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

JETSUMON SATTABONGKOT, TAKAFUMI TSUBOI, HAJIME HISAEDA, MAYUMI TACHIBANA,
NANTAVADEE SUWANABUN, THANAPORN RUNGRUANG, YA-MING CAO, ANTHONY W. STOWERS,
JEERAPHAT SIRICHAISSINTHOP, RUSSELL E. COLEMAN, AND MOTOMI TORII

Department of Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Ehime, Japan; Department of Immunology and Parasitology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan; Department of Molecular Parasitology, Ehime University School of Medicine, Shigenobu, Ehime, Japan; Department of Immunology, College of Preclinical Medicine, China Medical University, Shenyang, China; Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland; Office of Vector Borne Disease Control I, Saraburi, Thailand

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.1 Vaccines targeting antigens expressed on the surface of the sexual (gametocyte, gamete, zygote, ookinet) stages of malaria parasites are considered a promising strategy for malaria control.2–4 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.5,5

The ookinet surface proteins of P. falciparum, Pfs25 and Pfs28, are target antigens for a transmission-blocking vaccine.6–9 Homologous proteins have been cloned from other species of malaria parasites, to include P. vivax.10–15 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.16 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.17

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 protein17 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 (PI MSP-1)18 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.17 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 and Freund's incomplete adjuvant (Sal I strain-infected chimpanzee blood) boost each month using yeasted vaccine and alum. Whole blood was used for the experimental group and collected by cardiac puncture two weeks after the injection. 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
Medical and protocols reviewed and approved by the Animal Care and Use Committee. Rabbit immunization studies were done by the Spring Valley Company (Woodbine, MD) in compliance with National Institutes of Health guidelines and under the auspices of an Animal Care and Use Committee-approved protocol.

**Enzyme-linked immunosorbent assay (ELISA).** Mouse and rabbit serum were tested for antibodies to Pvs25 and Pvs28 using ELISAs specific for these antigens as described previously. Briefly, flat-bottom, 96-well microtiter plates (Immuno 4; Dynex Technology Inc., Chantilly, VA) were coated with antigen overnight at 4°C. The saturating concentration of antigen was determined to be 200 ng of Pvs25 or 100 ng of Pvs28 per well. The plates were blocked with 1% skim milk (Difco, Detroit, MI) in 0.05% Tween 20 in Tris-buffered saline (TBS-T) for one hour at room temperature. Mouse and rabbit sera were serially diluted in blocking buffer. One hundred microliters of diluted serum was added to antigen-coated wells in duplicate and incubated for two hours at room temperature. After extensive washing with TBS-T, the plates were incubated with 100 μL of alkaline phosphatase--conjugated goat anti-mouse IgG or anti-rabbit IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for one hour. Bound antibodies were visualized by adding 100 μL of the substrate solution (p-nitrophenylphosphate; substrate 104; Sigma Chemical Co., St. Louis, MO). The absorbance at 410 nm was read with a Spectra Max plus microplate reader (Molecular Devices, Sunnyvale, CA). Serum dilutions at an absorbance value of 0.5, on the linear part of the titration curve, were designated as the end point ELISA titers. The avidity index was determined as follows. Briefly, the diluted sera were added to antigen-coated plates in the presence of various concentrations of NH4SCN in blocking buffer and incubated for two hours at room temperature. The rest of the assay was performed as described in this report. The avidity index was then calculated as the molar concentration of NH4SCN to reduce the optical density to 50% from the original value.

**Immunofluorescence assay (IFA) titer against cultured ookinetes.** Gametocytemic blood was obtained from patients infected with *P. vivax* reporting to the Mae Sod malaria clinic in Tak Province in western Thailand, and ookinetes were cultured as previously described. Cultured ookinetes were used as the antigen source in an IFA that was used to determine antibody titer in the sera from mice and rabbits immunized with Pvs25 and Pvs28. Procedures for the IFA have been previously described. The IFA titer was determined by measuring the maximum dilution of immune mouse or rabbit sera that gave a positive signal.

**Study site and patients.** The study was conducted at the Mae Sod and Mae Kasa malaria clinics in Tak Province in western Thailand. Individuals who participated in the study were symptomatic volunteers 1 years of age and older seeking treatment. Informed consent was obtained from all adult participants (≥20 years old) and from parents or legal guardians of minors (16–19 years of age). Thick and thin blood smears were prepared from each individual and stained with 10% Giemsa by malaria clinic staff. Gametocyte and trophozoite densities were determined for all *P. vivax*-positive patients by counting the number of parasites per 500 leukocytes using oil-immersion microscopy, and converting raw counts to parasites/microliter by assuming a count of 7,000 leukocytes/μL. If gametocytes were present, the patient was asked to enroll in the study. After being briefed on the project and completing consent forms, approximately 20 mL of blood was collected by venipuncture into a heparinized tube. Volunteers were then released from the study and received antimalarial treatment from the malaria clinic staff. The complete process (entry into the malaria clinic for initial diagnosis until receiving malaria treatment) took approximately 60 minutes. All human subjects research conducted in these studies was reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army.

**Mosquitoes.** *Anopheles dirus* A (Bangkok colony) has been maintained at the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand for more than 20 years. Mosquitoes were transported from Bangkok to the Mae Sod malaria clinic and maintained at 26°C and a relative humidity of 70–80% until used in transmission-blocking assays.

**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 gametocytemic 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-druce 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 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.

**Determination of genetic polymorphism of Pvs25 and Pvs28.** Genomic DNA from the *P. vivax* isolates used for the transmission-blocking assay was obtained from dried filter paper blood spots as described by Sakihama and others. The full-length open reading frame of the Pvs25 gene from *P. vivax* infected human red blood cells. The Pvs25 DNA fragment was amplified by PCR using gene-specific 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 GAG CTT GCT GTT CC-3'; anti-sense: 5'-TGA CAT CAT GAA GAA GGC C 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	extsuperscript{27}, 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 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 ookinetes, as shown by the IFA. The IFA titers of these sera were similar to the ELISA titers for both Pvs25 and Pvs28 (mouse alum = 10^4, rabbit Freund's = 10^4, rabbit alum = 10^3). Serum from control mice vaccinated with PMSP-1 and pre-immune rabbit pooled sera did not interact with ookinetes (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.

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*-naive 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 Pvs25/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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dilution*</th>
<th>% of feeds infecting mosquitoes (positive feeds/total feeds)</th>
<th>Mean number of oocysts per positive mosquito (SEM)</th>
<th>Oocyst range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSP-1</td>
<td>1:2</td>
<td>100% (29/29)†</td>
<td>33.3% (155/465)†</td>
<td>0–137</td>
</tr>
<tr>
<td>Pvs25</td>
<td>1:2</td>
<td>10% (3/29)†</td>
<td>2.5% (11/435)†</td>
<td>0.18 (0.09)‡</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>81% (21/26)†</td>
<td>23.1% (93/403)†</td>
<td>1.26 (0.18)‡</td>
</tr>
<tr>
<td>Pvs28</td>
<td>1:2</td>
<td>10% (3/29)†</td>
<td>2.8% (12/436)†</td>
<td>0.11 (0.04)‡</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>65% (17/26)†</td>
<td>17.9% (68/380)†</td>
<td>1.31 (0.23)‡</td>
</tr>
</tbody>
</table>

* 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 (PMSP-1).
‡ *P* < 0.05 by Scheffe's F-test versus control (PMSP-1).
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 third EGF-like domain) were detected in the first three EGF-like domains, while 5

\[ L/I98, E/K105, V/E106 \] in the second EGF-like domain, \[ A/V53 \] in the first EGF-like domain, \[ T/K65, A/V81, G/N95 \] in the third EGF-like domain were distinct from Sal I type (E/I/Q).

The positions of the substitution are indicated with the amino acid position numbers from Pvs25 of the Sal I strain.

### 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>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-immune</td>
<td>1:2</td>
<td>100% (23/23)</td>
<td>50% (235/459)</td>
<td>16.14 (1.36)</td>
<td>0–174</td>
</tr>
<tr>
<td>Pvs25/A</td>
<td>1:2</td>
<td>70% (16/23)§</td>
<td>36% (177/495)§</td>
<td>4.25 (0.43)§</td>
<td>0–66</td>
</tr>
<tr>
<td>Pvs25/F</td>
<td>1:2</td>
<td>39% (9/23)‡</td>
<td>12% (58/486)‡</td>
<td>0.35 (0.07)‡</td>
<td>0–24</td>
</tr>
<tr>
<td>Pvs28/A</td>
<td>1:2</td>
<td>77% (17/22)‡</td>
<td>45% (208/459)‡</td>
<td>10.73 (1.00)</td>
<td>0–139</td>
</tr>
<tr>
<td>Pvs28/F</td>
<td>1:2</td>
<td>22% (5/23)‡</td>
<td>6% (26/457)‡</td>
<td>0.09 (0.02)‡</td>
<td>0–7</td>
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### Table 3

Genetic polymorphism of Pvs25 and Pvs28 and transmission-blocking efficacy of the mouse antisera against recombinant Pvs25 and Pvs28 on *Plasmodium vivax* human isolates from Thailand

<table>
<thead>
<tr>
<th>PIMSP-1 Efficacy (1:2)</th>
<th>Pvs25 Efficacy (1:2)</th>
<th>Amino acid substitutions</th>
<th>PIMSP-1 Efficacy (1:2)</th>
<th>Pvs28 Efficacy (1:2)</th>
<th>Amino acid substitutions</th>
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<tr>
<td>Cases</td>
<td>Infection rate</td>
<td>Mean oocyst</td>
<td>Cases</td>
<td>Infection rate</td>
<td>Mean oocyst</td>
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<tr>
<td>Sal I</td>
<td>—</td>
<td>—</td>
<td>E1</td>
<td>90</td>
<td>35.6</td>
</tr>
<tr>
<td>1</td>
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<td>35.6</td>
<td>20</td>
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<tr>
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<td>40</td>
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<tr>
<td>18</td>
<td>25</td>
<td>1.2</td>
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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 positions of the substitution are indicated with the amino acid position numbers from Pvs28 of the *Plasmodium vivax* Sal I strain.

§The number of repeats found in each Pvs28 genotype.

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 sera17, 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 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 adjuvants) 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* ookinetes 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 Pf25 molecules are in preparation (Saul A, unpublished data).

Received January 23, 2003. Accepted for publication July 7, 2003.

Acknowledgments: We thank the staffs of the Office of Vector Borne Disease Control 1 (Samut Sakhon, Thailand) for constant help in setting up the field sites, the staffs of the Department of Entomology at the Armed Forces Research Institute of Medical Sciences (Bangkok, Thailand), and Ayako Kurita for technical assistance. We also thank the Malaria Vaccine Initiative at Program for Appropriate Technology in Health for their constant help with the TBV development.

Financial support: This work was supported in part by Grants-in-Aid for Scientific Research 13576007 and 14570215 and Scientific Research on Priority Areas 13226087, 14021082, and 15019072 from the Ministry of Education, Culture, Sports, Science and Technology, and a Grant for Research on Emerging and Re-emerging Infectious Diseases (H12-Shinkou-17) from the Ministry of Health, Labor and Welfare, Japan. This work also received support from the National Science Foundation Committee of China (NSFC) 30070718, and the UNDP/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases (TDR).

Authors’ addresses: Jetsumon Sattabongkot, Nantavoode Suwanabun, and Russell E. Coleman, Department of Entomology, Armed Forces Research Institute of Medical Sciences, 3156 Rajvithi Road, Bangkok 10400, Thailand, Telephone: 66-2-644-5777, Fax 66-2-246-8832. Takaumi Tsuibo, Cell-Free Science and Technology Research Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan, Telephone: 81-89-927-8277, Fax: 81-89-927-9941. Hajime Hisaeda, Department of Immunology and Parasitology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan, Telephone: 81-92-642-6119, Fax: 81-92-642-6118. Mayumi Tachibana, Thapanorn Runguang, and Motomi Torii, Department of Molecular Parasitology, Ehime University School of Medicine, Shigenobu, Ehime 791-0295, Japan, Telephone: 81-89-90-
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5286, Fax: 81-89-960-5287. Ya-Ming Cao, Department of Immunology, College of Preclinical Medicine, China Medical University, 92 Bei Er Road, Shenyang 110001, China, Telephone: 86-24-23256666, Fax: 86-24-23264417. Anthony W. Stowers, Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5640 Fisher Lane, Rockville, MD 20852, Telephone: 301-455-2968, Fax: 301-455-6725. Jeeraphat Sirichaisinthop, Office of Vector Borne Disease Control 1, Phraabuddhabat, Saraburi 18120, Thailand, Telephone: 66-36-266142, Fax: 66-36-267586.

Reprint requests: Takafumi Tsuboi, Cell-Free Science and Technology Research Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan, Telephone +81-89-927-8277, Fax +81-89-927-9941, E-mail: tsuboi@m.ehime-u.ac.jp.

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