Strain-Specific Duffy Binding Protein Antibodies Correlate with Protection against Infection with Homologous Compared to Heterologous *Plasmodium vivax* Strains in Papua New Guinean Children[▽]

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Individuals repeatedly infected with malaria acquire protection from infection and disease; immunity is thought to be primarily antibody-mediated and directed to blood-stage infection. Merozoite surface proteins involved in the invasion of host erythrocytes are likely targets of protective antibodies. We hypothesized that Papua New Guinean children ($n = 206$) who acquire high antibody levels to two *Plasmodium vivax* merozoite proteins, Duffy binding protein region II (PvDBPII) and the 19-kDa C-terminal region of *P. vivax* merozoite surface protein 1 (PvMSP1₁₉), would have a delay in the time to reinfection following treatment to clear all blood-stage malaria infections. Ninety-four percent of the children were reinfected with *P. vivax* during biweekly follow-ups for 6 months. Since PvDBPII is polymorphic, we examined whether individuals acquired strain-specific immunity to PvDBPII. Children with high antibody levels to a prevalent PvDBPII allele (O) were associated with a delay in the time to reinfection with the same variant of *P. vivax* by 25% compared to parasites expressing other PvDBPII alleles (age-adjusted hazard ratio, 0.75 [95% confidence interval, 0.56 to 1.00 by Cox regression]) and 39% lower incidence density parasitemia ($P = 0.01$). Two other prevalent alleles (AH and P) showed a similar trend of 16% and 18% protection, respectively, against parasites with the same PvDBPII allele and reduced incidence density parasitemia. Antibodies directed to PvDBPII PNG-P and -O were both associated with a 21 to 26% reduction in the risk of *P. vivax* infections with higher levels of parasitemia (>150 parasites/ μ l), respectively. There was no association with high antibody levels to PvMSP1₁₉ and a delay in the time to *P. vivax* reinfection. Thus, anti-PvDBPII antibodies are associated with strain-specific immunity to *P. vivax* and support the use of PvDBPII for a vaccine against *P. vivax*.

Immunity to *Plasmodium vivax* has been shown to increase with age in communities where *P. vivax* is endemic, suggesting that a vaccine to *P. vivax* may be possible (1, 27). However, naturally acquired immunity does not prevent infection but instead limits parasite densities and reduces severe disease and clinical symptoms. Humoral immune responses against blood-stage antigens are believed to be an important component of naturally acquired immunity to *Plasmodium* (12, 29). Malaria blood-stage vaccines aim to disrupt the interactions between ligands on the *Plasmodium* merozoite and the receptors on the host erythrocyte by eliciting inhibitory antibodies that target the merozoite ligands. Humoral immune responses to the merozoite antigens Duffy binding protein region II (PvDBPII) and *P. vivax* merozoite surface protein 1 (PvMSP1) have been implicated in acquired immunity to *P. vivax*, are prevalent in communities where *P. vivax* is endemic, and are potential vaccine candidate antigens (2, 21, 22, 24, 27, 28). However, few prospective studies of immune responses to *P. vivax* antigens

have been performed on human populations in areas where *P. vivax* is endemic—we are aware of only one to PvMSP1 (16).

Since PvDBPII interaction with the N-terminal extracellular region of Duffy antigen (DA) on host erythrocytes is essential for *P. vivax* merozoite invasion, a prospective study of antibody responses to the PvDBPII antigen may lead to a better understanding of immune correlates of protection to *P. vivax*. Recently, we developed an assay that measures the functional antibodies that inhibit binding between PvDBPII and its receptor, the N-terminal extracellular region of DA (11). These binding inhibitory antibodies (BIAs) were shown to inhibit the invasion of *P. vivax* into host erythrocytes in vitro (8). Importantly, children that acquire high levels of BIAs show 55% reduction in the risk of *P. vivax* infection (11). Antibodies directed to PvDBPII as measured by enzyme-linked immunosorbent assay (ELISA) also correlated with protection but less strongly than BIAs (11). PvDBPII is highly polymorphic, however, and antibodies to different variants can inhibit the binding of homologous variants but have reduced ability to block the binding of heterologous PvDBPII protein variants in vitro (11, 25). Immune responses of children with BIAs that inhibit binding by $>90\%$ were usually strain transcending (11); however, responses of most children with BIAs that inhibit binding by $<90\%$ were strain specific (11). Only a quarter of

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the children had detectable BIABs using this assay (8), whereas more than 80% of the children had total antibody responses to PvDBPII. It is unknown whether antibodies to different PvDBPII haplotypes protect better against parasites with the same DBPII haplotype than parasites with a different PvDBPII haplotype. Since BIABs correlated with total antibodies to PvDBPII (11) and there were an insufficient number of children with BIABs, we examined the hypothesis that naturally acquired total strain-specific PvDBPII antibodies are associated with greater protection against the homologous versus heterologous strains.

In order to determine if host immunity toward a specific PvDBPII variant increases the time to reinfection with that variant, we followed 206 Papua New Guinean children (mean age, 9.4 years; range, 4 to 14 years) biweekly for 6 months after treatment to clear their blood-stage malaria infections. Prior to treatment, antibody levels were measured by ELISA to five different PvDBPII variants present in the population, and *P. vivax*-positive infections were genotyped for PvDBPII. Based on the three most prevalent PvDBPII alleles, we then determined whether the presence of the PvDBPII allele or high antibody levels to that allele affected the time to reinfection. We also examined the relationship of antibody levels to the 19-kDa C-terminal region of PvMSP1 (PvMSP1₁₉) and the F2 domain of *Plasmodium falciparum* erythrocyte membrane protein 1 (PFEBA175-F2, an ortholog to PvDBPII and an important invasion ligand that binds glycophorin A on host erythrocytes) with the time to reinfection with *P. vivax*.

MATERIALS AND METHODS

Prospective cohort study. The study population of 206 children for this treatment reinfection study has been described (15). This study was reviewed and approved by the institutional review boards of the Papua New Guinea Medical Research Advisory Council, the Veteran's Affairs Medical Center (Cleveland, OH), and the Walter and Eliza Hall Institute (Melbourne, Australia). Children enrolled in the study were primary school children between 5 and 14 years of age. Bed-net usage in the area is limited, and inhabitants with bed nets have not treated them. At the beginning of the study, peripheral venous blood was collected, and two thin and thick blood smear slides were made for determining malaria parasite infection. All children were treated with a 7-day course of artesunate monotherapy (4 mg/kg on day 1 and 2 mg/kg on days 2 to 7), with direct observation of at least five doses. Artesunate does not clear liver-stage *Plasmodium* infections. Following treatment, children were monitored for malaria through biweekly active follow-up visits at school for 25 weeks beginning in June 2004 for a total of 13 follow-up visits. Children that did not attend school on the day of the scheduled follow-up were checked the next day or at their homes at the earliest possible time within the next week. Children were monitored for the acquisition of new infections until they either withdrew from the study or did not provide two consecutive biweekly blood samples.

At each follow-up visit a 250- μ l blood sample was collected from each child into a potassium EDTA Microtainer tube (Becton Dickinson) by finger prick using a retractable lancet. Two thin and thick blood smears were made and stained with 5% Giemsa stain for 30 min to detect the presence of malaria parasites. If clinical malaria symptoms were detected while evaluating a child at a follow-up visit or during the intervening period (by passive case detection), a peripheral venous blood sample was taken, and it was treated according to the Papua New Guinea national treatment guidelines (chloroquine for 3 days plus sulfadoxine-pyrimethamine on day 1).

Two hundred of the 206 children in the study were genotyped for the Duffy gene using a ligase detection reaction (six of the samples did not amplify). All were Fya/Fya, Duffy positive. Additional sampling of individuals in this population failed to show any Fya^{null} alleles (P. Zimmerman, personal communication).

Detection of *Plasmodium* species infection. All blood slides were read by two experienced microscopists independently, and slides with discrepant results were read by a third microscopist. Standard procedures were used for reading the blood slides and estimating parasite densities as described previously (15). Par-

asitemia levels were normalized by the log transformation of $n + 1$. Parasitemia for each sample was determined from a geometric mean of two or more non-discrepant reads and converted to parasites per μ l of blood using a standard leukocyte count of 8,000 per μ l in whole blood. Finger-stick blood samples were separated into plasma and cell pellets. DNA was extracted from the cell pellet fraction by using a QIAamp96 DNA blood kit (Qiagen, Valencia, CA) for all samples.

Infection by each of the four malaria species was assessed in all blood samples collected using a semiquantitative post-PCR, ligase detection reaction-fluorescent microsphere assay (LDR-FMA) (6). To differentiate between treatment failures and newly established blood-stage infections, all *P. vivax* isolates that were detected (by PCR or light microscopy [LM]) between the pretreatment time point and 6 weeks posttreatment were genotyped for the highly polymorphic *P. vivax* Duffy binding protein alleles as previously described (5, 6).

The infection intensity of *P. vivax* was quantified using real-time quantitative PCR (RTQ-PCR) as previously described (17). Only *P. vivax*-positive samples identified by LDR-FMA were quantified using real-time PCR. All other samples were assumed to have a *P. vivax* quantity of zero.

PvDBP genotyping. The polymorphic *PvdbpII* gene was used as a marker for *P. vivax* strains. *PvdbpII* was genotyped at 14 polymorphic residues using a *PvdbpII* post-PCR LDR as previously described (5, 6). Single nucleotide polymorphisms were then assembled into distinct haplotypes using the predominant allele at each single nucleotide polymorphism as described previously (5, 6). This method was semiquantitative, and the dominant haplotype could usually be determined.

Measurement of antibody titers to recombinant PvDBPII variants, PvMSP1₁₉, and PFEBA175-F2 by ELISA. Five different variants of PvDBPII (Salvador-1 [Sal-1], PNG-O, PNG-P, PNG-AH, and PNG-C) observed in the Liksul, Papua New Guinea, population were expressed in *Escherichia coli* and refolded as previously described (8). The antibody titers to each of the recombinant PvDBPII variants PvMSP1₁₉ from *E. coli* expression (a gift from Anthony Stowers at the Malaria Vaccine Development Unit at the NIH) and *P. falciparum* EBA175F2 (a gift from Chetan E. Chitnis at the International Centre for Genetic Engineering and Biotechnology, New Delhi, India) for each study participant were determined pretreatment by ELISA. All PvDBPII variants tested were run on the same plate. Immulon 4 plates (Dynal, Inc.) were coated with 2 μ g/ μ l of recombinant protein in phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C and blocked with 1% bovine serum albumin in PBS (blocking buffer). Antigen-coated wells were incubated overnight at 4°C with 100 μ l of a 1:400 dilution of pretreatment serum samples in blocking buffer/well. To ensure that the same amount of properly refolded protein of each variant was added to each plate, the recombinant protein was tested for relative binding to chimeric nDA-Ig (a construct encoding the N-terminal 60 codons of the human DA ligated to the sequence encoding the Fc region of human immunoglobulin G [IgG]) using an ELISA-based format as previously described (11, 18). Each protein was adjusted such that each variant had the same endpoint binding titer. Plates were washed with PBS and 0.05% Tween 20 (wash buffer), followed by the addition of 100 μ l of alkaline phosphatase-labeled goat anti-human IgG (Jackson ImmunoResearch) at 0.5 μ g/ml that was incubated for 1.5 h at room temperature and then washed three times prior to development with *p*-nitrophenyl phosphate as the substrate. The reaction was terminated by the addition of 5% EDTA, and the optical density at 405 nm (OD₄₀₅) in each well was recorded using an ELISA microplate reader (Molecular Devices). For all test samples, both positive- and negative-control samples were added to each plate. Sera from at least four North American or Australian controls that had not been exposed to malaria were used as negative controls. The mean plus 3 standard deviations of the ODs from the negative controls was used as the cutoff for a positive response; cutoff values were as follows: 0.16 for Sal-1, 0.18 for C, 0.19 for O, 0.18 for P, and 0.20 for AH ($n = 14$). As a positive control, pooled sera from 10 individuals with strong antibody responses to all recombinant proteins were serially diluted twofold starting at a 1:400 dilution of the stock solution. The 1:400 starting dilution was given an arbitrary value of 1,000 antigen units (AU). Antibody responses for PvMSP1₁₉ and PFEBA175-F2 were performed differently. Here an AU was calculated by dividing the mean OD reading of the sample by the mean OD reading from the sera of 10 North Americans or Australians who had never been exposed to malaria plus an OD reading of 3 standard deviations; the cutoff values were as follows: 0.035 for PFEBA175 and 0.32 for PvMSP1₁₉. IgG to recombinant PFEBA175-F2 was tested in Nunc Maxisorb 96-well plates; wells were coated with antigen at 0.5 μ g/ml, serum was tested at 1/500, and IgG binding was detected using anti-human IgG-horseradish peroxidase and its substrate 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (5, 15). ELISA assays for PvMSP1₁₉ contained internal positive and negative controls, and ODs for the controls were within 20% of the coefficient of variation between

TABLE 1. Common *Plasmodium vivax* DBPII haplotypes identified from children residing in an area of Papua New Guinea where *P. vivax* is endemic

Haplotype	Amino acid residue ^a													Frequency (%) ^b
	308	333	371	375	384	385	386	390	417	424	437	447	503	
Sal-1	R	L	K	N	D	E	K	R	N	L	W	S	I	0.7
O	S				G			H		I		K		20.4
C	S				G		Q		K	I	R		K	1.3
AH	S		E		G		Q		K	I	R		K	26.0
P	S	F		D	G	K	N	H	K	I	R		K	10.4

^a Polymorphic residues present at a frequency of >5% in the population. Amino acid residues in italics are predicted to be surface exposed on PvDBPII according to homology model building based on the recently determined crystal structure of PkDBPαII (19).

^b *n* = 754.

test plates. AU were log transformed after adding 1 to each value to normalize the distribution for use in Cox regression.

Statistical analysis. The time to infection for *P. vivax* was calculated as the time between the date of the first treatment at enrollment and the detection of *P. vivax* infection by LDR-FMA and PvDBPII genotyping either through active or passive case definition during the 25 weeks of active surveillance. The time-to-infection data were analyzed using standard survival analysis techniques. A log-rank test was used to evaluate the difference in nonparametric survival curves. Because hazards for the different explanatory variables were generally proportional over the follow-up study, Cox regression was used to test for univariate and multivariate risk factors using PROC PHREG in the Statistical Analysis System (SAS) version 9.1 (SAS, Inc., Cary, NC). The best-fitting model was previously identified by backwards selection and likelihood ratio tests (15; unpublished observations). Village location was not included in the model since we had not observed the clustering of *P. vivax* infections during the study (15). Hazard ratios were adjusted for age since we had observed a decreased risk of *P. vivax* infections in children greater than 9 years old in this study (15).

Observations were right censored at the last follow-up date with no infection, when they withdrew from the study, or when they missed two consecutive follow-up bleeds. Since 101 of the children (49%) were re-treated according to Papua New Guinea national treatment guidelines (see above) during the course of the study for *P. falciparum* and later became positive for *P. vivax*, these observations were included in the survival analysis by adjusting the duration to infection by subtracting 28 days for each time the child was re-treated. Depending on the specific survival analysis performed, i.e., whether the child became positive for *P. vivax* or a specific *P. vivax* strain before retreatment (see Results), 12.1% to 42.2% of the children were re-treated. *P. vivax* infections observed during the first 14 days after the initial artesunate treatment were also censored.

As AU for antibody levels of PvDBPII variants were not normally distributed without log transformation, the correlation coefficients between PNG-AH, PNG-O, PNG-P, and Sal-1 were determined using Spearman's rank correlation in the SAS version 9.1 (SAS, Inc., Cary, NC) using PROC CORR. Since it is possible for correlation coefficients to be driven by double-negative samples, we removed all double-negative or very low samples (i.e., those in the lower tercile) before calculating the correlation coefficients.

Chi-square tests were used to test for differences in the number of PvDBPII variants recognized in different age groups of children using PROC FREQ in SAS version 9.1 (SAS, Inc., Cary, NC).

For the calculation of incidence density rates which incorporated repeated measures for the same individual, all disease episodes observed during active follow-up and passive morbidity surveillance were considered. A child was considered at risk until withdrawal or the end of the study with the exception of 14 days after initial treatment and 6 weeks following further antimalaria treatments. A linear mixed model was used in SAS 9.1 (PROC MIXED). The model was based on a restricted maximum likelihood model which estimates the applied maximum likelihood estimation techniques to the likelihood function associated with a set of error contrasts rather than to the original observations as previously described (13). The repeated option of PROC MIXED was used to specify the covariance structure of the error term to incorporate repeated measures for the same individual. PROC MIXED allowed us to incorporate subjects with incomplete data into the analysis. The compound symmetry covariance structure was used, as this structure gave the best fit based on Akaike's information criteria, the corrected Akaike's information criteria, and the Bayesian information criteria as provided in the SAS 9.1 output.

RESULTS

Malaria infection in the population. We have previously described the epidemiology of malaria infection in the current study population of 206 children in detail (15). Briefly, the median time to *P. vivax* reinfection following the initial artesunate treatment was 54 days as detected by LDR-FMA and 119 days by LM; for *P. falciparum*, the median time to reinfection occurred at 55 days as detected by LDR-FMA and 99 days by LM. The incidence rate for *P. vivax* during the follow-up period was 2.0 infections per person per year by LM (95% confidence interval [95% CI], 1.7 to 2.5) and 5.3 infections per person per year by LDR-FMA (95% CI, 4.5 to 6.1). The incidence rate for *P. falciparum* was 3.2 infections per person per year by LM (95% CI, 2.7 to 3.7) and 5.0 infections per person per year by LDR-FMA (95% CI, 4.3 to 5.8).

P. vivax infections detected in the first 6 weeks posttreatment by LDR-FMA were genotyped for the highly polymorphic PvDBPII (5). Only one *P. vivax* infection detected in the first 6 weeks posttreatment by LDR-FMA had the same *P. vivax* PvDBPII genotype as the infection at baseline, suggesting a possible treatment failure, and was excluded from further analysis.

One hundred and one (49%) children were re-treated during the course of the study for *Plasmodium* infections (mainly *P. falciparum*). Those children that were re-treated had a higher incidence rate for *P. vivax* blood smear infection than those children not re-treated (4.6 [95% CI, 4.0 to 5.5] versus 2.8 [95% CI, 2.4 to 3.4] per 100 person years).

A total of 715 of 788 *P. vivax*-positive samples were genotyped for PvDBPII and were ascribed to 1 of the 27 haplotypes identified during the course of the study (5); the remainder of the *P. vivax*-positive samples had haplotypes that could not be identified (*n* = 39) or did not amplify at the PvDBPII locus (*n* = 34). Table 1 shows some of most common PvDBPII haplotypes and relative frequencies determined over the course of the study. The AH, O, and P variants were the most common (26%, 20%, and 10% frequency, respectively), whereas the Sal-1 haplotype, a vaccine candidate antigen, was observed infrequently (0.7%). The details for additional PvDBPII haplotypes identified during the study have been described elsewhere (5).

The presence of a PvDBPII haplotype pretreatment does not affect the time to reinfection with the same haplotype. Since prior studies have suggested that PvDBPII is under immune selection, resulting in a high degree of polymorphism for the

gene encoding this protein, we hypothesized that an existing infection with one strain of the parasite will affect susceptibility to parasites with the same *PvdbpII* haplotype more than that to parasites with a different *PvdbpII* haplotype. Seventy of 206 (34%) of the children had *P. vivax* infections detected by post-PCR LDR-FMA pretreatment, and 57 were genotyped for the *PvdbpII* polymorphisms (*PvdbpII* did not amplify for 13 of the samples). The two most prevalent types of PvDBPII strains pretreatment were considered for analysis—PNG-AH (36.8%; $n = 21$) and PNG-O (15.7%; $n = 9$). Pretreatment infection status with the two most prevalent strains in the population (AH and O) did not alter the time to blood-stage reinfection with that same strain (adjusted hazard ratio [AHR] = 1.23 [95% CI, 0.60 to 2.53] and AHR = 0.77 [95% CI, 0.23 to 2.60] for AH and O, respectively).

We further examined whether the presence of *P. vivax* pretreatment with a similar PvDBPII allele—that is, in the same phylogenetic family or the same PvDBPII subdomain—could alter the time to reinfection with *P. vivax* with a similar PvDBPII allele. We previously found that the various PvDBPII alleles clustered into three distinct groups (4). We examined the two most prevalent PvDBPII phylogenetic groups since the third phylogenetic group (which includes Sal-1) had only two children that were infected with this type of *P. vivax* pretreatment. The pretreatment infection with *P. vivax* parasites with PvDBPII alleles corresponding to one or the other of the dominant phylogenetic groups did not alter the time to infection with the same PvDBPII phylogenetic group (AHR = 1.16 [95% CI, 0.62 to 2.18] and AHR = 1.55 [95% CI, 0.76 to 3.18] for phylogenetic groups B and A, respectively).

The recent crystal structure of a homologous protein to PvDBPII, *Plasmodium knowlesi* PkDBP- α , indicates that PvDBPII is comprised of three subdomains (9, 26). Subdomain 3 is the largest with the greatest number of cysteines; however, site-directed mutagenesis studies suggest that subdomain 2 contains the critical binding motif and is the most polymorphic region of the molecule (5). Therefore, we examined whether parasite strains that had identical amino acid residues for subdomain 2 (amino acid residues 303 to 406) or subdomain 3 (amino acid residues 407 to 528) prior to treatment correlated with the time to reinfection with *P. vivax* with the same or different PvDBPII subdomain. For subdomain 2, the two most common haplotypes were SLKNGEKH (haplotypes PNG-O, -U, -24, -25, and -T [Table 1]) and SLENGEQR (haplotype PNG-AH). For subdomain 3, KIRSK (including haplotypes PNG-AH, -C, -20, -17, -2, -23, -U, -26, -9, and -P) and NIWKI (including haplotypes PNG-O, -12, and -19) were the two most prevalent haplotypes as described (Table 1) (20, 21). Again, pretreatment infection status did not affect the time to reinfection with the same or different PvDBPII subdomain (AHR = 0.99 to 1.36 for all subdomains analyzed).

Strain-specific antibody responses to PvDBPII variants. To determine whether there are variant-specific antibody responses to PvDBPII, we measured levels of antibodies directed to five recombinant proteins corresponding to the different variants of PvDBPII (O, P, AH, C, and Sal-1) (Table 1) by ELISA. Of this cohort of 206 children, 72%, 90%, 90%, and 98% had detectable antibodies to the Sal-1, O, P, and AH variants of PvDBPII, respectively, prior to treatment. We observed a trend for more PvDBPII variants recognized by indi-

FIG. 1. Box and whisker plots for AU for five *Plasmodium vivax* merozoite antigens at the start of the study for 206 children before treatment. (A) PvDBPII PNG-AH, PvDBPII PNG-O, PvDBPII PNG-P, and PvDBPII Sal-1. (B) PvMSP1₁₉. All antigens except PvDBPII Sal-1 and PvMSP1₁₉ had individuals with maximum AU.

vidual sera with increasing age (e.g., sera from 49% of children less than 8 years old recognized all five PvDBPII variants; that increased to 68% for children aged 8.0 to 9.9 years, 64% for children aged 10.0 to 11.9 years, and 83% for children aged 12.0 to 14.4 years) but was not significant ($\chi^2 = 9.52$; degrees of freedom, 6; $P = 0.15$). Relative antibody levels were lowest for the Sal-1 and P strains and highest for the AH and O strains (Fig. 1), which exactly corresponded to the relative frequencies of these variants in the population (Table 1). Of note, 98% of the children had antibodies to the C strain with similar antibody levels to that for the AH strain, although the frequency of the C strain was 1.7% in the population. The C strain differed by only one amino acid at residue 371 from the AH strain (Table 1), indicating that these two variants are likely to be highly cross-reactive and that this amino acid substitution may not alter the protein's antibody-binding capacity. A high proportion of children also responded to the other malaria blood-stage antigens examined: 83.4% for PvMSP1₁₉ and 90.3% for PfEBA175-F2.

Serum samples from some individuals demonstrated different levels of antibody reactivity to each variant, while others demonstrated similar levels of antibodies to all variants. Serum from 65% of the children recognized all five PvDBPII variants assessed, 32% of the children recognized three of five PvDBPII variants, and six children (3%) responded to only one or two PvDBPII variants. To further evaluate variant-specific antibody responses, we performed competition ELISAs with sera from selected individuals with moderate to high titers to the AH variant and various antibody levels to other haplotypes (Fig. 2). The preincubation of sera with both the AH and C haplotypes at saturating levels almost completely blocked the subsequent detection of remaining antibody to AH. By contrast, the preincubation of sera with the recombinant P or O haplotype only partially blocked the detection of AH antibodies. The Spearman rank correlation coefficients between AH, O, P, and Sal-1 are indicative of a positive relationship between relative antibody levels as determined by AU even after removing double-negative or low samples (i.e., samples that were negative for both antigens or in the lower tercile) ($r = 0.24$ between O and AH [$n = 164$], $r = 0.44$ between P and AH [$n = 163$], $r = 0.47$ between Sal-1 and AH [$n = 160$] [Fig. 3], $r = 0.46$ between O and P [$n = 157$], $r = 0.24$ between O and Sal-1 [$n = 166$], $r = 0.46$ between P and Sal-1 [$n = 161$]). Overall, these results dem-

FIG. 2. Competition ELISA showing only partial cross-reactivity between the different variants. Plates were coated with 0.5 $\mu\text{g/ml}$ of the AH variant of PvDBPII. Plasma was preincubated with no protein (AH alone) or with 1 $\mu\text{g/ml}$ of recombinant AH, C, O, or P for 1 h at 37°C prior to being added to the ELISA plate. Results are shown for plasma obtained from one individual. Similar results were obtained from other individuals.

onstrate a variant-specific as well as a cross-reactive antibody response.

Variant-specific PvDBPII antibody levels correlate with delayed time to reinfection with homologous strains and high-density *P. vivax* infections. In order to test whether children with strain-specific PvDBPII antibodies would have longer times to reinfection with parasites expressing the same PvDBPII haplotype compared to parasites expressing other haplotypes, PvDBPII variant-specific antibody levels were used as a continuous variable for Cox regression. Hazard ratios were adjusted for age. Increased levels of PvDBPII variant-specific antibodies did not affect the susceptibility of any *P. vivax* infection based on LM or PCR diagnosis (Table 2). However, increased levels of PvDBPII PNG-O and -P antibodies significantly decreased the risk of any *P. vivax* infections with parasitemia of more than 150 parasites/ μl by 21 to 25%, and antibodies of PNG-AH and Sal-1 showed a similar trend (Table 2). The levels of PvDBPII antibodies to any of the variants tested did not affect the time to reinfection with *P. falciparum* (HR = 0.93 to 1.03; $P \geq 0.10$), and the antibody levels to PfEBA175-F2 did not decrease the hazard of *P. vivax* infections diagnosed by LM or LDR-FMA (Table 2). PfEBA175-F2 antibodies have been found to be associated with a reduced risk of symptomatic *P. falciparum* malaria and high-density parasitemia in this same study population (23).

Importantly, children with higher antibody levels to the *P. vivax* recombinant antigen corresponding to the AH, O, and P PvDBPII haplotypes showed a trend toward a greater delay in *P. vivax* reinfection assessed by LM with parasites expressing the same DBPII haplotype compared to parasites with heterologous PvDBPII haplotypes or no infection (from 16 to 25%) (Table 2). When the risk of *P. vivax* reinfection was assessed by LDR-FMA, only a small level of protection was observed that ranged from 4% to 14% for the homologous versus the heterologous strains or for no infection (Table 2). Since children with higher antibody levels to single PvDBPII variants showed a trend of protection, we investigated whether children with high antibody levels to all four variants would be at a reduced

FIG. 3. Scatter diagram for PvDBPII PNG-AH and PvDBPII Sal-1 AU for 160 children at the start of the study. R is 0.47, as determined by Spearman's rank correlation. A total of 46 children were not included in the correlation analysis since they either did not respond or had a response in the lower tercile for both antigens.

risk of *P. vivax* infection. Thus, positive antibody responses to the AH, O, P, and Sal-1 PvDBPII variants (based on an OD greater than the mean plus 3 standard deviations of the negative-control ODs) were summed and used as a discrete variable in Cox regression. Children with high antibody responses to more than one PvDBPII variant had a 30% decreased risk for *P. vivax* infections greater than 150 parasites/ μl (Table 2), but PvDBPII antibodies did not protect against any *P. vivax*-positive infections based on LM or LDR (Table 2).

Variant-specific PvDBPII antibody levels correlate with low incidence density parasitemia. The geometric mean incidence density parasitemia based on LM and RTQ-PCR tended to decrease with increasing PvDBPII antibodies (Fig. 4). Using a mixed linear model that adjusted for age, multiple strain *P. vivax* infections, and differing numbers of follow-up observations, we found no significant association between PvDBPII antibodies and *P. vivax* parasitemia as measured by LM ($F = 1.09$ to 2.48; $P = 0.09$ to 0.47). However, when measured by RTQ-PCR, this trend of decreasing parasitemia with a higher level of antibodies to the O variant of PvDBPII became significant ($F = 4.76$; $P = 0.01$). (F values ranged from 0.34 to 1.47, while P values ranged from 0.23 to 0.71 for the other PvDBPII variants.) This same trend of decreasing parasitemia with increasing PvDBPII antibodies was not observed for *P. falciparum* (data not shown).

Relationship of PvMSP1₁₉ antibody levels to susceptibility to reinfection with *P. vivax* and *P. vivax* parasitemia. Antibody levels to recombinant protein PvMSP1₁₉ did not significantly affect the risk of *P. vivax* over the course of the 6-month study (AHR = 0.92 [95% CI, 0.62 to 1.36] and $P = 0.68$ for LM; AHR = 1.07 [95% CI, 0.78 to 1.47] and $P = 0.69$ for LDR-FMA). Furthermore, anti-PvMSP1₁₉ antibodies did not significantly reduce the risk of moderate- to high-density *P. vivax* infections (i.e., >150 parasites/ μl) (HR = 0.69 [95% CI, 0.34 to 1.38]; $P = 0.29$). PvMSP1₁₉ also did not affect the risk of *P. falciparum* reinfection by LM or LDR-FMA over the course of the study (HR = 0.97 and $P \geq 0.86$ for PvMSP1₁₉).

As described above for PvDBPII, the geometric mean parasitemia based on LM and RTQ-PCR tended to decrease with increasing levels of PvMSP1₁₉ antibodies (Fig. 4). Using the

TABLE 2. Association of antibody levels to different variants of PvDBPII and PfEBA175-F2 and protection against *Plasmodium vivax* infection

Antibody	Infection	LM			LDR		
		AHR (95% CI) ^a	P value	No. (%) of children re-treated for <i>P. falciparum</i>	AHR (95% CI)	P value	No. (%) of children re-treated for <i>P. falciparum</i>
PvDBPII AH	Any <i>P. vivax</i> infection	0.98 (0.85–1.14)	0.81	46 (22.3)	1.02 (0.91–1.15)	0.74	25 (12.1)
	>150 Pv/μl	1.00 (0.80–1.27)	0.97	84 (40.8)	ND ^d	ND	
	AH variant ^b	0.84 (0.67–1.05)	0.13	87 (42.2)	0.96 (0.81–1.14)	0.65	72 (34.9)
PvDBPII O	Any <i>P. vivax</i> infection	0.96 (0.82–1.12)	0.60	46 (22.3)	1.00 (1.00–1.01)	0.06	25 (12.1)
	>150 Pv/μl	0.74 (0.57–0.97)	0.03	84 (40.8)	ND	ND	
	O variant ^b	0.75 (0.56–1.00)	0.05	83 (40.3)	0.86 (0.70–1.04)	0.12	65 (31.6)
PvDBPII P	Any <i>P. vivax</i> infection	0.96 (0.83–1.13)	0.65	46 (22.3)	0.92 (0.81–1.06)	0.25	25 (12.1)
	>150 Pv/μl	0.79 (0.63–0.99)	0.04	84 (40.8)	ND	ND	
	P variant ^b	0.82 (0.58–1.14)	0.24	87 (42.2)	0.86 (0.65–1.11)	0.24	86 (41.8)
PvDBPII Sal-1	Any <i>P. vivax</i> infection	0.98 (0.84–1.14)	0.77	46 (22.3)	0.96 (0.85–1.09)	0.54	25 (12.1)
	>150 Pv/μl	0.85 (0.64–1.14)	0.29	84 (40.8)	ND	ND	
	Sal-1 variant ^b	ND	ND		ND	ND	
PvDBPII (AH + O + P + Sal-1) ^c	Any <i>P. vivax</i> infection	0.96 (0.78–1.18)	0.68	46 (22.3)	1.05 (0.88–1.27)	0.58	25 (12.1)
	>150 Pv/μl	0.70 (0.51–0.95)	0.02	84 (40.8)	ND	ND	
PfEBA175	Any <i>P. vivax</i> infection	1.04 (0.90–1.12)	0.61	46 (22.3)	1.15 (1.01–1.30)	0.04	25 (12.1)

^a Hazard ratios were adjusted for age.^b Risk of reinfection to homologous versus heterologous variants or no infection.^c PvDBPII antibody responses to AH, O, P, and Sal-1 were coded as responders if greater than the mean + 3 standard deviations of the OD of the negative controls. Positive antibody responses were summed for all four variants tested—i.e., 4 for all positive, 3 to 1 for three to one positive, and 0 for all negative.^d ND, not determined.

same mixed linear model described above for PvDBPII, we found no significant association between PvMSP1₁₉ antibodies and *P. vivax* parasitemia as measured by LM ($F = 0.87$; $P = 0.46$) or RTQ-PCR ($F = 0.58$; $P = 0.63$).

DISCUSSION

The presence of strain-specific immunity to *Plasmodium vivax* infection has been clearly shown in early human studies of experimental human infections to treat neurosyphilis (7, 14). Two or three infections of one presumed strain of *P. vivax* resulted in significant reduction in the clinical disease and parasitemia to the homologous, but not heterologous, strains (7, 14). The question in the current study is whether polymorphisms in PvDBPII, an essential invasion ligand, could account for some of this strain-specific immunity. To study this question, we examined whether the presence of a *P. vivax* strain pretreatment would affect the time to reinfection with that same strain as defined by PvDBPII haplotype and whether the association of total antibody levels to two important blood-stage invasion ligands would protect against *P. vivax* infection. Our first hypothesis was that genotype-specific immunity develops only to the predominant blood-stage parasite detected prior to treatment and that it preferentially suppresses this genotype when it later emerges at a relapse of mixed infections. Since we observed no association between the presence of the initial predominant strain and the time to reinfection with the same or similar strain compared to different strains, this hypothesis is less likely, though there might be a threshold level of parasitemia or frequency of infection required for the induction of a protective immune response. Alternatively, since the overall *P. vivax* parasitemia levels were low and there was little clinical disease, some immunity to different strains may have already developed in the older children studied, obscuring detectable strain-specific immunity using this ap-

proach. It is also possible that since artesunate treatment does not eradicate liver-stage infection, relapsing latent parasite strains might be overrepresented and similar to the original parasite strain detected. Interestingly, this was not observed and is consistent with recent reports that genotypes that caused the initial infection are unrelated to the parasite isolates that caused the relapse (3, 10).

The second hypothesis studied was whether antibodies that recognized recombinant proteins corresponding to different variants of PvDBPII, a key invasion ligand that binds DA, correlated with protection against the same PvDBPII haplotype. More than 80% of the individuals acquired antibodies against PvDBPII. Antibody recognition was often strain specific, and antibody levels positively correlated with the relative frequencies of the most common PvDBPII haplotypes. High levels of PvDBPII-specific antibodies failed to correlate with a delay in time to any *P. vivax* infection. However, antibodies to the O and P variants protected against higher *P. vivax* parasitemia levels by 20 to 25%, and antibodies to the Sal-1 variant also support this trend by decreasing the risk of higher parasitemia infections by 15%. When antibody levels for the four most common variants were combined, protection to higher parasitemia levels increased to 30%, suggesting a component of strain-specific protection. This was demonstrated by showing that high levels of antibodies to the three most common PvDBPII variants tended to show a delay in the time to reinfection with homologous parasite strains, indicating strain-specific protection. This delay in the time to reinfection in children with high antibody levels to PvDBPII is further supported by incidence density parasitemia data. The mean parasitemia in children with high antibody levels to PvDBPII tended to be lower than that in those with lower antibody levels. After adjusting for repeated sampling and confounding factors, children with high antibody levels to the PvDBPII O variant had

FIG. 4. Geometric mean of *Plasmodium vivax* parasitemia determined by LM (A) and real-time PCR (B) based on the upper tertile (H), intermediate tertile (M), and lower tertile (L) of antibody responses in 206 children. Antibodies to four PvDBPII variants (AH, O, P, and Sal-1) and to PvMSP1₁₉ were measured at the start of the study. The geometric means of parasitemia are based on samples taken every 2 weeks throughout the 6-month study from 206 children ($n = 858$ to 941 for each geomean). Error bars show the standard errors. Significant P values are shown based on a linear mixed model which takes into account age, mixed *P. vivax* infections, and repeated measures on individual children.

significantly lower parasitemia based on real-time PCR. Although we detected antibodies to two other PvDBPII variants, their frequencies in the population were too low to detect protection against *P. vivax*. The protection against higher parasitemia levels likely includes both a strain-specific and strain-transcending component. Importantly, the protective effect was also species specific, as the children with high levels of PvDBPII antibodies were only protected against *P. vivax* infection and not *P. falciparum* infection, which had similar transmission intensity in the population. There was also no cross-protection against *P. vivax* with respect to the orthologous protein PfEBA175-F2. We were not able to determine if antibodies to PvDBPII variants protect against symptomatic *P. vivax* because there were very few *P. vivax* symptomatic episodes ($n = 5$) within the population during the 6 months of observation. Of note, there was no increased risk of reinfection with the same *P. vivax* parasites, which suggests that relapsing parasites are unlikely to have skewed reinfection results when examining the association of antibody levels to blood-stage invasion ligands.

We observed a stronger association between the presence of high-level anti-PvDBPII antibodies and a delay in the time to reinfection by homologous than by heterologous *P. vivax* strains, as measured by LM compared to the more sensitive PCR-based assay. This is not surprising since PCR will detect more infections with low-level parasitemia that are less likely to develop into clinical disease. It also suggests that PvDBPII-

specific antibodies decrease parasitemia by reducing *P. vivax* invasion into erythrocyte parasites but does not completely prevent infections that remain detectable by the much more sensitive PCR methods.

Recently, we developed a quantitative receptor-binding assay to distinguish between antibodies that merely recognize PvDBPII and those that inhibit binding to DA or BIABs. High levels of BIABs correlated with 50% protection against any *P. vivax* infection compared to 15 to 25% protection observed in the current study with total anti-PvDBPII antibodies detected by ELISA in this same cohort of children. The protection engendered by high levels of BIABs was strain transcending, whereas the protection against total anti-PvDBPII is primarily strain specific. Previously, we observed intermediate PvDBPII-specific BIABs in 25% of the children that showed little or no protection against *P. vivax* infection. Most of these children had not acquired strain-transcending BIABs. The number of children with strain-specific BIABs were too few to assess strain-specific immunity. By contrast, more than 80% of the children in the study had total PvDBPII antibodies that permitted the current analysis. It is possible that the measurement of total antibodies to PvDBPII may have functional significance because (i) the assay used to measure BIABs may be insufficiently sensitive or specific to measure all or low levels of BIABs or (ii) antibodies may be mediating protection by mechanisms other than by just blocking binding. Overall,

these results suggest that children first acquire strain-specific antibodies to PvDBPII that can confer better protection against homologous than heterologous strains. With repeated exposure to multiple PvDBPII variants, individuals acquire immunity to other strains, with a few individuals developing high levels of BIAs and strain-transcending immunity. The challenge will be the development of a vaccine that can achieve this strain-transcending immunity.

We also examined IgG to PvMSP1₁₉ with respect to the time to *P. vivax* infection. PvMSP1 is highly immunogenic since the C-terminal region of this protein is recognized by antibodies and T cells in individuals recently exposed to *P. vivax* (22). In addition, antibodies against the cysteine-rich epidermal growth factor-like domains of PvMSP1₁₉ increase with age in populations in areas where *P. vivax* is endemic, suggesting a role in acquired immunity (16). However, we found no evidence that antibodies to PvMSP1₁₉ correlate with protection against *P. vivax* infection. Only one other study, to our knowledge, has examined whether antibodies to PvMSP1 correlate with protection in populations where *P. vivax* is endemic, and similarly, the authors have found no protective effect for antibodies against the C-terminal PvMSP1₁₉ (16). They did observe a protective effect associated with antibodies against the N-terminal region of this protein.

An important limitation of the study is that almost half of the children (49%) acquired clinical *P. falciparum* infections and were re-treated prior to becoming infected with *P. vivax*. In order to retain these children in the study, we reduced the duration that each child was at risk by 28 days for each re-treatment. These re-treated children are not completely comparable to the children who received the initial treatment of 7 days with artesunate since the children with *P. falciparum* infections were treated for 3 days with artesunate and a single dose of sulfadoxine-pyrimethamine (SP). Pyrimethamine has a half-life of 3 to 4 days and sulfadoxine 6 to 8 days. Another potential confounding factor is that the children that received treatment for *P. falciparum* infection during the course of the study were also more likely to be infected with *P. vivax*. These children may be more susceptible to *Plasmodium* in general due to environmental or genetic factors.

Multiple statistical comparisons were performed, and some associations may have arisen by chance. However, the consistent trends of an association between elevated strain-specific antibody responses and protection against homologous versus heterologous strains for the most common variants suggest that these relationships did not arise just by chance.

In conclusion, the present study further demonstrates that parasite invasion ligand PvDBPII is an important target of naturally acquired immunity against *P. vivax* and that a component of this natural immunity is strain specific. By contrast, PvMSP1₁₉ failed to demonstrate any association with protection against *P. vivax* infection. Challenges for further studies include discovering how to generate high levels of protective strain-transcending antibodies, investigating whether multiple PvDBPII variants need to be included in a vaccine, and understanding better the role of other invasion ligands at the apical end of the merozoite in natural immunity to *P. vivax* with the aim of developing a multiantigen blood-stage vaccine.

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