Plasmodium vivax Ookinete Surface Protein Pvs25 Linked to Cholera Toxin B Subunit Induces Potent Transmission-Blocking Immunity by Intranasal as Well as Subcutaneous Immunization

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The nontoxic cholera toxin B subunit (CTB) was evaluated as a potential delivery molecule for the Plasmodium vivax ookinete surface protein, Pvs25. Recombinant Pvs25 was expressed as a secreted protein in the yeast Pichia pastoris, as a mixture of isoforms including multimers and the A and B monomers. The isoform with the presumed native protein fold was the most abundant, accounting for more than 40% of all expressed protein. The molecularly uniform A isoform was chemically conjugated to CTB via its primary amines, and the fusion protein, retaining GMI-ganglioside affinity, was administered to BALB/c mice by the subcutaneous (s.c.) or intranasal (i.n.) route. Immunization of mice with conjugated Pvs25 without supplemental adjuvant induced antisera that specifically recognized P. vivax ookinetes in vitro. Furthermore, the antisera, when mixed with parasitized blood isolated from P. vivax patients from Thailand, was found to reduce parasite transmission to mosquitoes, conferring a 93 to 98% (s.c.) or a 73 to 88% (i.n.) decrease in oocyst number. Unconjugated Pvs25 alone conferred only a 23 to 60% (s.c.) or a 0 to 6% (i.n.) decrease in oocyst number. Coadministration of extraneous adjuvants, however, further enhanced the vaccine efficacy up to complete blockade. Taken together, we conclude that a weakly immunogenic Pvs25 by itself, when linked to CTB, transforms into a potent transmission-blocking antigen in both i.n. and s.c. routes. In addition, the present study is, to the best of our knowledge, the first demonstration of the immune potentiating function of CTB for a vaccine antigen delivered by the s.c. route.

Malaria is one of the most serious infectious diseases, with high mortality and morbidity, especially in tropical regions of the world. The disease causes 350 to 500 million clinical cases every year, and the estimated annual mortality exceeds 1.1 million (28). Implementation of many malaria control measures, including chemotherapy and insecticide-treated bed nets, have made a significant contribution to the reduction of malaria cases worldwide; however, these control measures are suboptimal, and hence new tools, particularly vaccines, should be used for local elimination and the ultimate eradication of malaria from the globe (9, 10, 24). The development of effective and affordable malaria vaccines is therefore likely to benefit global public health (Malaria Vaccine Technology Roadmap [MVTR], 2006 [http://www.malariavaccineroadmap.net/pdfs/Malaria_Vaccine_TRM_Final.pdf]). Although Plasmodium falciparum causes the highest mortality rates among the four Plasmodium species known to infect humans (18), P. vivax malaria has the highest morbidity and is an important cause of recurrent malaria. This species is therefore an important target of malaria control efforts (4–6; MVTR). Furthermore, because global malaria eradication is the ultimate goal, the value of developing vaccines against P. vivax cannot be underestimated (4–6; MVTR). Several promising vaccine candidates have been intensively investigated, such as those targeting the asexual stages, i.e., the sporozoite, hepatic and erythrocytic stages, which are designed to prevent infection and to reduce disease severity. On the other hand, transmission-blocking vaccines (TBVs) that target the sexual stage, in which the parasite undergoes sporogonic development in anopheline mosquitoes, prevent parasite transmission from mosquitoes to humans (7, 14, 25). TBVs induce antibodies that react with the ookinete surface proteins (OSPs) of malaria parasites within the mosquito midgut, and as such they do not directly protect vaccinated individuals from infection. They could, however, contribute to elimination of the disease by lowering the parasite transmission frequency below the threshold at which the parasite can maintain its life cycle (4, 6). In addition, TBVs, when combined with vaccines targeting other stages of the infection, could prevent transmission of parasites that have escaped the immune response. Furthermore, TBVs could also prevent transmission of drug-resistant
parasites, which will likely to emerge when mass administration of primaquine is initiated. Therefore, TBVs might function as a “safety net” for pre-erythrocytic and erythrocytic vaccines, as well as other nonvaccine interventions.

We have recently tested whether a mucosal vaccination regimen can be applied to TBVs, on the premise that noninvasive and easy-to-administer mucosal vaccines are advantageous in a mass vaccination campaign in a region where malaria is endemic (1–3). In these animal studies, we have demonstrated the potential of mucosal vaccines to block parasite transmission, but enhancement of the mucosal immunogenicity of recombinant antigens was found to be critically dependent upon the use of cholera toxin (CT) adjuvant. CT is well known for its high immune potentiating function for admixed antigens administered through the mucosal, particularly the intranasal (i.n.), route (13). However, its clinical application is hampered by its severe toxicity (26). Thus, alternative vaccine formulations not using the CT holotoxin are highly desirable.

Here, we extended our previous studies to test our hypothesis that the immunogenicity of a Plasmodium vivax malaria OSP, Pvs25, becomes substantially augmented when physically linked to the nontoxic B subunit of CT (CTB), even without supplementation with exogenous adjuvants. This should, in theory, effectively reduce parasite transmission to mosquitoes. Furthermore, we tested the TBV efficacy of the engineered fusion complex in a subcutaneous (s.c.) immunization regimen to test the immune potentiating function of CTB with this particular immunization route.

MATERIALS AND METHODS

Expression of Pvs25H protein from the metathrophic yeast Pichia pastoris.

The Pvs25 coding region (Ala23 to Leu195) was amplified by PCR from plasmid BMGY medium with vigorous shaking in a baffled flask at 30°C until the optical density was 0.6. Immediately after electroporation, the cells were plated on minimal medium containing increasing concentrations of Geneticin (G418; 1 to 5 mg/ml; Nacalai Tesque, Inc., Kyoto, Japan) for yeast extract-peptone-dextrose (YPD) medium containing increasing concentrations of monohydrate standard were used to estimate the amounts of free sulfhydryls. The sulfhydryl groups for recombinant protein samples. In addition, 5,5'-dithio-ditoluidide (DTT; Pierce, Rockford, IL) was used to generate a reduced form of the sulfhydryl groups for recombinant protein samples. In addition, 5,5'-dithio-DTDL (2-nitrobenzoic acid) (Ellman’s reagent; Pierce) and a cysteine hydrochloride monohydrate standard were used to estimate the amounts of free sulfhydryls. The N-terminal protein sequences were analyzed by the Edman degradation method as described elsewhere, using a protein sequencer (Shimadzu, Kyoto, Japan).

All recombinant DNA experiments were conducted according to the Safety Guidelines for Gene Manipulation Experiments of the University of the Ryukyus.

Chemical conjugation between Pvs25H-A and CTB. Recombinant CTB was expressed and purified as described previously (11), and purified Pvs25H-A was chemically conjugated to CTB by using the heterobifunctional cross-linker N-succinimidyl 3-(2-pyridyl)dithio)propionate (SPDP; Thermo Scient., Inc., Rockford, IL). One milligram of Pvs25H-A (2 mg/ml in PBS-EDTA) was incubated with SPDP (0.6 mM, final concentration) for 1 h at room temperature and then desalted and buffer exchanged to PBS by a size exclusion membrane filter (Amicon Ultra-15, MWCO 10,000; Millipore, Billerica, MA) to remove excess reagent and by-products (pyridine 2-thione). Similarly, 1 mg of CTB (2 mg/ml in PBS-EDTA) was incubated with SPDP (0.6 mM, final concentration) for 1 h at room temperature and then desalted and buffer exchanged to PBS. Pyridyldithio-activated CTB was then incubated with dithiothreitol (DTT; 50 mM, final) for 30 min at room temperature to expose the sulphydryl groups and then desalted and buffer exchanged to PBS as before. Finally, equal amounts of pyridyldithio-activated Pvs25H-A and sulfhydryl-activated CTB were mixed, flicked, and incubated at 37°C overnight for conjugation. The conjugated sample was desalted as before, and the endotoxin content was measured to confirm that there had been no significant contamination during the conjugation process.

To evaluate the conjugation efficiency, untreated Pvs25H-A was separately incubated with either untreated or DTT-treated CTB (CTBDTT or CTBTreat, respectively) (see Results and Fig. 2a for details). Each conjugation sample was analyzed by GM1–enzyme-linked immunosorbent assay (GM1-ELISA) as described previously (11). Briefly, 5 μg of monosialoganglioside GM1 (Sigma-Aldrich, St. Louis, MO)/ml, a receptor for CT, diluted with bicarbonate buffer (15 mM Na2CO3, 55 mM NaHCO3, pH 9.6) (100 μl) was coated onto a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and the plate was incubated at 4°C overnight. After washing the plate twice with PBS containing 0.05% Tween 20 (PBS-T) and once with PBS, the plate was blocked with PBS containing 10% skim milk for 2 h at 37°C. Each conjugation sample (2 μg of total protein/well) was then applied to the wells, and the plate was incubated for 2 h at 37°C, followed by incubation with rabbit anti-CT antiserum (1/4,000; Sigma-Aldrich) or mouse anti-Pvs25 antiserum (1/500) for 2 h at 37°C. This was followed by the addition of anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (1/4,000; Sigma-Aldrich). Finally, the plate was incubated in a biochip reader (Bio-Rad) was added, and the plate was incubated for 20 min at 37°C. The OD415 was measured by using a microplate reader (Bio-Rad). To evaluate the conjugation state of the fusion complex, 1 mg of conjugation sample was subjected to size exclusion chromatography (HiLoad 16/60 Superdex 200 26/60, GE Healthcare, Piscataway, NJ).

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75 pg column; GE Healthcare). Molecular weight standards (Gel Filtration Calibration kits LMW; GE Healthcare) were used to estimate the molecular weights of CTB, Pvs25H-A, and their fusion complex by calculating the partition coefficient (Kav) values for each protein standard and sample protein.

**Immunization with CTB-Pvs25H-A fusion protein and analysis of induced antibodies.** Eight-week-old female BALB/c mice were purchased from Japan SLI (Chiba, Japan). Mice (four or eight mice per group) were immunized via the s.c. or i.n. route with 30 μg of Pvs25H-A, a mixture of CTB and Pvs25H-A (30 μg each) or 60 μg of CTB-Pvs25H-A fusion protein. Where indicated, incomplete Freund’s adjuvant (IFA; Difco Laboratories, Detroit, MI) or 0.1 to 1.0 μg of CT (List Biological Laboratories, Campbell, CA) was used for s.c. or i.n. adjuvants, respectively. The mice were immunized three times, at weeks 0, 2, and 3.

Mice were anesthetized 1 week after the third immunization (week 4) by intraperitoneal injection of pentobarbital sodium salt (Nacalai Tesque, Inc.) and were sacrificed by exsanguination to collect serum. For specific serum antibody analysis, ELISA plates (Sumilon; Sumitomo Bakelite Co.) were coated with Pvs25H-A (5 μg/ml) in bicarbonate buffer by incubating the plate at 4°C overnight. The plate was washed twice with PBS-T and once with PBS. The plate was blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. A serial dilution of the antisera starting with a 50-fold dilution with 0.5% BSA in PBS was applied to the wells in duplicate, which were then incubated for 2 h at 37°C. The plate was then incubated with anti-mouse IgG conjugated to alkaline phosphatase (1/4000; Sigma-Aldrich) for 2 h at 37°C. p-Nitrophenylphosphate (Bio-Rad) was added to the plate for color development, and the absorbance at OD415 was measured after 20 min of incubation at 37°C, using a microplate reader (Bio-Rad). The antibody titer was defined as the serum dilution that gave an OD415 value equal to 0.1 or as the serum dilution where a one magnitude higher dilution gave an OD415 value of <0.1.

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of the Ryukyus, and the experiments were conducted according to the Ethical Guidelines for Animal Experiments of the University of the Ryukyus.

**Mosquito membrane feeding assay.** Heparinized syringes were used for peripheral blood collection, with written informed consent from P. vivax patients. Infections were obtained by a malaria clinic in the Mae Sod district in the Tak province of northwestern Thailand. Single species infection with P. vivax was confirmed by Giemsa stain of thick and thin blood smears. The levels of parasitemia and the number of oocysts per mosquito were counted by confocal scanning laser microscopy (LSM5 Pascal; Carl Zeiss Micro Imaging, Thornwood, NY).

**Expression and purification of recombinant Pvs25H-A isoform from P. pastoris.** Ookinete surface proteins (OSP) contain several intramolecular disulfide bonds, e.g., the 11 disulfide bonds in Pvs25, which are important for overall structural integrity and native antigenicity. For this reason, E. coli expression systems are unsuitable, and therefore yeast Saccharomyces cerevisiae expression systems are used (12, 15, 19, 22). We explored other, more efficient recombinant protein expression systems for Pvs25 and found a higher production efficiency in the yeast P. pastoris. We constructed a plasmid for secretory expression of Pvs25 as a C-terminal hexahistidine-tagged protein (Pvs25H1) (Fig. 1a). The culture supernatant of recombinant P. pastoris was found to contain several protein species that were not present in the culture supernatant of vector-transformed clones (Fig. 1b), and these protein bands specifically reacted with anti-Pvs25 antiserum, as well as with an anti-hexahistidine tag monoclonal antibody (data not shown). Secreted Pvs25H1 was affinity purified on a nickel Sepharose column and further separated into large (fractions 16 to 19) and small (fractions 21 to 23) protein species by size exclusion chromatography (Fig. 1c and d). At least five protein bands were identified on SDS-PAGE (Fig. 1b) and their corresponding peaks by size exclusion chromatography (fractions 16, 17, 18-19, 21-22, and 22-23 in Fig. 1c and d). The apparent molecular masses of these protein species based on gel mobility on SDS-PAGE were estimated to be 63.2, 47.3, 33.2, 16.1, and 28.3 kDa, respectively. The calculated molecular mass of Pvs25H1 (based on its deduced amino acid sequence) is 20.2 kDa, to which the fraction 21 and 22 protein species correspond most closely. We therefore concluded that this was monomeric Pvs25H1. Furthermore, the apparent molecular masses of protein species found in fractions 18 and 19, 17, and 16 corresponded very well to multiples of the molecular mass of the apparent monomer, so we concluded that they represented the dimer, trimer, and tetramer of Pvs25H1, respectively. The protein species that appeared in fractions 22 and 23 exhibited markedly different gel mobility (28.3 kDa) from that of the monomeric protein (16.1 kDa) (Fig. 1d), although these two showed extensively overlapping chromatographic peaks on size exclusion chromatography (Fig. 1c). We concluded that the protein species in fractions 22 and 23 was a monomeric protein with a distinct hydrophobic character, and thus decided to further separate these two monomers based on hydrophobicity profile by HIC. HIC clearly separated the two monomeric isoforms (Fig. 1f), and we named the less hydrophobic protein with a distinct hydrophobic character, and thus decided to further separate these two monomers based on hydrophobicity profile by HIC. HIC clearly separated the two monomeric isoforms (Fig. 1f), and we named the less hydrophobic isoform the A and B monomeric isoforms, respectively (19). The observed lower gel mobility for the A isoform than the B isoform on SDS-PAGE under nonreducing conditions (Fig. 1d) was consistent with the results of HIC: the more hydrophobic the molecule, the fewer SDS molecules bind, and the protein becomes less negatively charged, resulting in reduced gel mobility on SDS-PAGE.
**Molecular characterization of Pvs25H isoforms.** The A isoform appeared as a single sharp band, but the other isoforms, including the multimers and the B monomer, appeared as diffuse bands on SDS-PAGE (Fig. 1b and d to g), indicating that the A isoform is constrained to a more uniform molecular configuration than the other isoforms. In addition, all of the isoforms, except the A isoform, generated at least three identical protein bands when samples were heat treated in the presence of SDS and mercaptoethanol (2-ME) prior to SDS-PAGE. The A isoform appeared as a single protein band with a slightly higher molecular mass than that for the largest protein band among the three bands observed for the other isoforms (Fig. 1e).

To characterize the molecular configuration of each isoform furthermore, mixtures of the multimers, including dimers, trimers, and tetramers and the A and B monomers, were subjected to SDS-PAGE after various treatments, as indicated in Fig. 1g. The gel mobility of all of the isoforms did not show any noticeable changes after boiling (100°C, 2 min), but 2-ME treatment (10% [vol/vol]) resulted in a banding pattern very similar to the pattern observed for complete denaturation (2-ME and boiling), except that the A isoform seemed to be slightly more resistant to the reducing agent than the other isoforms. This became particularly notable when lower concentrations of the reducing agent were used (data not shown). These results suggest that the multimers comprised B monomers self-cross-linked by intermolecular disulfide bonds and that the A isoform has a higher molecular rigidity than the other isoforms. Because all of the isoforms exhibited a strong resistance to the boiling and SDS, but not to the 2-ME and SDS treatment, it is plausible that the physical integrity of the protein is critically, if not completely, maintained by disulfide bonds.

Next, to evaluate the status of the covalent disulfide bonds in...
the Pvs25H protein, Ellman's test was conducted for each isoform. No isofrm reacted with the Ellman's reagent, suggesting either that no reduced sulfhydryls were present or that the molecules were inaccessible to the reagent. However, treatment with TCEP immobilized on agarose prior to the Ellman's test resulted in the detection of 4 to 6 molecules of reduced sulfhydryls per molecule of B monomer or multimeric isoforms, but fewer than 0.3 molecules of reduced sulfhydryls were detected per molecule of A monomer. These results indicated that the B and multimeric isoforms have disulfide bonds that are more accessible to surface-immobilized TCEP than those of the A isoform, indicating that the A isoform has a more deeply buried disulfide bond than the other isoforms. It is indicative of the higher molecular flexibility of the B isoform and the multimers than of the A isoform. Interestingly, denaturation of proteins with 2% SDS or 6 M guanidine hydrochloride, or TCEP agarose treatment in the presence of these denaturants prior to the Ellman's test, did not further increase the level of free sulfhydryls. These results strongly support the notion that all of the isoforms are tightly packed molecules and that their rigidity is maintained by intramolecular disulfide bonds and other noncovalent interactions.

Finally, the N-terminal protein sequences determined by the Edman degradation method for each isoform supported the results of SDS-PAGE, in that the multimers and the B isoform contain a mixture of polypeptide species with multiple N termini; however, the A isoform comprises a single polypeptide with a longer, unique N terminus (Fig. 1h). These results suggested that the multimers and the B isoform contain the same set of polypeptide species, with multiple primary structures and presumably various folding configurations. By using different combinations of structurally heterologous polypeptides, different isoforms might be generated.

Taken together, we concluded that the A isoform, which has a more uniform protein configuration, is less hydrophobic and has higher molecular rigidity than the B isoform, and perhaps it most closely resembles the native Pvs25 protein at the structural level. The proportions of each isoform expression were estimated to be 42% (A isoform), 27% (B isoform), 16% (dimer), 10% (trimer), and 5% (tetramer). Thus, the A isoform was produced most abundantly among all of the isoforms. The final protein yields of the total Pvs25H and the A isoform using our expression and purification method were 30 to 50 mg and 12 to 20 mg/liter of culture medium, respectively. We confirmed that the purified Pvs25H-A contained endotoxin at levels less than 0.05 EU/μg of protein. Based on these observations, we decided to use the A isoform (Pvs25H-A) as a TBV antigen to be linked to CTB.

**Chemical conjugation of Pvs25H-A to CTB and its molecular evaluation.** Recombinant CTB was expressed by *P. pastoris* strain GS115 and purified as previously reported (11). Pvs25H-A was chemically conjugated to CTB by using the heterobifunctional cross-linker SPDP (Fig. 2). Because CTB contains two cysteine residues per monomeric subunit, which, in the native form, are involved in an intramolecular disulfide bond, the existence of reduced sulfhydryls in our recombinant CTB was determined by using Ellman's reagent, and none was detected (data not shown).

Various conjugation schemes were evaluated for efficiency of linking Pvs25H-A to CTB via SPDP (Fig. 2a and b). Consistent with the results of Ellman's test for Pvs25H-A and CTB, SPDP modification of only one protein failed to generate the CTB-Pvs25H-A fusion complex (Fig. 2b). Thus, at least one partner protein had to be treated with the reducing agent to expose free sulfhydryls and make it reactive toward the pyridydithiol groups added to the partner protein. Because intact disulfide bonds might be important for the overall structural integrity and native antigenicity of Pvs25 (12, 15, 19, 22), we avoided treating it with reducing agents. Therefore, the CTB or SPDP-modified CTB (CTB<sub>SPDP</sub>) was treated instead with DTT (designated as CTB<sub>DTT</sub> or CTB<sub>SPDP/DTT</sub> in Fig. 2a). Although both CTB<sub>DTT</sub> and CTB<sub>SPDP/DTT</sub>, when reacted with SPDP-modified Pvs25H-A (Pvs25H-A<sub>SPDP</sub>), generated substantial levels of fusion complex with retained affinity for GM1-ganglioside, sequential treatment of CTB with SPDP and then with DTT resulted in an even higher specific reactivity toward Pvs25 antiserum (Fig. 2b).

Second, to evaluate the homogeneity of the fusion complex and the stoichiometry of each component within the complex, proteins before and after the conjugation process were analyzed by size exclusion chromatography (Fig. 2c). The two chromatographic peaks for Pvs25H-A and CTB in a mixed sample disappeared, and a new single peak emerged with an apparent molecular mass of 97.2 kDa. Because the molecular masses of Pvs25H-A and CTB are 29.8 and 53.4 kDa, respectively, based on the K<sub>m</sub> values of chromatography standard proteins, the average stoichiometric ratio for CTB and Pvs25H-A was calculated to be 1:1.5, indicating that one CTB pentamer molecule carries one to two molecules of Pvs25H-A on its surface. Alternatively, if it is assumed that the fusion complex is highly homogeneous and its stoichiometric ratio is 1:1, the 14-kDa discrepancy between the observed and calculated fusion complex mass may be explained by irregularities in the molecular shape, resulting in a higher apparent molecular mass.

Taking all of the results together, we decided to use the Pvs25H-A<sub>SPDP</sub> + CTB<sub>SPDP/DTT</sub> conjugation method (Fig. 2d) to generate the fusion complex for all immunization experiments.

**Immunogenicity in mice of Pvs25H-A and its fusion protein with CTB when administered by the s.c. or the i.n. route.** BALB/c mice were immunized with Pvs25H-A (designated as “S” in Fig. 3 and 4), a mixture of Pvs25H-A and CTB (designated as “M” in Fig. 3 and 4), or CTB-Pvs25H-A fusion protein (designated as “L” in Fig. 3 and 4), by the s.c. or the i.n. route, with or without the indicated adjuvants, at weeks 0, 2, and 3. Antisera were collected at week 4, and the Pvs25H-A-specific IgG titers were determined (Fig. 3a). We demonstrated that: (i) s.c. immunization tended to induce a higher response than i.n. immunization in both the absence and the presence of adjuvants; (ii) the fusion protein (L) consistently induced a higher response than antigen alone (S) or the mixture of proteins (M), regardless of adjuvant supplementation; (iii) supplementation with adjuvants was required for substantial augmentation of the IgG response for both immunization routes; (iv) IFA significantly augmented the response elicited by unfused or CTB-mixed antigen, but CT only marginally affected the response elicited by these antigens; and (v) CT did not exhibit a dose-dependent augmentation effect on the IgG response in the dose range used in the present study (0.1 to 1.0...
Finally, we confirmed that the antisera specifically recognized the \textit{P. vivax} ookinete surface by immunofluorescence (Fig. 3b).

Transmission-blocking effect of the induced mouse antisera against field strains of \textit{P. vivax} parasites. The TBV efficacy of the induced mouse antisera against \textit{P. vivax} parasites in infected blood samples from patients was evaluated by the membrane feeding assay. The same experiments were performed twice, using blood samples from two volunteer donors (Fig. 4). The average number of oocysts observed per mosquito fed on patient blood mixed with antisera induced by s.c. immunization of mice with Pvs25H-A/IFA was reduced by \(99.9\%\) compared to the naive control serum (N). Omission of the adjuvant significantly abated the effect down to \(20\) to \(60\%\) reduction; however, conjugating the antigen to CTB resulted in a dramatic restoration of the vaccine efficacy back to \(90\%\). A similar tendency, albeit with significantly lower efficacy, was observed for i.n. immunization, in that antisera induced by i.n. immunization with the fusion protein decreased the oocyst number by \(70\) to \(90\%\), whereas unfused antigen conferred only a \(0\) to \(6\%\) blocking effect. As expected, CT supplementation augmented the effect for i.n.-administered antigens, in that both unfused and CTB-fused antigens conferred a blocking effect of \(>90\%\). Interestingly, however, addition of CTB to the mixture of antigen and CT significantly abated the vaccine efficacy down to \(40\) to \(50\%\). The reason for this is unknown, and it could not have been predicted from the antibody titers (Fig. 3a). Taken together, we concluded that chemical coupling of Pvs25 to CTB is a potentially promising strategy to enhance the transmission-blocking efficacy in i.n. and s.c immunization regimes.

DISCUSSION

Pvs25 is one of the top-priority \textit{P. vivax} TBV candidates, and the production of stable and functional forms of the antigen in the most appropriate formulation is crucial (4; MVTR). In the present study, we investigated the methylotrophic yeast \textit{P. pas-
toris as a production host for Pvs25. The yield of Pvs25H was comparable to that reported previously for its expression in S. cerevisiae (19). When expressed in S. cerevisiae, this protein was also produced as a mixture of various isoforms (19). Although we observed a similar protein expression pattern, i.e., multimers and the A and B monomers, higher proportions of the molecularly homogeneous A isoform than the heterogeneous B and multimeric isoforms were produced when Pvs25H was expressed in P. pastoris and not in S. cerevisiae. This might present an advantage of using P. pastoris expression system for Pvs25 vaccine production rather than S. cerevisiae system. The P. pastoris-derived A isoform could be as conveniently and efficiently purified from the culture supernatant as reported for S. cerevisiae-derived A isoform, by a combination of affinity, size exclusion, and hydrophobic interaction chromatographies.

The next critical step in vaccine generation is the optimization of vaccine antigen formulations; a search for the optimal antigen formulation is often considered to be as important as choosing the best antigen among many vaccine candidate antigens. Pvs25H antigen adsorbed onto Alhydrogel (Brentag Biosector, Frederikssund, Denmark) has recently been shown to induce antibody effectively in human volunteers in a phase 1 clinical trial, and the antigen was found to be efficacious, as evidenced by significant transmission-blocking activity observed in the membrane feeding assay (17). That study confirmed that Pvs25 is a very promising TBV candidate; however, it is highly desirable to induce higher levels of transmission-blocking immunity for practical vaccine development (17). Another phase 1 clinical trial using Montanide ISA 51, a water-in-oil emulsion, has recently been completed; however, due to an unexpected frequent local reactogenicity, the vaccine efficacy has not been verified (29). Therefore, there seems to be an increasing demand for the development of a new immune-enhancing vaccine platform technology for malaria OSPs, because they are low-molecular-weight proteins that are by themselves not sufficiently immunogenic.

There have been several reports showing examples of chemical conjugation of Plasmodium falciparum OSPs with potential antigen carrier molecules such as the outer membrane protein of Neisseria meningitidis (30), exoprotein A of Pseudomonas
...them to other proteins. In the present study we evaluated CTB as a potential carrier for Pvs25. First, to extend our previous study where CT was used as adjuvant (1–3), we tested our hypothesis that the mucosal immunogenicity of Pvs25 would increase when the protein was coupled to the nontoxic CTB subunit, even in the absence of CT supplementation. Second, to explore CTB’s less-characterized immune potentiating properties for s.c.-delivered antigens, we immunized mice with the CTB-Pvs25H-A fusion protein by an s.c. route, in the presence or absence of IFA. Our principal finding was that the coupling of the antigen to CTB profoundly enhanced its immunogenicity in i.n. as well as in s.c. immunization regimes, without supplementation with extraneous adjuvants. However, the membrane feeding assay revealed that there was still much room for improvement (Fig. 4). For instance, although i.n. administration of the fusion protein alone conferred a relatively high transmission-blocking immunity (88 and 73% decreases in oocyst number for blood samples from donors 1 and 2, respectively) compared to unfused antigen alone or the unimmunized control group, only a few mosquitoes (0/20 to 3/20) were free of parasites. Supplementation of CT to the fusion protein increased the efficacy close to complete blockade (>99.9%), significantly increasing the number of mosquitoes free of parasites (19/20 to 20/20). Because supplementation of CT to unfused antigen resulted in an intermediate level of protection (97% [9/20] and 92% [11/20] decrease in oocyst number for blood samples from donors 1 and 2, respectively), we concluded that both the CT supplementation and the CTB-coupling strategies contributed to the increased vaccine efficacy, although the former was more efficient than the latter. A similar tendency was observed for the s.c. immunization regime: s.c. administration of the fusion protein alone conferred a more than 90% decrease in oocyst number, in which approximately half of the mosquitoes were free of parasites (9/20 to 11/20); however, the use of IFA with unfused antigen contributed more than the fusion method. We observed that the efficacy of the fusion protein administered alone by the s.c. route was almost equal to that attained by the unfused antigen administered i.n. with CT supplementation, in terms of the average numbers of oocyst per mosquito (>90%) as well as the number of mosquitoes free of parasites (9/20 to 11/20). Similarly, the vaccine efficacy for unfused antigen administered s.c. with IFA was almost equal to the level attained by the fusion protein administered i.n. with CT in terms of the average number of oocysts per mosquito (>90%) as well as the number of mosquitoes free of parasites (9/20 to 11/20). Although we did not assess the vaccine efficacy of the fusion protein emulsified in an oil adjuvant such as IFA, CTB was very effective in enhancing transmission-blocking immunity, but supplementation with extraneous adjuvants is expected to induce an even higher immunity (Fig. 5).

The clinical use of CT, particularly as a nasal adjuvant, is hampered by its toxicity (26). Furthermore, the nontoxic CTB has yet to be proven a safe nasal vaccine delivery molecule.

![Graph](http://iai.asm.org/)

**FIG. 4. Transmission-blocking vaccine (TBV) effects of the induced mouse antisera on *Plasmodium vivax* oocyst development in the *Anopheles dirus* A mosquito midgut. TBV effects on oocyst numbers induced by antiserum (1/2 dilution) obtained from mice immunized with each antigen formulation (S, M, and L) as described in Fig. 3. N, nonimmune serum. Either IFA or CT was used as an adjuvant, as indicated. The data are expressed as the median values of oocyst number found per mosquito (bar within the box) with the 25 and 75% quartiles (the box) and ranges (whiskers above and below the box). The percent reduction was calculated as the reduction in the average oocyst number for the unimmunized control group compared to the average oocyst number for the unimmunized control group (N). The number of parasite-free mosquitoes per total number of mosquitoes examined (20 mosquitoes) is provided. The analysis was performed twice, using different blood samples, as indicated in the upper panel (*P. vivax* [Pv]-infected blood donor 1) and the lower panel (*P. vivax* [Pv]-infected blood donor 2). M groups without adjuvant supplementation, M and L groups with IFA supplementation, and all groups with 0.1- and 0.5-µg CT supplementations were excluded from membrane feeding analysis. *, *P* < 0.001 versus the nonimmune (N) group as determined by the Wilcoxon-Mann-Whitney test; **, *P* < 0.001 between the S and L groups as determined by the Wilcoxon-Mann-Whitney test; ††, *P* < 0.001 among the three groups (S, M, and L) as determined by the Kruskal-Wallis test; †, *P* < 0.005 versus the nonimmune (N) group as determined by the chi-square test; ††, *P* < 0.005 between the indicated two groups as determined by the chi-square test.

**aeruginosa** (16), ovalbumin (16), and a *P. falciparum* OSP itself by chemical crosslinking (16). All of these were demonstrated to increase TBV efficacy, but no attempts have yet been made to enhance the immunogenicity of *P. vivax* OSPs by coupling...
Therefore, an alternative approach for using CTB as a vaccine antigen carrier is highly desirable. Although there have been numerous reports demonstrating enhanced mucosal immunogenicity of various antigens by coupling to CTB (13), very few systematic studies have been conducted to assess CTB’s antigen carrier capacity for s.c.-delivered antigens. Our present study clearly demonstrated the potential of CTB in s.c. vaccine platform design. Furthermore, it is notable that, unlike antigens emulsified with oil adjuvant such as IFA or antigens administered with an aluminum hydroxide adjuvant, the protein-only CTB-coupled antigens are likely to be much less reactogenic. It is believed that recent innovations in effective but much less locally reactogenic and safer oil adjuvants such as MF59 (Chiron Corp., Emeryville, CA) (20, 21), the Montanide ISA series (Seppic, Inc., Fairfield, NJ) (20, 29), and the GlaxoSmithKline adjuvant systems (GlaxoSmithKline, Brentford, United Kingdom) (8, 27), will expedite malaria vaccine development. It is also possible that protein delivery molecules will ultimately be combined with effective oil or other adjuvants, including aluminum hydroxide. This is supported by our recent unpublished study in which a recombinant malaria antigen administered with an alum adjuvant only marginally enhanced its immunogenicity, whereas the same antigen loaded onto carrier molecules became highly immunogenic when applied together with the alum.

In the present study, we did not assess the molecular mechanisms of the immune potentiating function of CTB. However, our observation that simple mixing of Pvs25H-A with CTB did not produce a profound immune enhancement implies that its immunogenicity results from the antigen delivery, rather than a physiological cell activation, as occurs for CT. Further experiments are ongoing to characterize the immune potentiating function of CTB using the C-terminal 19-kDa fragment of CTB, cholera toxin B subunit; CT, cholera toxin; IFA, incomplete Freund’s adjuvant.

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