois cortes transversais da população. No primeiro (linha de base) os indivíduos foram convidados a participar do estudo e aqueles que deram consentimento por escrito foram entrevistados por questionário para obtenção de dados demográficos, epidemiológicos e clínicos (Silva-Nunes *et al.*, 2008). Em todas as visitas, ao longo dos 12 meses (demanda ativa e passiva), os indivíduos foram examinados por médicos da equipe, sendo os sintomas classificados de acordo com Karunaweera e colaboradores (1998) como: assintomático, leve, moderado e severo; no momento do exame o diagnóstico foi realizado através da confecção de gotas espessas e, mais tarde, pela *nested* PCR (Kimura *et al.*, 1997). Diante disso, foram selecionados para o primeiro corte (linha de base) 366 indivíduos com idade entre 5-90 anos (mediana = 24,5 anos) e tempo de exposição à transmissão de malária variando de 0 a 72 anos (mediana = 14 anos). Dos 366 participantes do primeiro corte, 287 foram incluídos no segundo corte, o que possibilitou a obtenção de

amostras pareadas. Em cada visita cerca de 5 mL de sangue foi coletado, sendo esta coleta realizada tanto de forma passiva, na qual o indivíduo procurava o centro de saúde, quanto de forma ativa, na qual o grupo de pesquisa visitou as residências (segundo corte transversal).

Os aspectos éticos e metodológicos deste estudo foram aprovados pelo Comitê de Ética de Pesquisas em Seres Humanos do Instituto de Ciências Biomédicas da Universidade de São Paulo (USP), (318/CEP, 19 de julho de 2002 e 538/CEP, 7 de janeiro de 2004) acordo com a resolução do Conselho Nacional de Saúde 196/96.

4. 2 Obtenção de plasma e DNA

Para a obtenção das amostras, o sangue total dos indivíduos a serem testados foi coletado em tubos a vácuo contendo EDTA (Becton Dickinson, Rutherford, NJ), e após centrifugação (150 x *g* por 10min a 4°C), os soros/plasmas foram aliquotados e conservados a -20°C até o uso. O DNA das amostras foi extraído com um kit para purificação de DNA genômico (Puregene DNA Purification System, Gentra Systems, Minneapolis, MN), sendo os procedimentos realizados segundo instruções recomendadas pelo fabricante. As amostras de DNA extraídas também foram estocadas a -20°C até que fossem utilizadas.

4. 3 Ensaio Imunoenzimático (ELISA)

Os ensaios de ELISA foram realizados segundo protocolo padrão, sendo a concentração do antígeno recombinante (PvDBP) e a diluição dos anticorpos primários e secundários determinados previamente por titulação (Cerávolo *et al.*,2005). Os 96 poços das placas de ELISA (Maxysorp, Nunc, Denmark) foram sensibilizados por 12h a 4°C com 100µL dos antígenos PvDBP (5µg/mL) e controle, glutathione S-transferase de *Schistosoma japonicum* (GST) (5µg/mL). Posteriormente, as placas foram lavadas com tampão de lavagem [(0,05% tween 20 em PBS, (PBS-T)] e a cada poço foram adicionados 200µL de tampão de bloqueio [5% de leite em pó (Molico) diluído em PBS-T]. Após 1h de bloqueio a 37°C, as placas foram lavadas três vezes com PBS-T, e 100µL dos soros testes foram adicionados aos poços, em duplicatas, na diluição de 1:100 (PBS-T adicionado de 1,5% de leite em pó). Após incubação por 1h a 37°C, as placas foram lavadas com PBS-T por 10 vezes e incubadas novamente, nas mesmas condições, com 100µL/poço do anticorpo anti-IgG conjugado a peroxidase, diluído a 1:1.000 (PBS-T com

1,5% de leite). Após lavagem, a reação foi revelada com a adição do substrato OPD (*o*-phenylenediamine dihydrochloride substrate - Sigma-Aldrich, USA). A reatividade dos anticorpos anti-PvDBP foi determinada pelo valor obtido da absorbância de 492nm (OD_{492}), a partir da leitura das placas em um leitor de ELISA (Stat Fax-2.100, Awareness Technology, Palm City, FL). Para cada soro, as absorbâncias foram corrigidas do valor obtido para a proteína controle, GST. O limite de positividade entre os resultados positivos e negativos foi estabelecido entre a média observada pelos soros de 20 indivíduos nunca expostos à malária, acrescida de dois desvios-padrão, resultando em um limite de positividade variando de 0,1 a 0,2 (*cut-off*) dependendo do lote da proteína utilizada.

4.4 Reação em Cadeia da Polimerase para Amplificação da PvDBP_{II} e Sequenciamento

Reações em cadeia da polimerase (PCR) foram realizadas para a amplificação de um fragmento correspondente aos nucleotídeos 870-1545 (aminoácidos 290-515) dentro do domínio II da proteína *Duffy Binding Protein* de *P. vivax* (PvDBP_{II}). As reações de PCR foram realizadas utilizando a enzima *platinum Taq DNA polimerase high fidelity* (Invitrogen, California, USA). As amplificações foram conduzidas em termocicladores automáticos *Mastercycler Gradient* (Eppendorf, Hamburg, Alemanha) e PTC100TM *Programmable Thermal Controller* (MJ Research Inc., Massachusetts, USA). Após a amplificação dos fragmentos, os amplicons foram tratados com o sistema de purificação de PCR GFX-96 (Amersham Biosciences), seguindo as recomendações do fabricante. Seguindo a purificação, as amostras foram amplificadas com iniciadores e marcadores e, posteriormente, a reação foi precipitada para o sequenciamento.

As reações de sequenciamento das amostras foram realizadas no sequenciador automático de DNA *MegaBACE 500* (Amersham Biosciences, Little Chalfont, UK). O produto das reações de sequenciamento foram analisadas utilizando o programa *Bioedit* (*Biological Sequence Alignment Editor for Windows*, Ibs Therapeutics, Carlsbad, CA) e alinhadas pelo programa *ClustalW* (EMBL-EBI, European Bioinformatics Institute, Cambridge, UK) identificando os polimorfismos da PvDBP_{II} relativas à sequência da variante de referência Sal-1 (Fang *et al.*, 1991).

4.5 Plasmídeos

Como a região do ligante da PvDBP (região II, DBP_{II}) é polimórfica foram utilizadas quatro construções para transfectar as células COS-7: Sal-1 (Fang *et al.*, 1991), PNG 7.18, PNG 27.16 (Vanbuskirk *et al.*, 2004a) – gentilmente cedidas pelo Dr. John Adams (Notre Dame University, USA) - e Acre-1. A primeira construção codifica a região II da cepa de referência Sal-1, as construções PNG 7.18 e PNG 27.16 codificam para a região II de isolados da Papua Nova Guiné e Acre 1 codifica a região II de isolados do estado do Acre (AC), identificados no presente trabalho. Para a obtenção do plasmídeo Acre 1, um fragmento correspondente aos aminoácidos 198-522 da região II da DBP (DBP_{II}) da cepa de referência de laboratório Sal 1 de *P. vivax* (Fang *et al.*, 1991), foi subclonado em um plasmídeo pEGFP-N1 (Clontech, Mountain View, CA), que codifica uma proteína de fluorescência verde (*Green fluorescent protein* - GFP) utilizada como marcador de transfecção, associada a glicoproteína D1 do vírus herpes simples (HSVgD1) (Michon *et al.*, 2000).

Para a clonagem foram usados um par de iniciadores para amplificar a região II da DBP além dos sítios para as enzimas de restrição Apal e EcoRI, por PCR. Os iniciadores utilizados foram: 5'-ACTAGTGGGCCCTGTCACAACTTCCTGAGT -3' e 5'-GCGGAATTCACGATCTCTAGTGCTATT -3', para Apal e EcoRI, respectivamente (Chitnis & Miller, 1994). Após a reação de PCR, os amplicons e o vetor foram digeridos com as endonucleases EcoRI e Apal. Os insertos digeridos e os vetores pEGFP-HSVgD1 foram unidos através de ligases. Os plasmídeos recombinantes foram purificados a partir da utilização de um *kit* de purificação livre de endotoxina (Qiagen, Valencia, CA), conforme sugerido pelo fabricante.

4.6 Culturas Celulares

Para realização dos ensaios de citoaderência foi utilizada uma linhagem de células de mamíferos permissíveis a transfecção, COS-7, originalmente isolada a partir de células de rim de primata africano, e modificada pelo vírus SV 40 (American Type Culture Collection - ATCC, Manassas, VA). Para realização dos ensaios de citoaderência, células COS-7 foram mantidas em garrafas de cultura de 75cm² (Corning Incorporated, EUA) contendo 10mL do meio de cultura Dulbecco's Minimal Eagle Medium (DMEM) (Gibco, Invitrogen Corporation Rockville, MD, EUA). O meio para manutenção das células (meio DMEM completo) continha 5% de soro bovino fetal (SBF) inativado (Invitrogen Life

Technologies, Rockville, MD, EUA), 25mM de bicarbonato de sódio, 2mM de L-glutamina (Gibco, Invitrogen Corporation Rockville, MD, EUA), 100UI/mL de penicilina (Gibco, Invitrogen Corporation Rockville, MD, EUA), 100µg/mL de estreptomicina (Gibco, Invitrogen Corporation Rockville, MD, EUA), 25mM de HEPES (Sigma). As culturas foram mantidas em estufa a 37°C com 5% de CO₂ e 95% de umidade, sendo os repiques realizados a cada dois dias utilizando-se solução de tripsina e EDTA a 0,25% (Gibco), conforme protocolo padrão (Phelan, 2003).

4.7 Transfecção de células COS-7 e ensaio funcional

A transfecção de células COS-7 foi realizada utilizando-se lipofectamina e reagente *Plus* (Invitrogen Life Technologies, Carlsbad, CA), nas concentrações e protocolos indicados pelo fabricante. Resumidamente, as células COS-7 foram adicionadas às placas de cultura de seis poços (Nunc, Denmark) ($1,5 \times 10^5$ células/poço) e então transfectadas com 0,5µg/poço de DNA plasmidial e complexos de lipossomos (5% de reagente *Plus* e 3% de lipofectamina) em meio de cultura DMEM (Gibco-BRL Life Technologies, Rockville, MD) sem soro bovino fetal (SBF) (meio DMEM incompleto). Este meio incompleto continha 25mM de bicarbonato de sódio, 2mM de L-glutamina (Gibco, Invitrogen Corporation Rockville, MD, EUA) e 25mM de HEPES (Sigma). Após 6h de incubação do complexo lipossoma-DNA (37°C, 5% de CO₂ e 95% de umidade), o meio de transfecção foi substituído por meio DMEM contendo 10% SBF (Gibco), 2mM de L-glutamina (Gibco), 25mM de HEPES (Sigma-Aldrich), 25mM de bicarbonato de sódio (Merck, Darmstadt, Germany), 100UI/mL de penicilina e 100µg/mL de estreptomicina (Gibco), sendo as placas incubadas a 37° C. Após 24 h, o meio de cultura foi novamente substituído por meio DMEM completo, e a eficiência da transfecção verificada por meio da visualização das células em um microscópio de fluorescência. Quarenta e oito horas após a transfecção, as placas foram lavadas com meio DMEM incompleto, e as células incubadas com os soros/plasmas-testes (37°C, 1h, 5% de CO₂) diluídos em meio DMEM incompleto. A diluição inicial utilizada foi de 1:40, pois em ensaios prévios, essa diluição apresentou a melhor inibição interação ligante-receptor quando diferentes soros foram testados. Posteriormente foram realizadas diluições sucessivas para cada amostra que apresentava anticorpos anti-PvDBP no ELISA. Em seguida, foram adicionados 200µL/poço de uma solução a 10% de eritrócitos humanos O/DARC positivos em meio DMEM completo e as placas incubadas à temperatura ambiente por 2h. Ao final da incubação as placas foram

lavadas, três vezes com meio DMEM incompleto, para que os eritrócitos não aderentes fossem retirados.

As rosetas formadas a partir da interação ligante-receptor (**Figura 3**) foram quantificadas (20 campos/poço) em um microscópio estereoscópio de fluorescência invertido e com contraste de fase (200x) (Nikon, Melville, NY, USA). As rosetas só foram quantificadas quando eritrócitos aderentes cobriram mais que 50% de sua superfície celular (Michon *et al.*, 2000). Para cada ensaio um *pool* de soros de indivíduos da área sem anticorpos anti-PvDBP (avaliados através do método de ELISA) foi utilizado como controle negativo. Adicionalmente um *pool* de soros de indivíduos expostos a um longo período de transmissão de malária na Amazônia e que apresentavam anticorpos inibitórios previamente testados nos ensaios funcionais, foi incluído nos ensaios, funcionando como um controle positivo. A porcentagem de inibição foi calculada de acordo com a fórmula $100 \times (R_c - R_t)/R_c$, onde R_c é a média do número de rosetas presente no controle e R_t a média do número de rosetas presentes nos soros-testes.

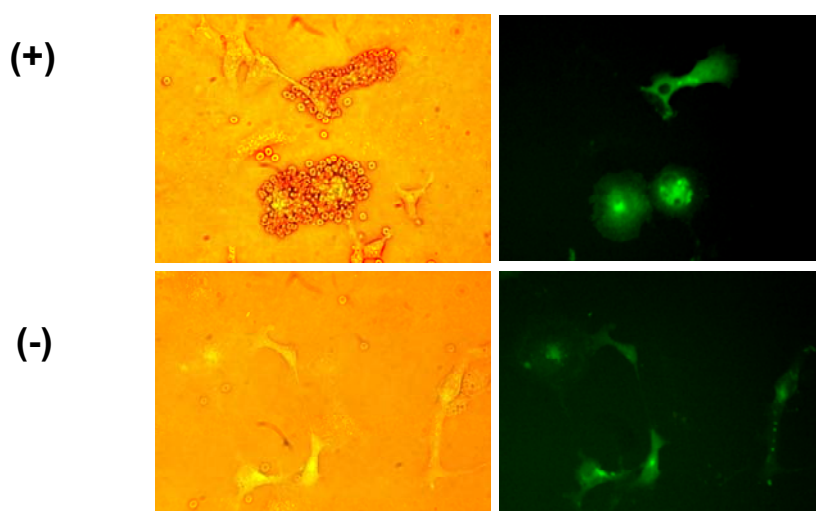


Figura 3: Resultado do ensaio funcional *in vitro* mostrando a presença (+) ou ausência (-) de rosetas após a incubação com soro contendo anticorpos não inibitórios e inibitórios, respectivamente.

4.8 Análise estatística

As análises estatísticas do estudo de corte transversal foram realizadas no programa Epi-Info 2002 (*Centers for Disease Control and Prevention*, Atlanta, GA, USA) ou no Mini-Tab versão 13.1 (MiniTab Inc., State College, PA). Como se tratavam

de dados não paramétricos, ou seja, que não apresentavam distribuição normal foram utilizados testes não-paramétricos incluindo: 1) *Wilcoxon Rank Sum test (Mann-Whitney)* para análises de comparação de medianas dos resultados obtidos em diferentes diluições de soros de indivíduos; 2) coeficiente de correlação de *Spearman* (ρ) para verificar a força da associação entre as variáveis. Em todos os testes realizados foi considerado um nível de 5% de significância.

Para as análises estatísticas do estudo de coorte foi criado um banco de dados pelos nossos colaboradores (grupo de pesquisa do Dr. Marcelo Urbano – USP) utilizando-se o programa SPSS 13.0 (SPSS Inc., Chicago, IL). As proporções foram comparadas em tabelas 2X2, utilizando-se o teste qui-quadrado (X^2) com correção de continuidade de *Yates*, ou teste Exato de *Fisher* quando necessário. Para avaliar a correlação entre as amostras pareadas utilizou-se o coeficiente de correlação de *Spearman* (ρ). Modelos de regressão Logística múltipla foram construídos para descrever associações independentes entre as covariáveis (idade, gênero, tempo de residência na área, setor de residência e infecção aguda ou recente) e a presença de anticorpos anti-PvDBP durante o estudo de coorte. Foram utilizados dois níveis de modelo logístico, o primeiro correspondente a cada observação por indivíduo e o segundo correspondente a cada indivíduo.

A transmissão de malária em Granada não é homogênea, pois a utilização da terra e a taxa de desmatamento são desiguais ao longo do seu território. Desta forma, para evitar que este fator interferisse no modelo logístico, a área de estudo foi dividida em quatro setores relativamente homogêneos com relação à incidência de malária. Os setores foram divididos da seguinte forma: 1) setor A (92 indivíduos, 0,46 episódios por *P. vivax*/100 pessoas por mês sobre o risco de infecção); 2) setor B (97 indivíduos, 0,79 episódios por *P. vivax*/100 pessoas por mês sobre risco de infecção); 3) setor C (130 indivíduos, 3,44 episódios /100 pessoas sobre risco de infecção); e 4) setor D (47 indivíduos, 9,71 episódios por *P. vivax*/100 pessoas por mês sobre risco de infecção). O programa HML (versão 6.03, *Scientific Software International*, Lincolnwood, IL) foi utilizado para as análises com mais de um nível. Em todos os testes realizados foi considerado um nível de 5% de significância. As análises referentes ao estudo de coorte foram realizadas pelo nosso colaborador Dr. Marcelo Urbano da Universidade de São Paulo (USP).

5 Resultados

5.1 Artigo 1

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Inhibitory Properties of the Antibody Response to *Plasmodium vivax* Duffy Binding Protein in an Area with Unstable Malaria Transmission

I. P. Ceravolo*, F. A. Souza-Silva*, C. J. F. Fontes†, E. M. Braga‡, A. P. Madureira*, A. U. Krettli*, J. M. Souza§, C. F. A. Brito*, J. H. Adams¶ & L. H. Carvalho*

Abstract

*Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, MG, Brazil; †Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil; ‡Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; §Instituto Evandro Chagas, Belém, PA, Brazil; and ¶University of South Florida, Tampa, FL, USA

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Correspondence to: Dr Luzia H. Carvalho, Malaria Laboratory, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ-MG), Av. Augusto de Lima 1715, 30190-002 Belo Horizonte, MG, Brazil. E-mail: lhcarvalho@cpqrr.fiocruz.br

The function of the *Plasmodium vivax* Duffy binding protein (DBP) during the erythrocyte invasion process is critical for successful parasite growth and pathogenesis in human infections. Although DBP is the subject of intensive malaria vaccine research, investigations on the functional proprieties of anti-DBP antibodies in the human population have been limited [*Infect Immun* 68 (2000) 3164]. In the present study, we examined the ability of sera from different populations of the Brazilian Amazon – an area of markedly unstable malaria transmission – to inhibit the erythrocyte-binding function of the DBP ligand domain (region II, DBP_{II}). We found that long-term exposure to malaria in the Amazon area elicits DBP-specific antibodies that inhibit the binding of different DBP_{II} variants to erythrocytes. Despite the great variability of inhibitory antibody responses observed among study participants, we observed a positive correlation between erythrocyte binding-inhibitory activity and enzyme-linked immunosorbent assay anti-DBP antibodies. Of importance, there was a non-significant tendency towards increased levels of anti-DBP antibodies among individuals with asymptomatic *P. vivax* infections.

Introduction

Plasmodium merozoites initiate erythrocyte invasion through an active process mediated by parasite ligands that interact with erythrocyte receptors. A key step during host cell invasion is the irreversible commitment of the merozoite to the selected host cell by formation of a tight junction between the parasite and the erythrocyte [1, 2]. In the case of *P. vivax*, junction formation requires that Duffy binding protein (DBP) binds to its cognate receptor on erythrocytes, the Duffy blood group antigen [3–5] also known as Duffy antigen receptor for chemokines [6]. Individuals that lack the Duffy antigen on their erythrocytes are highly resistant to *P. vivax* infection [7]. Thus, DBP represents one of the most promising vaccine candidate antigens against *P. vivax* infection.

Plasmodium vivax DBP is a 140-kDa protein that belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL-EBP) located within the micronemes of *Plasmodium* merozoites [8]. The simi-

larity among DBL-EBPs is most prominent in two conserved Cys-rich domains, region II and VI. The functional binding domains of DBL-EBP lie in region II, which for *P. vivax* DBP is 330-aa, and the critical binding residues map to a central 170-amino-acid stretch that includes cysteines 5–8 [9–11]. DBP is likely to be exposed on the merozoite surface during invasion, enabling it to bind to its receptor and, thus, making it accessible to serum antibodies. Currently, the available data regarding humoral immune responses to DBP in the human population are limited, and studies have been essentially restricted to areas where malaria is highly endemic [12, 13, 14]. Recently, we demonstrated that DBP is naturally immunogenic in the Brazilian Amazon area, where malaria transmission rates are low-to-medium and clinical symptoms affect people of all ages [15]. In the Amazon, the proportions of DBP IgG-positive subjects increased with exposure to malaria, reaching a peak in those subjects with long-term exposure in the endemic area (>15 years). This observation was subsequently confirmed in malaria-endemic populations of Rondônia State,

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Brazil, where specific antibodies to DBP were biased towards the cytophilic subclasses IgG1 and IgG3 [16].

Many field studies examining immunity to malaria have focused on measuring the concentrations of antibodies to vaccine candidate antigens, while less attention has been paid to complementary approaches defining the functional relevance of these antibodies [17]. For example, measuring antibodies to recombinant merozoite antigens by enzyme-linked immunosorbent assay (ELISA) is a simple, robust procedure widely used in population studies, but provides little qualitative information when used alone [18]. In the present study, we hypothesized that part of the humoral response to malaria infection in the Amazon area is mediated by antibodies that inhibit the interaction between DBP and its receptor on erythrocytes. To date, this inhibitory antibody response was demonstrated only in highly endemic regions of Papua New Guinea [12], which is representative of only some tropical regions where *P. vivax* is prevalent. To characterize these inhibitory antibodies in our study, we used an *in vitro* erythrocyte-binding assay, in which the putative ligand domain of DBP (region II, DBP_{II}) was expressed on the surface of cultivated mammalian cells [12, 19]. To assess the potential effects of DBP polymorphisms, three different DBP_{II} variants were used in the erythrocyte-binding assays.

Materials and methods

Study areas and subjects. We analysed subjects from three previously well-characterized areas of the Brazilian Amazon [15, 20] who had been exposed to different levels of malaria transmission (Table 1). The first group consisted of 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, where levels of malaria transmission are low and

unstable. The second group was composed of 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. vivax* and/or *P. falciparum*. The third group, named the Apiacas group, consisted of migrant miners who had lived for approximately 17 years in several gold-mining areas of the Brazilian Amazon where *P. vivax* and *P. falciparum* malaria are endemic. At the time of blood collection, the three groups (Belém, TNN and Apiacas) consisted of asexual individuals, as assessed by microscopic examination.

A previous study on the prevalence of malaria in Apiacas (MT) has suggested that gold-miners develop partial resistance to clinical malaria [21]. At the time of the field survey, 10% of the gold-miner population (36 out of 369) had *P. vivax* parasites, as detected by microscopy or species-specific PCR [22], 19 of which were classified as asymptomatic and 17 as symptomatic. Asymptomatic malaria infection was defined as absence of classical malaria symptoms, such as fever, headache, muscle and/or joint pain, for at least 72 h after parasite detection. In the current study, we selected 15 of the 19 gold-miners with asymptomatic *P. vivax* infection and 10 of the 17 with symptomatic *P. vivax* infection.

Human plasma. Human plasma samples were collected from *P. vivax*-exposed residents of the Brazilian Amazon area, as described earlier [20, 23]. Initially, the DBP-erythrocyte binding assay relied on pooled samples. For each area, samples were pooled as following: (i) from individuals who had anti-DBP antibodies, as detected by ELISA (responders), and (ii) from individuals who had not developed anti-DBP antibodies (non-responders) (Table 1). The criterion for classifying individuals as responders or non-responders to DBP was based on the ELISA cutoff point, as described in the 'Antibody measurement' section; individuals whose plasma samples had an optical density ≥ 0.1 (OD value at 492 nm) were classified as responders, and those whose plasmas had an OD value < 0.1 were classified as non-responders. For further experiments, individual samples from these Amazonian communities were used; these individuals were subsequently classified as high, low and non-responders, according to their ELISA antibody titre. Additional plasma samples were obtained from individuals infected with *P. vivax* that had developed asymptomatic ($n = 15$) or symptomatic ($n = 10$) malaria infection after a short follow-up period [21]. Negative control plasma samples were collected from Brazilian volunteers ($n = 20$) with no previous malaria exposure.

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

Table 1 Characteristics of the three communities studied in the Brazilian Amazon area.

Characteristics	Area		
	Belém	TNN	Apiacas
Antibodies anti-DBP, n /total (%)	5/36 (14%)*	18/47 (38%)	24/37 (65%)
Age (mean \pm SD) [†]	32 \pm 19	40 \pm 28	32 \pm 8
Exposure to malaria transmission	Sporadic	Constant	Constant
Time of malaria exposure	<1 month	~10 years	~17 years
Number of past malaria episodes	1	1–10	>10
Previous malaria episodes by	Pv	Pv and/or Pf	Pv and/or Pf

Pv = *P. vivax*, Pf = *P. falciparum*.

*As previously detected by ELISA [15].

[†]Differences not significant ($F = 1.47$, $P = 0.234$).

Rachou/FIOCRUZ (Report 002/2002 and Report 07/2006), 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).

Antibody measurement. Specific DBP antibody reactivities were determined by an ELISA against a recombinant protein containing region II–IV of the DBP. Detailed construction of this GST fusion protein, which represents amino acids 177–815 of DBP has been described elsewhere [13, 15]. The ELISA for total IgG antibodies was performed as described previously [15] and the recombinant protein was used at a concentration of 5 µg/ml. All samples were initially diluted 1:100 and evaluated for total IgG using peroxidase-conjugated anti-human IgG antibodies (Sigma, St Louis, MO, USA). 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 non-exposed subjects.

DBP_{II}-pEGFP constructs. Region II of DBP from a *P. vivax* laboratory reference clone Sal-1 (DBP_{II}V1) was previously subcloned into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) along with a flanking signal sequence from the herpes simplex virus glycoprotein D1 [12, 19]. This plasmid allows for expression of a recombinant fusion protein to the N terminus of the enhanced-green fluorescent protein (GFP), which is used as a transfection marker. Additional GFP constructs with two DBP region II variants, PNG-7.18 (DBP_{II}V2) and PNG-27.16 (DBP_{II}V3) [24], were also used in the erythrocyte-binding assays. All DBP_{II}-pEGFP plasmids were purified by use of an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA, USA).

COS-7 cultures and erythrocyte-binding assays. Recombinant plasmids were transfected into Green monkey kidney cells (COS-7, American Type Culture Collection, Manassas, VA, USA) by use of lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocols. Briefly, COS-7 cells seed in six-well culture plate (1.5×10^5 cells/well) were transfected with plasmid (0.5 µg/well)-liposome complexes (5% Plus-reagent and 3% lipofectamine) in Dulbecco's Modified Eagle Medium (DMEM, Sigma) without serum. After 6 h of exposure to the DNA-liposome complexes (37 °C, 5% CO₂), the transfection medium was replaced with DMEM plus 10% foetal bovine serum (Gibco-BRL Life Technologies, Rockville, MS, USA). 24 h after transfection, the culture medium was replaced again and the efficiency of transfection was assessed using an inverted fluorescence microscope (Nikon TE2000E, Melville, NY, USA). Over 80% of the cells demonstrated green fluorescence, indicating that they were successfully transfected.

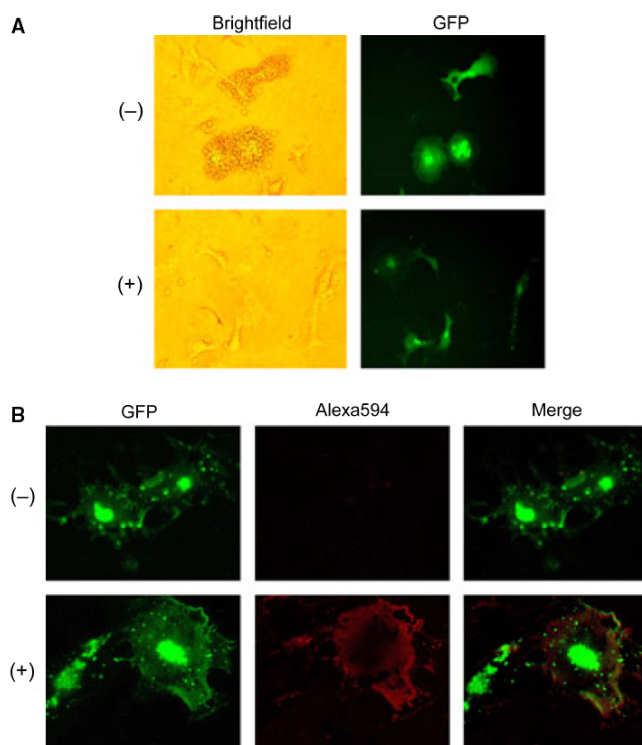
Forty-eight hours after transfection, the erythrocyte-binding assays were performed as previously described

[12]. For this, transfected COS-7 cells were incubated for 1 h at 37 °C in 5% CO₂ with various dilutions of the individual and/or pooled human plasmas in DMEM without serum. Human O⁺ erythrocytes in a 10% suspension were added to each well (200 µl/well), and plates were incubated for 2 h at room temperature. Unbound erythrocytes were then removed by washing the wells three times with PBS. Binding was quantified by counting positive rosettes around GFP-positive cells within 20 fields at a magnification of $\times 200$ (Fig. 1A). Rosettes were counted as positive when adherent erythrocytes covered more than 50% of the cell surface. To assess their inhibitory activity, all human plasmas were first tested (duplicate) at a 1:40 dilution. Serial dilutions were then tested for positive samples. Binding for each plasma dilution was compared to binding of transfected COS-7 cells incubated with negative control plasma at a 1:40 dilution (100% binding). The per cent inhibition was calculated as $100 \times (R_c - R_t)/R_c$, where R_c was the average of the number of rosettes in the control wells and R_t was the average of the number of rosettes in the test wells. The threshold of positivity was a 50% rosette-inhibitory activity at the 1:40 plasma dilution; this dilution was chosen because in preliminary experiments this dilution provided a wide range of inhibitory activity among different plasmas.

Confocal fluorescence microscopy. Under the same culture conditions as above, transfected COS-7 cells growing on glass coverslips (25 mm diameter; Fisher Scientific, Slangerup, Denmark) were placed into six-well culture plates and incubated either with plasma samples that inhibit or do not inhibit erythrocyte-binding to DBP_{II} (1:80, 1 h at 37 °C). After washing the wells with PBS, cells were incubated (30 min at 4 °C) with goat anti-human IgG conjugated to Alexa Fluor[®] 594 dye (1:500; Molecular Probes, Invitrogen). Cells were then washed and coverslipped images were acquired with a LSM 510 Zeiss confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA). The signal from Alexa594-labelled conjugate is in the red region of the spectrum (absorption/emission maximum: 590/619 nm), and the signal from DBP_{II}-GFP expressing COS-cells is in the green region (absorption/emission: 488/509 nm). The detector sensitivity and emission wavelength range were set independently for optimal special resolution of each fluorescence signal. Images were acquired using LSM 510 Zeiss software.

Statistical analysis. Statistical analyses were performed using the Epi-Info 2002 software (Centers for Disease Control and Prevention, Atlanta, GA, USA) and/or Mini-Tab statistical software (Minitab Inc., State College, PA, USA). Differences between two medians were compared by the non-parametric Wilcoxon rank sum test. The strength of the association between variables was analysed by the non-parametric Spearman rank correlation coefficient (r_s). Statistical significance was defined as $P < 0.05$.

Figure 1 Binding of human anti-DBP antibodies to the surface of COS cells expressing DBP_{II}. (A) Erythrocyte-binding assay; *Upper panels* illustrate rosettes of erythrocytes on the surface of COS-7 cells transfected with DBP_{II}-GFP after incubation with plasma, at 1:40 dilution, of a Brazilian resident characterized as a non-responder to DBP (-); *lower panels* illustrate a complete inhibition of erythrocyte binding (no-rosettes) by a human plasma characterized as an anti-DBP responder (+). Cells were observed using either brightfield or conventional epifluorescence microscopy (×200). (B) Localization by confocal microscopy of anti-DBP_{II} IgG antibodies (red fluorescence) on the surface of COS cells expressing DBP_{II}-GFP (green fluorescence). Cultures were incubated with plasma that either inhibited (+) or did not inhibit (-) erythrocyte-binding to DBP_{II}, as described in (A), followed by incubation with Alexa594-labeled goat antibody to human IgG (red fluorescence). The last panels on the right illustrate overlapped images, which were acquired with a LSM 510 Zeiss confocal microscope (×630).



Results

Natural exposure to *P. vivax* in the Amazon area induces an inhibitory antibody response

A preliminary analysis was used to screen individuals in the Amazon area, which is an area with unstable malaria transmission, for exposure to *P. vivax* and to assess whether their acquired antibodies had the ability to inhibit DBP binding to erythrocytes. To measure inhibition of DBP–erythrocyte binding activity, assays were performed using transiently transfected COS-7 cells expressing DBP_{II} variants as GFP fusion proteins (Fig. 1A). Confocal microscopy immunofluorescence analysis illustrates the specificity of the inhibitory human anti-DBP IgG, but not the non-inhibitory antibody, in transfected COS cells (Fig. 1B). Our results demonstrate that individuals with long-term exposure to malaria in the Amazon area (TNN and Apiacas) acquire inhibitory antibodies to the various DBP_{II} variants (Fig. 2). In contrast, plasma samples from residents who had been sporadically exposed to *P. vivax* (<1 month) did not inhibit the ligand–receptor interaction. In this group, a low level of inhibition (<50%) was detected, but only for the DBP_{II}V3 allele (Fig. 2). Taken together, these results suggest that an efficient inhibitory antibody response requires long-term exposure to malaria. No inhibition was observed using pooled plasmas at the same dilution from individuals

living in endemic areas (Belém, TNN or Apiacas) but who did not have anti-DBP antibodies (classified as non-responders), as detected by conventional serology.

Anti-DBP antibody levels and inhibition of binding

We sought to determine whether the level of anti-DBP IgG antibodies, as detected by ELISA, correlated with the levels of binding-inhibitory antibodies to DBP_{II}. For this, we analysed plasma samples from 17 individuals with long-term exposure to malaria in the Amazon area (TNN or Apiacas) who had been characterized as high or low responders by endpoint ELISA experiments (Fig. 3A). The efficacy of these samples to inhibit DBP–erythrocyte interactions was measured utilizing COS-7 cells expressing the DBP_{II}V3 (Fig. 3B), and we observed a wide range of DBP–erythrocyte inhibition activity among the 17 selected individuals. Importantly, samples from the high-responder group showed significantly greater anti-DBP inhibition than samples from the low-responder group (Fig. 3C). This difference was estimated statistically as a moderate positive correlation between anti-DBP antibodies and inhibitory activity (Spearman's coefficient: 0.49; $P < 0.05$) (Fig. 3D). Of great interest in our study was the fact that the levels of these inhibitory antibodies were relatively high in the Amazon area, with titres at 640–1280 in some individuals (Fig. 3B–C).

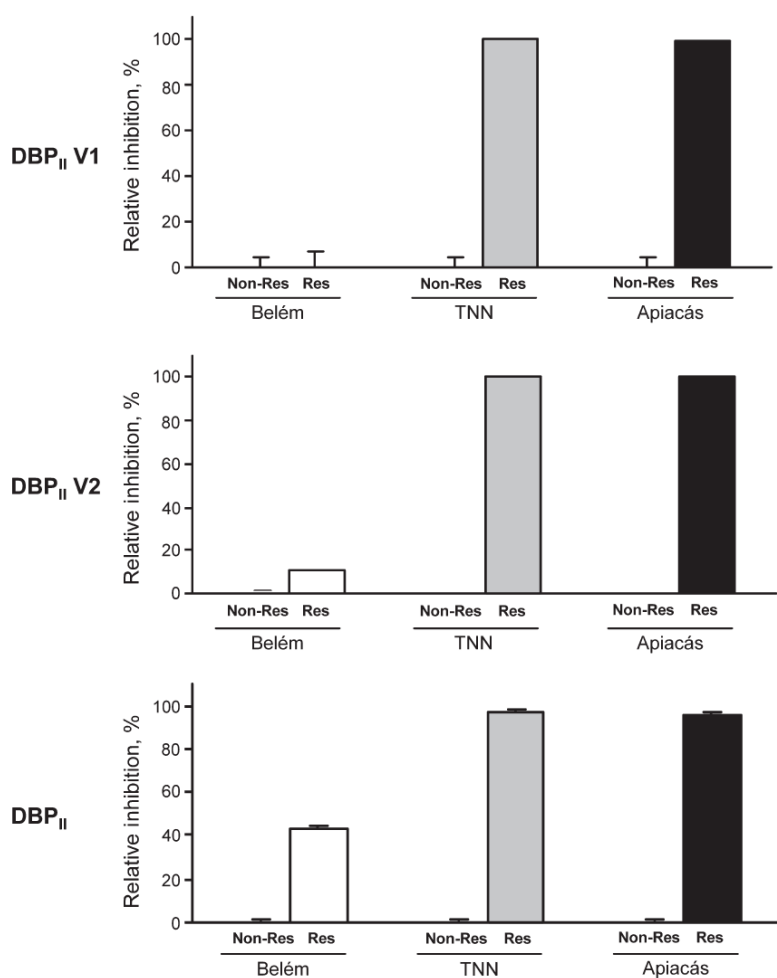


Figure 2 Inhibition of erythrocyte binding to DBP_{II} variants expressed in COS cells by plasma from individuals who had been sporadically (Belém) or continuously exposed to malaria (TNN and Apiacás) in the Amazon area. COS-7 cells expressing each variant (DBP_{II}V1, DBP_{II}V2 or DBP_{II}V3) were incubated with different pooled plasma samples, at 1:40 dilution, prior to the addition of human erythrocytes. Binding was quantified by counting rosettes around GFP positive cells within 20 fields at a magnification of $\times 200$; per cent inhibition was determined relative to pooled plasma from unexposed Brazilian donors (negative control). Plasmas from Amazonian residents were designated as non-responders (Non-Res) or responders (Res) to DBP according to their previous ELISA positivity [15]. Bars correspond to the mean \pm standard deviation of two independent experiments.

Asymptomatic *P. vivax* infection and inhibition of binding

To determine whether there is a relationship between the prevalence of anti-DBP and clinical illness, we analysed plasma samples from acutely infected *P. vivax* individuals who had ($n = 10$) or had not ($n = 15$) developed clinical malaria after a short follow-up period in the Apiacás area. ELISA results demonstrated that the frequency of anti-DBP antibodies was similar between symptomatic (7/10; 70%) and asymptomatic (9/15; 60%) infections, but there was a tendency towards increased levels of anti-DBP antibodies among asymptomatic individuals (Fig. 4A). Plasmas from those individuals who were ELISA positive for anti-DBP antibodies (seven symptomatic and nine asymptomatic infections) were analysed for the efficacy of these antibodies to inhibit erythrocyte-DBP_{II} binding. Despite the variability of individual responses, there was a non-significant tendency towards increased levels of inhibition among asymptomatic infections (Fig. 4B).

Discussion

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. Unfortunately, due to the methodological constraints on performing functional assays with the limiting amount of test sera typically obtained from field studies [18], few reports have examined functional antibodies in the malaria-exposed population. To date, a single study carried out in highly endemic areas of Papua New Guinea demonstrated that individuals naturally exposed to malaria acquire antibodies that inhibit DBP binding to erythrocytes [12].

In the present study, our goal was to characterize the anti-DBP inhibitory antibody response in individuals from an area of markedly unstable malaria transmission. The transmission of malaria in the Amazon area is not homogeneous, similar to most of Latin America (where

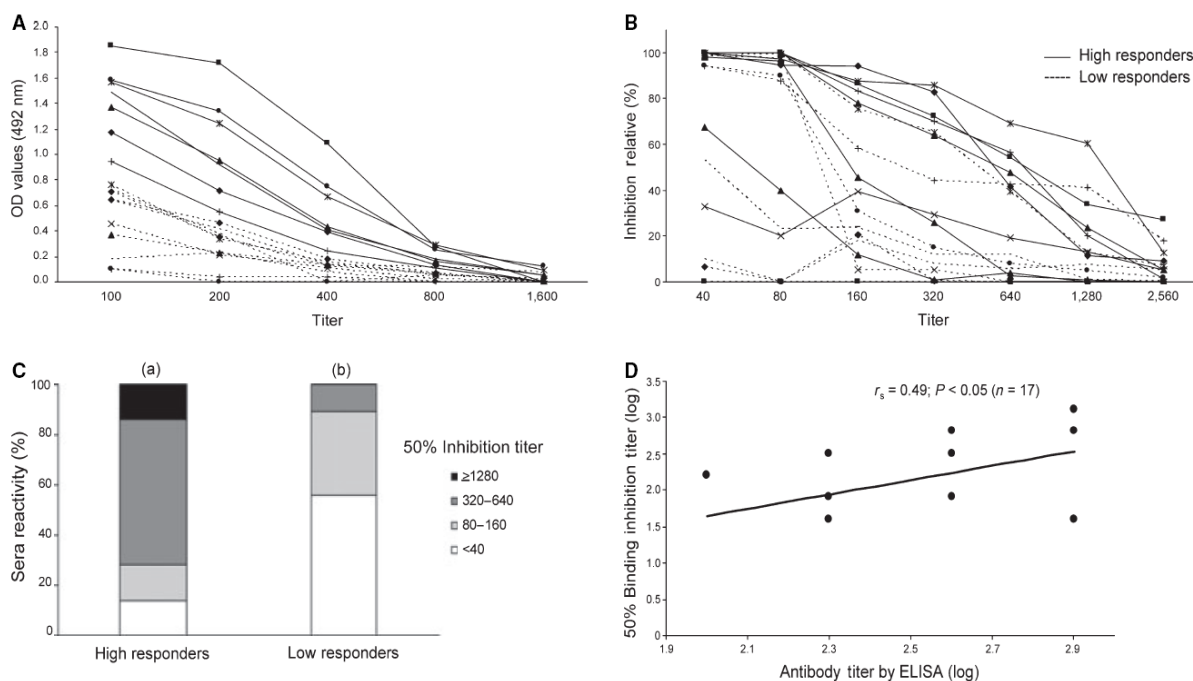


Figure 3 Comparison between anti-DBP antibodies detected by ELISA and rosette-inhibitory activity. (A) After titration anti-DBP IgG antibodies in ELISA, individuals continuously exposed to malaria in the Amazon area (TNN and Apiacas) were classified as high ($n = 8$) or low ($n = 9$) responders. IgG antibody responses are expressed as optical density (OD values at 492 nm). (B) Titration of the rosette-inhibitory activity in the same plasma samples classified in (A). For this, COS cells expressing the DBP_{II}V3 variant were incubated with different plasma dilutions of these individuals and the per cent inhibition was determined as described in Fig. 1. (C) Fifty per cent binding inhibition titres were determined from curves obtained in (B) plotting the percentage of inhibition at different plasma dilutions. Different letters on the top of (C) indicate significantly different medians ($P < 0.05$), as determined by the Wilcoxon Rank sum test. (D) Scatter plot showing the correlation between rosette-inhibitory activity and total anti-DBP IgG (Spearman's $r_s = 0.49$). For each assay, titres were defined as the reciprocal of the plasma dilution, and log-transformed titres were used for statistical analysis.

about 1 million cases are reported per year) [25]. Vivax malaria in the Amazon basin tends to be concentrated in areas with uncontrolled establishment of rural and mining settlements, being associated with poorly maintained dwellings and favorable transmission conditions [26, 27]. In general, the exposed populations consist of migrants who are mostly from malaria-free areas. Under such a pattern of transmission, the infection is generally accompanied by clinical symptoms that vary from mild to severe. Recently, we and others have demonstrated that antibodies to DBP, as measured by ELISA, are prevalent in the Amazon area [15, 16, 28].

By using an *in vitro* assay to quantify inhibition of DBP–erythrocyte binding [12, 19], we demonstrated that natural exposure to *P. vivax* in the Amazon area induces anti-DBP antibodies that inhibit DBP–erythrocyte interactions. An efficient inhibitory antibody response was only detected in plasmas from individuals having a long-term exposure to malaria in that area (>10 years). Plasma samples from individuals sporadically exposed to *P. vivax* (Belém) could not inhibit DBP–erythrocyte binding. These results suggest that DBP is normally a poor

immunogen when presented through the 'natural' exposure of an infection and that chronic exposure is needed to induce a more effective inhibitory antibody response that recognizes different DBP_{II} variants. This finding is in accordance with our previous work in the Amazon area, in which we clearly demonstrated that conventional serological responses to DBP correlate with the time of exposure to malaria transmission [15]. Although our current data suggest that an efficient inhibitory antibody response can be developed only in individuals demonstrating a long-term exposure to malaria, a longitudinal study is required to properly address this question, especially to evaluate the effect of further malaria episodes in the inhibitory antibody responses. Alternatively, it is possible that the people sporadically exposed to malaria in the Belém area were infected with different variants of DBP_{II} that are antigenically distinct from the three alleles used in the present study. Recent findings with rabbit immune-sera support the idea that distinct strain-specific antibody response profiles can be a general phenomenon that restricts the functional immune response against DBP in an allele-specific manner [24]. These

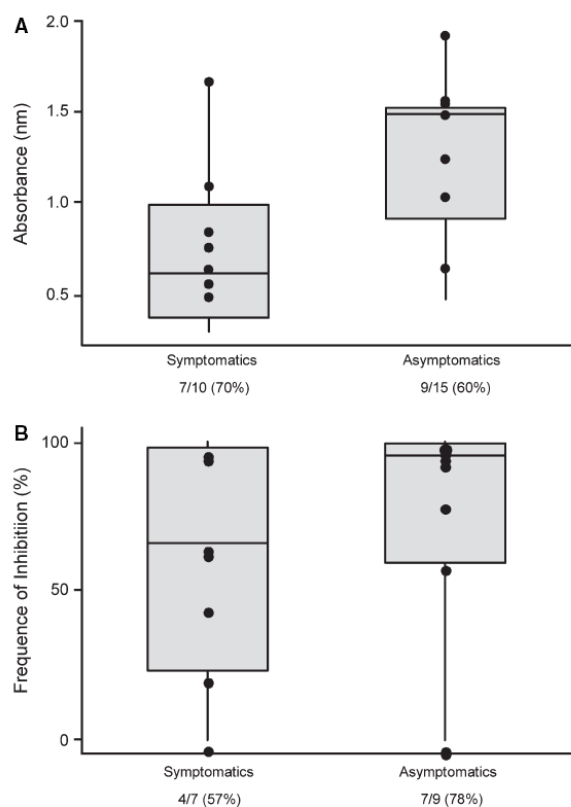


Figure 4 ELISA anti-DBP IgG antibodies (A) and rosette-inhibitory activity (B) among 25 individuals who were grouped based on the presence (symptomatic) or absence (asymptomatic) of clinical malaria. Initially, samples were assayed for the presence of conventional anti-DBP antibodies by ELISA (at 1:100 dilution) (A), and positive samples were evaluated for the presence of inhibitory antibody responses (1:80 dilution) (B). For each specific assay, the lines in the box-plots represent medians. Values on the bottom of figures represent the overall frequency of the response for each assay.

results point towards strain specificity in the natural immune response against DBP, which has also been suggested to another microneme protein and vaccine candidate, the apical membrane antigen 1 (AMA1) [29]. As expected, these factors can have consequences for the current efforts on vaccine development. However, the success of vaccines such as DBP will depend on how these immune obstacles can be overtaken, including strategies for improving vaccine efficacy.

In an attempt to determine whether the level of serological responses correlates with the anti-DBP inhibitory activity, we analysed plasma samples from 17 individuals chronically exposed to malaria who had been classified by conventional serology (ELISA) as high or low responders. As determined by titration experiments, a positive correlation was observed between the anti-DBP antibody level and the ability to inhibit erythrocyte binding. This is consistent with the results of the Papua New Guinea study [12], in which the titre of naturally acquired

antibodies against DBP region II correlated with their rosette-inhibitory activity. However, it is of relevance that our study clearly showed a wide range of inhibitory antibody responses among the study participants. In fact, there were a number of samples with low anti-DBP antibodies and high rosette-inhibitory activity and vice versa. Although it is possible that this individual variability in the inhibitory immune response may be a characteristic of unstable malaria transmission areas, this conclusion remains uncertain as pooled samples were used in the PNG study [12], which may have masked individual variability in the inhibitory antibody response. The variability in anti-DBP inhibitory activity observed here is consistent with the results of Ohas et al. [30] using region II of *P. falciparum* EBA-175; these authors demonstrated a wide range of functional antibody responses among lifelong residents of a malaria holoendemic area in western Kenya.

Despite a wide range in the anti-DBP inhibitory activity among study participants, the titres of inhibitory antibodies in the Amazon area were relatively high in some individuals whose inhibitory activity could still be detected at a 1280 titre. Given the scarcity of data from the human population, this profile of inhibitory immune responses to DBP_{II} can only be compared with those results obtained from experimental models. In mice, studies of the immunogenicity of a recombinant DPB_{II} formulated with human compatible adjuvant demonstrated that maximum inhibitory activity was obtained with immune sera diluted up to 1:1800 [31]. So far, vaccination of monkeys with a formulation of DPB_{II} suitable for human use (montanide ISA-720) did not induce significant levels of inhibitory antibody response; the 50% inhibition titres ranged from 1:16 to 1:366 [32]. Therefore, it is very intriguing that in the Amazon and PNG a strong inhibitory immune response to DBP is acquired under natural conditions.

The occurrence of asymptomatic malaria in Latin America is unusual, and only recently has been documented in detail among Amazonians living in remote riverine settlements [33, 34]. Considering the vastness of the Amazon region and the remoteness of some of its riverine settlements, the plasma samples available from a small group of asymptomatic *P. vivax*-infected individuals, previously identified in the Apicacas area [21], offered an exceptional opportunity to investigate the relationship between malaria symptoms and the functional immune response to DPB_{II} *in vitro*. Although the percentage of responders was similar between groups, there was a trend in the levels of conventional as well as inhibitory antibodies to be higher in the asymptomatic group than in the symptomatic group; however, these differences did not reach the level of significance. Unfortunately, the size of our sample could not be increased in the Apicacas area; geographical access and political conflicts – which seems

to be common in the Brazilian Amazon area – hamper these studies in the endemic Apiacas area. A long-term prospective study is needed to determine the protective nature of the inhibitory anti-DBP_{II} antibodies in terms of anti-disease immunity.

Taken together, our results demonstrate that individuals from unstable malaria transmission areas acquire anti-DBP antibodies that inhibit *in vitro* binding of different DBP_{II} variants to erythrocytes. It is not yet clear whether this finding correlates with increased levels of protection. We believe that this first study on the profile of immune response to DBP_{II} variants in the population of the Amazon area will contribute to current efforts on vaccine development, and may facilitate future clinical trials in areas of unstable malaria transmission.

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Authors' contributions

I. P. Ceravolo and F. A. Souza-Silva were involved in all stages of this study. E. M. Braga and A. U. Krettl were involved in the initial design of the field study. C. J. F. Fontes and J. M. Souza participated in the coordination of the field work. C. A. Brito and J. H. Adams helped to compose the manuscript and gave substantial constructive advice during the project. A. P. Madureira performed statistical analysis and contributed to the interpretation of data. L. H. Carvalho accepts direct responsibility for the conception, design and coordination of the study. All authors read and approved the manuscript.

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5.2 Artigo 2

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

Flávia A. Souza-Silva, Mônica da Silva-Nunes, Bruno A. M. Sanchez, Isabela P. Ceravolo, Rosely S. Malafronte, Cristiana F. A. Brito, Marcelo U. Ferreira, and Luzia H. Carvalho*

Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais, Brazil;
Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo (USP), São Paulo, Brazil;
Instituto de Ciências Biomédicas, USP, São Paulo, Brazil

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)

*Address correspondence to Luzia H. Carvalho, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Malaria Laboratory, Av. Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil. E-mail: lhcavvalho@cpqrr.fiocruz.br

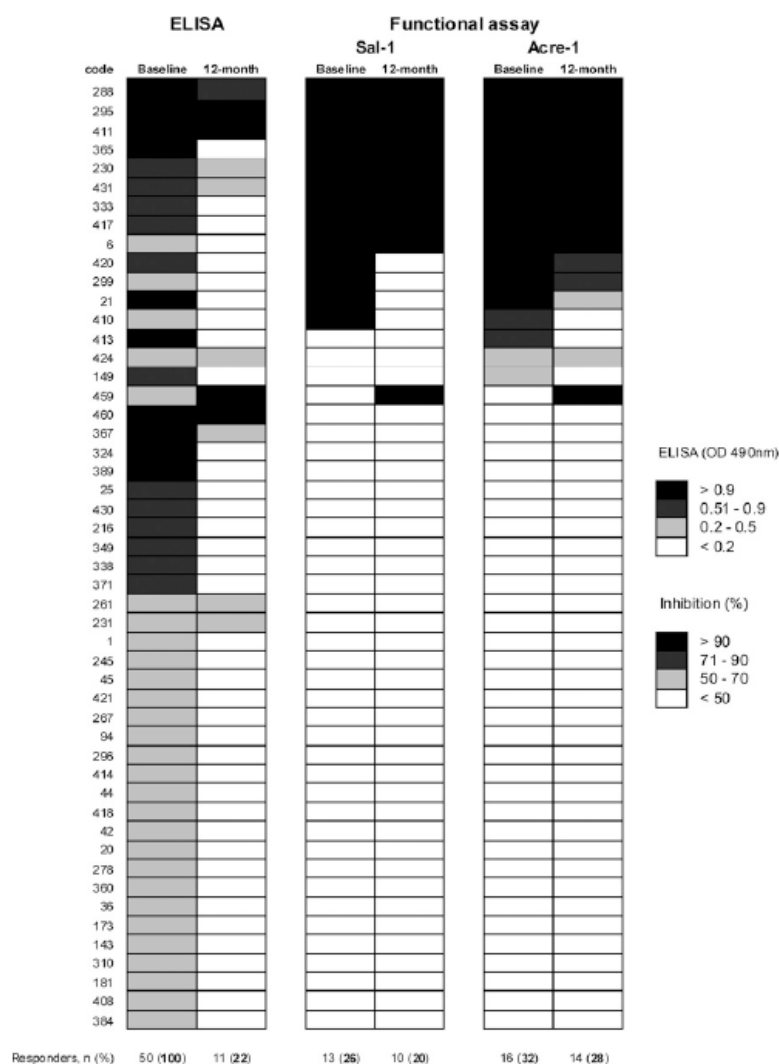


FIGURE 1. Inhibition of DBP_{II}-DARC binding in sequential samples from 50 individuals who had conventional anti-DBP antibodies by enzyme-linked immunosorbent assay (ELISA) at the time of enrollment. Each sample was assayed at baseline and \cong 12-month latter. Conventional anti-DBP antibodies were 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 × 10⁶ 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 × (Rc - Rt)/Rc, where Rc is the average of the number of rosettes in the control wells and Rt 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)	N ^o	February–March 2005		<i>P</i> (yes vs. no)
	No.	Subjects with IgG antibodies	(%)			No.	(%)	
Current								
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.	P†	
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

$\approx 50\%$ 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 contribute to the relatively low frequency ($\approx 30\%$) 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-I, 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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Note: Supplemental figures appear at www.ajtmh.org.

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Authors' addresses: Flávia A. Souza-Silva, Bruno A. M. Sanchez, Isabela P. Ceravolo, Cristiana F. A. Brito, and Luzia H. Carvalho, Laboratory of Malaria, Institute René Rachou, Fundação Oswaldo Cruz (FIOCRUZ-MG), Belo Horizonte, Minas Gerais, Brazil, E-mails: praflavia@cpqrr.fiocruz.br, bruno@cpqrr.fiocruz.br, ceravolo@cpqrr.fiocruz.br, cristiana@cpqrr.fiocruz.br, and lhcarvalho@cpqrr.fiocruz.br. Mônica da Silva-Nunes and Marcelo U. Ferreira, Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, E-mails: msnunes1@yahoo.com.br and muferrei@usp.br. Rosely S. Malafrente, Laboratory of Protozoology, Institute of Tropical Medicine of São Paulo, Cerqueira César, São Paulo, São Paulo, Brazil, E-mail: rmalafrente@usp.br.

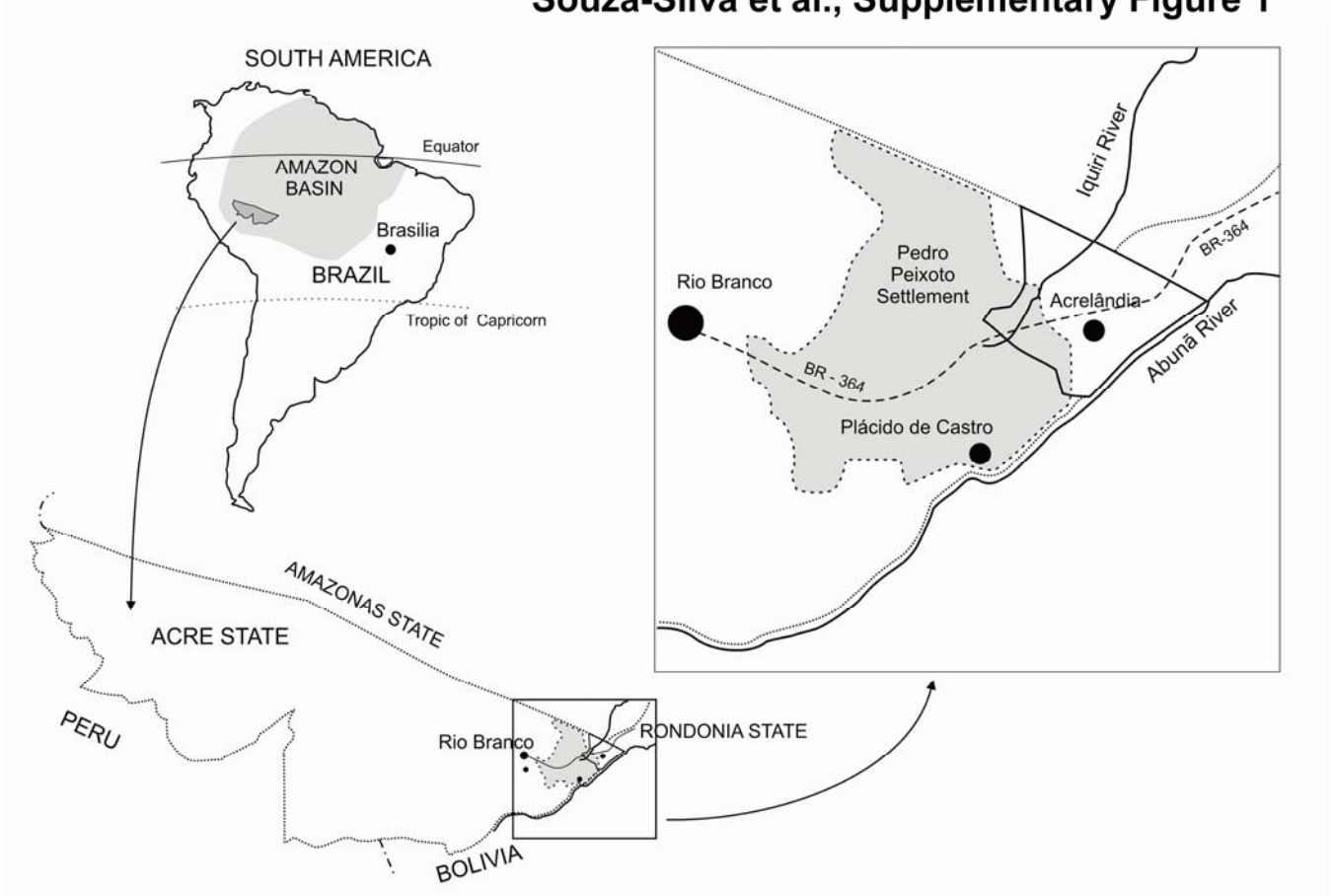
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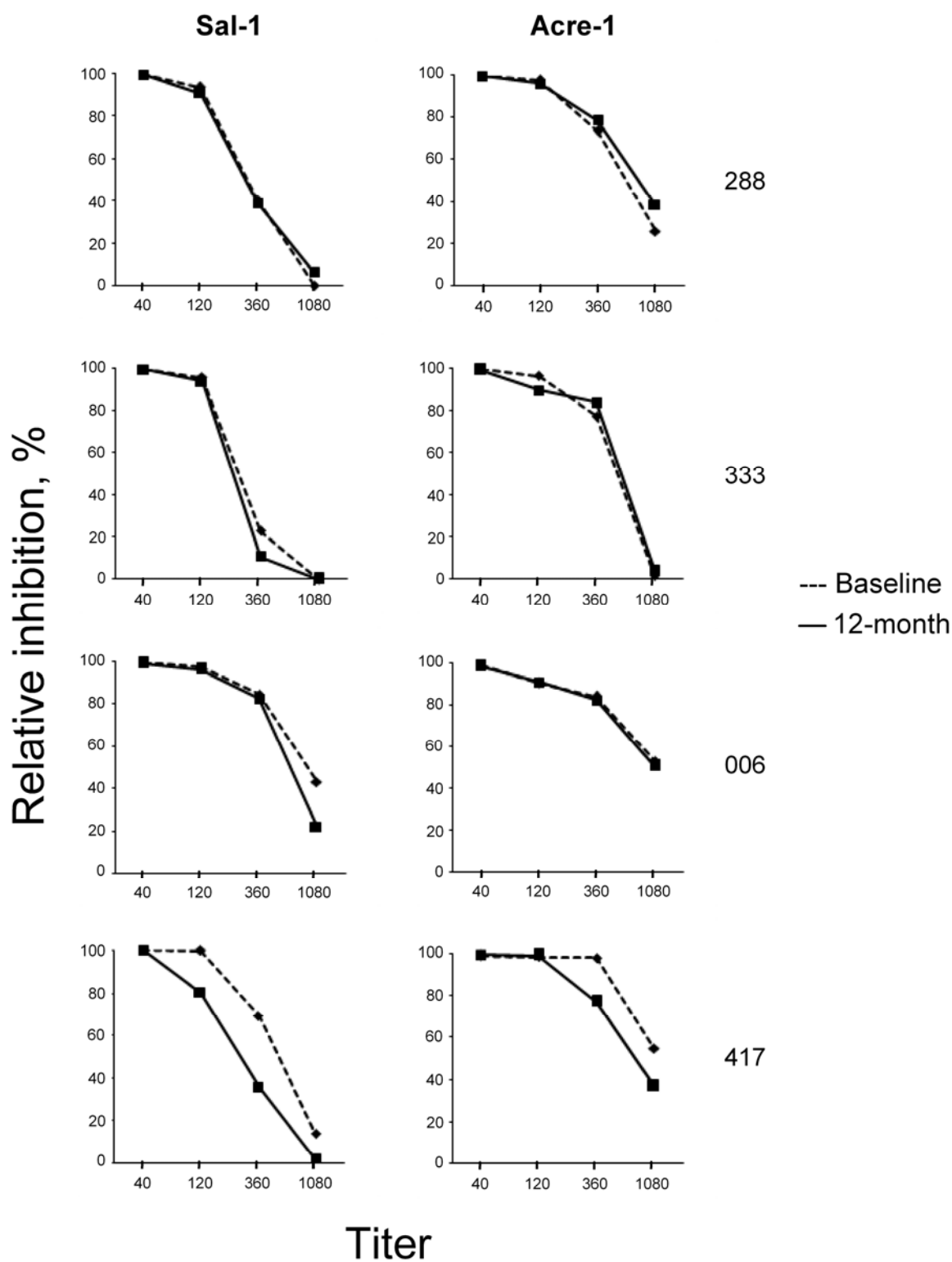
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Souza-Silva et al., Supplementary Figure 1



Souza-Silva *et al.*, Supplementary Figure 2



6 Considerações finais

Neste estudo, foi caracterizada a resposta de anticorpos anti-PvDBP_{II} em áreas da Amazônia brasileira, onde os indivíduos estavam expostos a diferentes níveis de transmissão de malária.

Em uma etapa inicial, os resultados obtidos permitiram demonstrar que indivíduos com história de longa exposição à malária na Amazônia brasileira desenvolvem anticorpos inibitórios, isto é, aqueles capazes de inibir a interação entre o ligante do parasito (DBP_{II}) e seu receptor presente na superfície da célula hospedeira (**Artigo 1**). Este dado foi relevante, já que estes anticorpos haviam sido demonstrados apenas em áreas hiperendêmicas da Papua Nova Guiné (Michon *et al.*, 2000; King *et al.*, 2008), onde as crianças na idade escolar já desenvolveram imunidade adquirida contra a malária. Com a realização deste estudo inicial foi possível ainda observar dois pontos importantes: 1) a presença de anticorpos inibitórios dependia de uma longa exposição à malária, e 2) os níveis de anticorpos inibitórios eram, aparentemente, mais altos no grupo de assintomáticos do que nos sintomáticos.

Os achados preliminares - que a resposta anti-PvDBP incluía anticorpos inibitórios - estimulou o nosso grupo de pesquisa a dar continuidade ao estudo visando com isto descrever melhor esta resposta imune através de um estudo prospectivo de base populacional (**Artigo 2**). Para isso, foi escolhida uma área (ramal do Granada) cujo perfil epidemiológico tinha sido recentemente descrito pelo grupo de pesquisa do Dr. Marcelo Urbano, USP, São Paulo (Silva-Nunes *et al.*, 2006, 2008). Nesta área, as medianas do tempo de exposição e idade dos indivíduos era de 14 e 24,5 anos, respectivamente.

O estudo realizado no ramal Granada permitiu observar que na linha de base apenas cerca de 20% da população apresentava anticorpos anti-PvDBP, detectados pelo ensaio de ELISA, e que os níveis destes anticorpos diminuíram por ocasião do segundo corte (\cong 12 meses depois). Para verificar se fatores individuais tais como, idade, sexo, tempo de residência na área e infecção malárica (aguda ou recente) estavam relacionados com a presença de anticorpos anti-PvDBP, fez-se necessário a construção de modelos de regressão logística múltipla. Através desta análise foi possível observar que o fator mais fortemente relacionado com a presença desses anticorpos foi o tempo de residência do indivíduo na região amazônica (em média 19 anos). Mais especificamente, foi possível determinar que a cada ano de exposição à

malária aumentava em 2% a chance do indivíduo apresentar anticorpos anti-PvDBP. Nesta área, a idade não se correlacionou com a presença de anticorpos anti-PvDBP; entretanto este achado já era esperado, pois a população exposta consistia, principalmente, de migrantes originados de áreas do Brasil onde a malária não é endêmica (Camargo *et al.*, 1994).

Com a realização dos ensaios funcionais foi possível observar que apenas um terço dos indivíduos com anticorpos anti-PvDBP (ELISA), apresentou anticorpos inibitórios. Resultados semelhantes foram encontrados em um estudo realizado com crianças assintomáticas em áreas hiperendêmicas de malária na Papua Nova Guiné (King *et al.*, 2008), onde cerca de 30% das crianças desenvolveram anticorpos inibitórios. Os resultados aqui descritos permitiram concluir que o perfil da resposta de anticorpos inibitórios é semelhante em áreas de transmissão instável, como é o caso da Amazônia brasileira, e áreas de transmissão estável (PNG). Portanto, uma baixa resposta à PvDBP deve ser esperada em populações naturalmente expostas à malária.

Embora os mecanismos pelos quais a PvDBP induz uma baixa resposta de anticorpos ainda sejam desconhecidos, é possível especular fatores que poderiam influenciar nesta resposta. Um dos fatores que provavelmente dificulta a aquisição de uma resposta imune funcional contra a PvDBP é a baixa imunogenicidade da proteína e/ou sua limitada exposição ao sistema imune. Isto porque a PvDPB está localizada nos micronemas do merozoíto, sendo liberada somente no momento da invasão (teoria do “*Just in time*”) (Singh *et al.*, 2006). Desta forma, o *P. vivax* minimizaria a exposição direta do ligante funcional aos anticorpos inibitórios, servindo como um mecanismo de escape deste parasito ao sistema imune do hospedeiro. Este mecanismo de evasão poderia explicar, parcialmente, o motivo pelo qual a população estudada no Acre necessita estar exposta por um longo período à transmissão de malária antes de adquirir anticorpos anti-PvDBP. Corroborando com esta hipótese, um estudo realizado anteriormente na área da Amazônia brasileira demonstrou que altos níveis de anticorpos anti-PvDBP foram adquiridos por trabalhadores de garimpo de ouro (garimpeiros) (Cerávolo *et al.*, 2005), cuja atividade profissional os coloca altamente expostos à infecção malárica (Carvalho *et al.*, 1999; Barbieri & Sawyer, 2007). De fato a prevalência de malária no ramal Granada (3,6%) (Silva-Nunes *et al.*, 2006) foi muito mais baixa do que aquela encontrada em áreas de mineração (14 – 35%) (Barbieri & Sawyer, 2007; Santos *et al.*, 1992; Silbergeld *et al.*, 2002).

É possível que o alto polimorfismo presente na região do ligante possa também influenciar na baixa resposta imune contra a PvDBP (McHenry & Adams, 2006; Vanbuskirk *et al.*, 2004a). A primeira evidência de que os polimorfismos comumente observados na PvDBP_{II} podem alterar o seu caráter antigênico, interferindo assim no bloqueio da ligação PvDBP-DARC por anticorpos inibitórios, foi demonstrado por Vanbuskirk e colaboradores (2004). Os autores demonstraram que soros de coelhos experimentalmente imunizados inibiam a interação ligante-receptor de uma maneira dependente da cepa do parasito. Mais recentemente, nosso grupo demonstrou que indivíduos residentes em área não-endêmica de malária - em um pequeno foco de transmissão autóctone de *P. vivax* em Minas Gerais - desenvolvem anticorpos inibitórios que são cepa-específicos (Cerávolo *et al.*, 2009). Visando reduzir influência do polimorfismo na capacidade do soro em inibir a interação PvDBP-DARC, no presente trabalho utilizou-se duas variantes comumente encontradas na população de estudo (Sal-1 e Acre-1). Entretanto, foi observada uma resposta inibitória muito semelhante para as duas variantes utilizadas, sugerindo a interferência de outros fatores na resposta de anticorpos inibitórios anti-PvDBP.

Os resultados também nos permitiram verificar, uma correlação, ainda que moderada, entre os níveis de anticorpos detectados no ELISA e a porcentagem relativa de inibição, como já descrito por outros pesquisadores (Michon *et al.*, 2000; Cerávolo *et al.*, 2009). Apesar disso, é importante destacar que um número considerável de indivíduos, cujos soros mantiveram alta capacidade inibitória da interação ligante-receptor, perderam seus anticorpos convencionais, detectados no ELISA, ao longo dos 12 meses de estudo. Isso corrobora a hipótese de que os ensaios sorológicos não permitem avaliar a capacidade funcional destes anticorpos. Isto pode ser explicado pelo fato de que a conformação da PvDBP é de grande importância para avaliar a sua atividade funcional, o que não pode ser detectado nos ensaios de ELISA.

Embora tenha sido observado que os anticorpos inibitórios da interação ligante-receptor se mantiveram estáveis ao longo dos 12 meses, a associação entre a presença desses anticorpos e a proteção - como sugerido por um estudo recente com crianças da Papua Nova Guiné (King *et al.*, 2008) - não pôde ser avaliada no presente trabalho. Isto ocorreu devido ao pequeno número de indivíduos que apresentaram anticorpos inibitórios, impedindo, desta forma, a análise estatística. Diante disso, novos estudos devem ser conduzidos para avaliar o papel protetor e para que se possa entender porque apenas poucos indivíduos expostos à infecção

pela malária desenvolvem anticorpos inibitórios. Entretanto, apesar de essenciais, estudos como estes encontram grandes desafios, como a diversidade genética entre os indivíduos e o fato de que a resposta imune não parece ser direcionada apenas para um antígeno específico (Richie, 2006; Gray *et al.*, 2007; Langhorne *et al.*, 2008).

7 Conclusões

1) A maioria dos indivíduos expostos ao *P. vivax* na Amazônia brasileira não é capaz de desenvolver anticorpos capazes de inibir a interação entre o ligante do parasito (DBP_{II}) e seu receptor presente na superfície da célula hospedeira;

2) Quando os anticorpos anti-PvDBP funcionais são adquiridos, os mesmos tendem a se manter estáveis;

3) A presença de anticorpos anti-PvDBP está relacionada com o tempo de exposição dos indivíduos à malária;

4) O perfil de resposta de anticorpos inibitórios em áreas de transmissão instável é semelhante ao perfil descrito em áreas de transmissão estável.

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