BLOCKING OF TRANSMISSION TO MOSQUITOES BY ANTIBODY TO PLASMODIUM VIVAX MALARIA VACCINE CANDIDATES PVS25 AND PVS28 DESPITE ANTIGENIC POLYMORPHISM IN FIELD ISOLATES

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Abstract. We have previously demonstrated that mouse antisera against yeast-produced recombinant forms of the ookinet surface proteins of Plasmodium vivax (Pvs25 and Pvs28) blocks transmission of the homologous P. vivax (Sal I strain). In this study, we developed mouse and rabbit antisera against Pvs25 and Pvs28 and evaluated the efficacy of these vaccine candidates against natural isolates of P. vivax in Thailand. Although both Pvs25 and Pvs28 genes are polymorphic, sera from mice immunized using alum adjuvant completely inhibited oocyst development for most human isolates, whereas sera from rabbits immunized with either alum or Freund’s adjuvant were partially inhibitory. All inhibition occurred in an antibody dose dependent fashion. Data from this study clearly demonstrates that antibodies raised against Sal I-based vaccines overcome the genetic polymorphism of Pvs25 and Pvs28 present in natural isolates of P. vivax, suggesting the wide range applicability of Sal I based vaccines.

INTRODUCTION

Malaria causes high morbidity and mortality in human populations throughout much of the world. Of the four species of human malaria parasites, Plasmodium falciparum is responsible for most of the mortality; however, P. vivax causes high morbidity for millions of people in tropical and subtropical countries outside of sub-Saharan Africa. Vaccines targeting antigens expressed on the surface of the sexual (gametocyte, gamete, zygote, ookinete) stages of malaria parasites are considered a promising strategy for malaria control. These vaccines induce antibodies in the human host that inhibit the development of the parasite within the mosquito vector and consequently block parasite transmission from the mosquito to a susceptible human. These transmission-blocking vaccines (TBVs) may effectively interrupt parasite transmission in areas with relatively low transmission rates, and may also prevent the spread of parasites that are resistant to other vaccines or drugs, thereby prolonging their effective life.

The ookinete surface proteins of P. falciparum, PfS25 and PfS28, are target antigens for a transmission-blocking vaccine. Homologous proteins have been cloned from other species of malaria parasites, to include P. vivax. Pfs25, Pfs28 and all their homologs have a conserved structure consisting of four tandem epidermal growth factor (EGF)-like domains, anchored to the parasite surface by a glycosylphosphatidylinositol moiety. The P. vivax orthologs, Pvs25 and Pvs28, have been isolated from P. vivax Sal I strain. Mice vaccinated with the yeast-produced Pvs25 and Pvs28 adsorbed to aluminum hydroxide (alum) developed strong antibody responses against the immunogens. The development of oocysts in mosquitoes was completely inhibited when the antisera were ingested with P. vivax Sal I strain-infected chimpanzee blood.

In this study, we evaluated the ability of antisera raised against yeast-produced Pvs25 and Pvs28 to prevent transmission of naturally circulating parasites of P. vivax in western Thailand.

MATERIALS AND METHODS

Production of antisera. Seven-week-old female CAF1 (H-2d) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). An experimental group of mice received three intraperitoneal injections (at three-week intervals) of 500 μL of phosphate-buffered saline (PBS, pH 7.2) containing 50 μg of yeast-produced recombinant Pvs25 or Pvs28 protein adsorbed with 800 μg of alum (aluminum hydroxide; Superfos Biosector, Vedbeak, Denmark). A control group of mice received 50 μg of yeast-produced recombinant C-terminal 19-kD portion of merozoite surface protein-1 of P. falciparum (PfMSP-1) using the same procedures described in this report. Adsorption of these antigens to alum under these conditions has been shown previously to be particularly effective. Two groups of rabbits were used in this study. An experimental group of rabbits consisted with three rabbits received three subcutaneous injections (at two-week intervals) of 500 μL of PBS (pH 7.2) containing 50 μg of yeast-produced recombinant Pvs25 or Pvs28 protein adsorbed with 800 μg of alum (Pierce). A second experimental group consisted with three rabbits received the same amount of immunogen emulsified with Freund’s complete adjuvant in the primary immunization followed twice (at two-week intervals) by boosting with Freund’s incomplete adjuvant. The preimmune pooled sera of these rabbits were used as negative controls. Whole blood from both mice and rabbits was collected by cardiac puncture two weeks after the final immunization. The whole blood was allowed to clot for one hour at room temperature and incubated overnight at 4°C. Sera were collected by centrifugation and stored at -80°C until transmission-blocking assays were conducted. The mouse experiments in this study were carried out in compliance with the Guide for Animal Experimentation at Ehime University School of
sites/microliter by assuming a count of 7,000 leukocytes/oil-immersion microscopy, and converting raw counts to parasite densities were determined for all Giemsa by malaria clinic staff. Gametocyte and trophozoite sera that gave a positive signal.

Participants (20 years old) and from parents or legal guardians of children who were symptomatic volunteers 1 years of age and older seeking malaria treatment. Informed consent was obtained from all adult participants. The study was conducted at the Study site and patients. The chi-square test was used to determine if there were differences in the proportion of mosquitoes infected after feeding on blood from each experimental group. Scheffe’s F-test was used to determine if there were significant differences in oocyst numbers in the different experimental groups.

Transmission-blocking assays. Sera from mice and rabbits immunized with Pvs25 and Pvs28 were diluted (1:2 and 1:8) with normal human AB serum from a malaria-naive donor. Mouse anti-PIMSP-1 and pre-immune rabbit sera were used as negative controls. Three hundred microliters of gametocyte whole blood from a human volunteer was placed in a tube and the plasma was removed after a brief centrifugation. One hundred fifty microliters of diluted animal sera was then added to the P. vivax-infected human red blood cells. The blood mixture was incubated for 15 minutes at room temperature and then placed in a glass feeder covered with a bau-druche membrane. The temperature of the feeder was maintained at 37°C using a water jacket circulation system. One hundred mosquitoes were fed on each glass feeder for 30 minutes, after which all unengorged mosquitoes were removed. Mosquitoes were provided 10% sucrose daily until they were dissected for oocysts seven days after feeding. The number of oocysts per mosquito was recorded for up to 20 mosquitoes fed on each glass feeder.

Statistical analysis. The full-length open reading frame of the Pvs25 gene from P. vivax genomic DNA obtained from Thai isolates was amplified with a polymerase chain reaction (PCR) using gene-specific PCR primers (sense: 5′-ACT TTC GTT TCA CAG CAC-3′; anti-sense: 5′-AAA GGA CAA GCA GGA TGA TA-3′) based on the Pvs25 DNA sequences of P. vivax Sal I strain. The reaction mixture contained 2 μL of 10× KOD-Plus buffer, 2 μL of 2 mM dNTPs, 0.8 μL of 25 mM MgSO4, 0.2 μL of 100 μM solutions of each primer, 0.2 μL (0.2 units) of KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), 0.2 μL of genomic DNA template, and 14.6 μL of water, yielding a final volume of 20 μL. Amplification conditions were 94°C for two minutes, followed by 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for two minutes, with a final extension at 68°C for five minutes. The Pvs25 DNA frag-
ments were sequenced directly using Pvs25-specific PCR primers as sequencing primers (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA). A pair of gene-specific PCR primers (sense: 5'-CTA CCA CTG CTT GCT GTT CC-3'; anti-sense: 5'-TGA CAT CAT GAA GAA GGC G-3') at each end of the gene sequence were used to amplify full-length Pvs28 gene from P. vivax genomic DNA obtained from Thai isolates by using KOD-Plus DNA polymerase in the same PCR condition described in this report. After the purification of the specific DNA fragment, we directly sequenced the DNA fragment by using Pvs28-specific PCR primers as sequencing primers.

Nucleotide sequence data reported in this paper are available in the GenBank™, European Molecular Biology (EMBL), and the DNA Data Bank of Japan (DDBJ) databases under the accession numbers AB091729–AB091745.

RESULTS

Immunogenicity of recombinant Pvs25 and Pvs28. We assessed the immunogenicity of Pvs25 and Pvs28 by vaccinating CAF1 mice and rabbits with recombinant Pvs25 or Pvs28 and subsequently testing the immune sera for antibody using the ELISA and IFA. Pooled serum from mice vaccinated with Pvs25 with alum had a six-fold higher ELISA titer (22,360) than did that of rabbits vaccinated with alum (3,852), while pooled serum from rabbits vaccinated with Pvs25 with Freund’s adjuvant had a titer between the two (14,206). Similarly, pooled serum from mice vaccinated with Pvs28 with alum had a 16-fold higher ELISA titer (61,112) than that of rabbits vaccinated with alum (3,784), while pooled serum from rabbits vaccinated with Pvs28 with Freund’s adjuvant had an antibody titer of 15,837.

Sera from mice and rabbits vaccinated with Pvs25 or Pvs28 bound to the surface of mature ookinete, as shown by the IFA. The IFA titers of these sera were similar to the ELISA titers to both Pvs25 and Pvs28 (mouse alum = 10⁴, rabbit Freund’s = 10⁴, rabbit alum = 10³). Serum from control mice vaccinated with PMSP1 and pre-immune rabbit pooled sera did not interact with ookinete (IFA) and had no specific antibody response by the ELISA. In contrast, there were no significant differences of the avidity index among the mouse and rabbit immune sera against recombinant Pvs25 or Pvs28, respectively.

Effects of immune sera on P. vivax transmission. Blood from 39 patients infected with P. vivax successfully infected mosquitoes. Packed blood cells from 29 and 23 of these patients were reconstituted with mouse and rabbit sera, respectively. Blood from 13 of these patients was used in experiments with both rabbit and mouse sera.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dilution*</th>
<th>% of feeds infecting mosquitoes (positive feeds/total feeds)</th>
<th>% of mosquitoes developing oocysts (positive mosquitoes/total mosquitoes)</th>
<th>Mean number of oocysts per positive mosquito (SEM)</th>
<th>Oocyst range</th>
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<tr>
<td>PMSP1</td>
<td>1:2</td>
<td>100% (29/29)</td>
<td>33.3% (155/465)</td>
<td>5.81 (0.77)</td>
<td>0–137</td>
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<td>Pvs25</td>
<td>1:2</td>
<td>10% (3/29)†</td>
<td>2.5% (11/435)†</td>
<td>0.18 (0.09)‡</td>
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<td>1:8</td>
<td>81% (21/26)†</td>
<td>23.1% (93/403)†</td>
<td>1.26 (0.18)‡</td>
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<td>Pvs28</td>
<td>1:2</td>
<td>10% (3/29)†</td>
<td>2.8% (12/436)†</td>
<td>0.11 (0.04)‡</td>
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<td></td>
<td>1:8</td>
<td>65% (17/26)†</td>
<td>17.9% (68/380)†</td>
<td>1.31 (0.23)‡</td>
<td>0–41</td>
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* Sera from immunized mice were diluted with human AB sera from a malaria-naive donor and then mixed 1:1 with packed red blood cells from patient infected with P. vivax.
† P < 0.05 by chi-square test versus control (PfMSP1).
‡ P < 0.05 by Scheffe’s F-test versus control (PMSP1).

There was a significant reduction in both mosquito infection rates (percentage of oocyst-positive mosquitoes in each group) and the mean number of oocysts per midgut in mosquitoes feeding on blood reconstituted with sera from mice immunized with Pvs25/alum or Pvs28/alum compared with those feeding on blood reconstituted with sera from mice receiving PMSP1/alum (Table 1). Reconstitution of blood with a 1:8 dilution of sera resulted in a lesser reduction in infection rates and oocyst numbers than for sera diluted 1:2, indicating that the transmission-blocking activity of both Pvs25 and Pvs28 was dose dependent. Oocyst development was completely inhibited in mosquitoes fed on 90% (26 of 29) of the patients whose blood was reconstituted with mouse sera (Pvs25 or Pvs28) that was diluted 1:2 with P. vivax-naïve human AB serum, whereas complete inhibition of oocyst development was only recorded in 19% and 35% of patients whose blood was reconstituted with a 1:8 dilution of sera from Pvs25 and Pvs28 immunized mice, respectively (Table 1).

Use of sera from rabbits immunized with Pvs25/Freund’s adjuvant and Pvs28/Freund’s adjuvant produced results similar to that observed with mice immunized with Pvs25/alum and Pvs28/alum. There was a significant reduction in the mean number of oocysts per midgut of mosquitoes fed on blood mixed with sera of rabbit immunized with Pvs25/Freund’s adjuvant and with Pvs28/Freund’s adjuvant compared with pre-immune sera (Table 2). The use of alum as an adjuvant in rabbits was less effective than use of Freund’s adjuvant, both in terms of effect on percent of mosquitoes that were infected as well as in oocyst loads (Table 2). The transmission-blocking activity of rabbit sera was dose-dependent when animals were immunized with Pvs25/Freund’s adjuvant or Pvs28/Freund’s adjuvant, whereas dose-dependent transmission-blocking activity was not observed in mosquitoes fed on blood reconstituted with sera from rabbits immunized with Pvs25/alum or Pvs28/alum (Table 2).

Genetic polymorphism of Pvs25 and Pvs28 and transmission-blocking efficacy. We then analyzed the relationship between the transmission-blocking activity of immune mouse serum and the genetic polymorphism of the Pvs25 and Pvs28. A total of 18 patients whose blood infected at least 25% of mosquitoes (in the control group) were included in this analysis. A summary of the sequence polymorphism of Pvs25 and Pvs28 genes is presented in Table 3. Although the Pvs25 gene was highly conserved in these 18 isolates, three amino acid substitutions were found. One amino acid substitution (E/Q97) was located in the second EGF-like domain of Pvs25, while two amino acid substitutions (I/T130 and Q/K131) were located in the third EGF-like domain. Although the genotype of Pvs25 in three cases where complete transmission-blocking
immunity was not achieved was identical (E/T/K), complete transmission-blocking efficacy was derived in 10 other cases where the genotype was identical to E/T/K. Moreover, complete transmission-blocking efficacy was also demonstrated in five other cases where the genotypes were E/T/Q or Q/T/Q, as distinct from Sal I type (E/I/Q).

The Pvs28 gene was more polymorphic than the Pvs25 gene. A total of 10 amino acid substitutions (M/L52 and A/V53 in the first EGF-like domain, T/K65, A/V81, G/N95, L/I98, E/K105, and V/E106 in the second EGF-like domain, A/V53 in the first EGF-like domain, T/K65, A/V81, G/N95, L/I98, E/K105, and V/E106 in the second EGF-like domain, A/V53 in the first EGF-like domain) were detected in the first three EGF-like domains, while 5 and L/V116 and T/S140 in the third EGF-like domain) were L/I98, E/K105, and V/E106 in the second EGF-like domain, A/V53 in the first EGF-like domain, T/K65, A/V81, G/N95, L/I98, E/K105, and V/E106 in the second EGF-like domain, A/V53 in the first EGF-like domain.

The positions of the substitution are indicated with the amino acid position numbers from Pvs25 of the Plasmodium vivax of the Sal I strain, and showed complete transmission-blocking with anti-Pvs28 mice sera. Although the genotypes of Pvs28 in these 18 isolates were highly polymorphic, the Sal I-based Pvs28 vaccine successfully induced transmission-blocking efficacy against each of the 16 isolates except for cases 2 and 3.

**DISCUSSION**

Yeast-produced recombinant Pvs25 and Pvs28 were highly immunogenic in mice. Although sera from mice immunized with Pvs25 or Pvs28 derived from the Sal I strain significantly inhibited oocyst development when mosquitoes ingested *P. vivax* (Sal I strain)-infected chimpanzee blood reconstituted with the sera, it is not clear whether antibodies raised against these allelic forms of Pvs25 and Pvs28 will be equally effective against the full spectrum of naturally circulating parasites.

### TABLE 2

Transmission-blocking activity of sera from rabbits immunized with Pvs25 or Pvs28 on *Plasmodium vivax* human isolates from Thailand

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Dilution†</th>
<th>% of feeds infecting mosquitoes (positive feeds/total feeds)</th>
<th>% of mosquitoes developing oocysts (positive oocysts/total mosquitoes)</th>
<th>Mean number of oocysts per positive mosquito (SEM)</th>
<th>Oocyst range</th>
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<tr>
<td>Pre-immune</td>
<td>1:2</td>
<td>100% (23/23)</td>
<td>50% (235/469)</td>
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<td>Pvs25/A</td>
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<td>70% (16/23)‡</td>
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<td>Pvs25/F</td>
<td>1:2</td>
<td>79% (11/14)‡</td>
<td>34% (73/218)‡</td>
<td>4.06 (0.75)§</td>
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<td>Pvs28/A</td>
<td>1:2</td>
<td>77% (17/22)‡</td>
<td>45% (208/459)‡</td>
<td>10.73 (1.00)</td>
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<td>Pvs28/F</td>
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<td>22% (5/23)‡</td>
<td>6% (26/457)‡</td>
<td>0.09 (0.02)§</td>
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* Pre-immune sera were used as a control. Pvs25 and Pvs28 were administered using either alum (/A) or Freund’s (/F) adjuvant.
†Sera from immunized rabbits were diluted with human AB sera from a malaria-naive donor and then mixed 1:1 with packed red blood cells from a patient infected with *P. vivax*.
‡P<0.05 by chi-square test versus control (Pre-immune).
§P<0.05 by Scheffe’s F-test versus control (Pre-immunes).

### TABLE 3

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* PIMSP-1 = *P. falciparum* merozoite surface protein-1.
†The positions of the substitution are indicated with the amino acid position numbers from Pvs25 of the *Plasmodium vivax* Sal I strain.
‡The number of repeats found in each Pvs28 genotype.

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parasites of *P. vivax*. In this study, we tested the immunogenicity of recombinant Pvs25 and Pvs28 in mice (with alum as an adjuvant) and rabbits (with alum or Freund's adjuvant) and evaluated the ability of sera from vaccinated animals to block transmission of *P. vivax* from humans to mosquitoes. We found no differences in the responses of Sal I and naturally circulating parasites of *P. vivax* to Pvs25 and Pvs28. Sera from immunized mice completely inhibited oocyst development for most isolates. Although sera from rabbits immunized with recombinant Pvs25 or Pvs28 adsorbed to alum was less inhibitory than the analogous mouse sera, sera from rabbits immunized with recombinant Pvs25 or Pvs28 emulsified in Freund's adjuvant was almost as effective as sera from the mice immunized with alum. Thus, although it is difficult to compare antibody titers across species, these differences in inhibitory activity appear to reflect antibody titer (e.g., Pvs25 titers for mice immunized with alum, rabbits immunized with Freund's adjuvant, and rabbits immunized with alum were 22,360, 14,206, and 3,852, respectively). Importantly, regardless of species, roughly equivalent levels of antibody are required to achieve complete blocking. For example, with anti-Pvs25, mice antiserum with a titer of, 22,360 gave complete blocking in 90% of the cases. Similarly, rabbit antiserum with a titer of 14,206 gave complete blocking in 61% of the cases. Since all of the transmission-blocking assays were performed at a minimum 1:2 dilution in a species-independent fashion, serum titers of 10,000 against the Sal I allelic form of Pvs25 could be expected to provide complete blocking against 90% of field infections (and lower the mean oocyst numbers in the remainder of cases). Therefore, complete transmission-blocking efficacy can be achieved if higher concentration of the antiserum is used for testing in the incomplete blocking cases (1, 2, and 3 in Pvs25, and 2 and 3 in Pvs28 experiments).

A variety of studies have reported that TBV candidate antigens are less polymorphic than candidate antigens expressed by hepatic and erythrocytic stage parasites. Since TBV candidate antigens are predominantly expressed by parasite stages found in the mosquito and not in humans, they will presumably avoid immune selection pressure from the human host. Although *P. falciparum* ookinete surface proteins are highly conserved, polymorphism has been detected in Pvs25 and Pvs28 molecules isolated from malaria-endemic areas. In the current study, we detected only three amino acid substitutions in Pvs25. In contrast, a total of 10 amino acid substitutions and a variety of different numbers of repeats at the end of the fourth EGF-like domain were found in Pvs28. Because Pvs28 is expressed during later mosquito stages, the polymorphism found in Pvs28 may result from something other than immune selection by the human host. Additionally, the polymorphism does not seem to have reduced the efficacy of a single allele vaccine, arguing against an immune-evasion purpose.

Although we have not determined the mechanism that results in Pvs28 polymorphism, it will be necessary to evaluate the effect of this polymorphism on the efficacy of Sal I-based TBV. The transmission-blocking experiments conducted in this study clearly demonstrated that antisera to recombinant Pvs25 and Pvs28 based on the Sal I strain of *P. vivax* recognized corresponding molecules expressed by field-isolated parasites in Thailand, and that the antisera blocked transmission of these field isolates. We believe that the efficacy of the Sal I-based vaccine against the polymorphic target antigens in these field isolates results from the fact that almost all of the amino acid substitutions we detected were conservative substitutions (except for I/T130 in Pvs25 and V/E106 in Pvs28), suggesting that the tertiary structure and epitopes of the transmission-blocking antibodies were conserved. In addition, the low substitution frequencies, the conservative nature of the substitutions, and the likelihood that these substitutions have not been selected as a result of immune pressure suggests that antigenic diversity will not limit the efficacy of Pvs25 and Pvs28 based TBVs.

Emergence of drug-resistant parasites is a major factor affecting the control of malaria. Although TBV will not prevent the emergence of drug-resistant mutants, these vaccines may prevent the spread of drug-resistant parasites. Thus, even in high endemic areas where a TBV may not have sufficient impact to reduce the parasite prevalence, it is expected that it would have significant impact on spread of escape mutants. Although it would be difficult to introduce a transmission-blocking vaccine for this purpose alone, we anticipate that the problems observed with the spread of drug-resistant malaria will be mimicked by the spread of vaccine-resistant parasites. Genton and others recently demonstrated that an erythrocytic stage malaria vaccine induced selection pressure on parasite populations in Papua New Guinea. These data suggest that vaccines should comprise conserved antigens and/or multiple components representing all dominant allelic types. Therefore, incorporation of a TBV with vaccines targeting the other stages may be an important strategy to preserve their effective life. Accordingly, for many malarious regions outside of Africa, development of effective TBV will require coverage against both *P. falciparum* and *P. vivax*. At this moment, Phase I clinical trials and formulations of both Pvs25 and Pvs25 molecules are in preparation (Saul A, unpublished data).

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