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Saccharomyces cerevisiae Recombinant Pfs25 Adsorbed to Alum Elicits Antibodies That Block Transmission of *Plasmodium falciparum*

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Antibodies to Pfs25, a cysteine-rich 25-kDa protein present on the surface of *Plasmodium falciparum* zygotes, can completely block the transmission of malaria parasites when mixed with infectious blood and fed to mosquitoes through a membrane feeding apparatus. Recently, a polypeptide analog, Pfs25-B, secreted from recombinant *Saccharomyces cerevisiae* was found to react with conformation-dependent, transmission-blocking monoclonal antibodies and to elicit transmission-blocking antibodies in experimental animals when emulsified in either Freund's or muramyl tripeptide adjuvant. In this study, Pfs25-B adsorbed to alum induced transmission-blocking antibodies in both rodents and primates. Bacterially produced Pfs25, however, did not elicit complete transmission-blocking antibodies in rodents. Furthermore, unlike monoclonal antibodies to Pfs25, which block transmission only after ookinete development, antisera to Pfs25-B adsorbed to alum appeared to block the in vivo development of zygotes to ookinetes as well.

The resurgence of malaria, due in part to the spread of chloroquine-resistant *Plasmodium falciparum* and to the reduced efficacy of vector control campaigns, has intensified the search for alternative interventions to slow or reverse the reemergence of malaria in many developing countries (9). Much of the recent effort has focused on the development of effective subunit vaccines for malaria. Several stages of the complex life cycle of *P. falciparum* are being examined in an attempt to identify parasite proteins that elicit immunity to malaria (9). A number of proteins expressed during the sexual stage of the parasite have been shown to be target antigens of antibodies that prevent the transmission of *P. falciparum* from host to mosquito (5). Antibodies to a cysteine-rich 25-kDa surface protein, Pfs25 (8), expressed predominantly on zygotes and ookinetes (15) completely prevent mosquitoes from becoming infected with *P. falciparum* (1, 6, 7, 14). Since humans constitute the sole reservoir for *P. falciparum*, an effective program for immunizing persons residing in malaria endemic regions with a multivalent vaccine containing Pfs25 might help control or even eradicate malaria in some regions of the world.

Although a number of promising malaria vaccine candidate antigens induce immunity to malaria, none has been shown to be completely effective when delivered in adjuvants currently licensed for use in humans. The number of adjuvants currently approved for use in humans is quite limited. Of those adjuvants that have been tested in humans, alum has had the greatest clinical use and is relatively nonreactogenic. The initial immunization trials with recombinant Pfs25 derived from *Saccharomyces cerevisiae* (Pfs25-B) in experimental laboratory animals were conducted with Freund's and muramyl tripeptide-based adjuvant systems (1, 6). Pfs25-B in either adjuvant effectively induced transmission-blocking activity as assayed by a membrane feeding assay. Here we report that yeast-produced, but not bacterially produced, Pfs25 adsorbed to alum (Pfs25/alum), given three times, is an effective formulation for inducing complete transmission-blocking activity in mice and primates.

MATERIALS AND METHODS

Parasites. Mature gametocytes of strains 3D7 (a cloned laboratory line derived from the Amsterdam Airport strain NF54) (16) and 7G8 (a laboratory line adapted to culture from a Brazilian isolate) (2) of *P. falciparum* were obtained by in vitro culture as previously described (11).

Yeast recombinant Pfs25. As previously described, a synthetic Pfs25 gene with common yeast codons was cloned into yeast vector pBS24 and expressed in *S. cerevisiae* (1). Pfs25-B was recovered from culture supernatants to near homogeneity by ion-exchange chromatography and then gel filtration chromatography (1). The purified, lyophilized product was dissolved in phosphate-buffered saline (PBS) (pH 7.4) at a final concentration of 1 mg/ml and stored at −20°C until formulated with adjuvant.

Bacterial recombinant Pfs25. Pfs25 lacking the presumptive signal and anchor sequences (8) was amplified by PCR with synthetic oligonucleotides (sense primer, 5′-cc aat tct GCG AAA GTT ACC GTG GAT; antisense primer, 5′-agt cga cta AGA GCT TTC ATT ATC TAT) and cloned into the *Eco*RI and *Sal*I sites of the TrpE fusion protein bacterial expression vector pATH 3 (kindly provided by T. J. Koerner) (13). Inclusion bodies containing recombinant Pfs25 fused to the carboxy terminus of TrpE were purified from *Escherichia coli* (SCS1 strain; Stratagene, La Jolla, Calif.) induced by overnight growth in the presence of 10 mg of indole acrylic acid per liter, as follows: cells were recovered by centrifugation for 20 min at 5,000 × *g*, resuspended in 300 mM NaCl–0.5 mM EDTA–50 mM Tris (pH 8.0) with 1 mg of lysozyme per ml, incubated for 15 min on ice,

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mixed with Triton X-100 (to a final concentration of 0.2%), further incubated on ice for 10 min, adjusted to 1 M NaCl, 6 mM $MgCl_2$, and 20 μg of DNase I per ml, and then incubated on ice for 60 min. The insoluble protein recovered by centrifugation was washed with PBS (pH 7.4) and stored at −70°C until formulated with adjuvant.

Adjuvant components and formulations. For the studies with mice, a stock of Pfs25/alum was made by adding 100 μl of Pfs25-B (1 mg in PBS) to 100 μl of 2.0% Rehydragel and rocking the suspension at room temperature (RT) for 20 min. The material was stored at 4°C until used.

Animals and immunizations. CAF1 mice were immunized by intraperitoneal injection on days 0, 21, and 42. The mice were bled on days 0, 14, 21, 28, 42, 49, and 56. *Aotus vociferans* monkeys were immunized by intramuscular injection on days 0, 21, and 42 and bled on days 21, 28, 42, 49, 56, 105, and 131.

ELISA for Pfs25-specific antibodies. Enzyme-linked immunosorbent assays (ELISAs) were performed as described previously (6). Briefly, Immunlon II 96-well microtiter plates (Dynatech) were coated with Pfs25-B at 2 μg/ml in 50 mM borate buffer at pH 9.0 and were stored overnight at 4°C. The plates were washed with PBS, blocked for 1 h at room temperature with 5% normal goat serum and 0.5% Nonidet P-40 in PBS, and then washed with PBS. A 1:4 dilution of test serum in blocking solution was applied to the first well of a column and then serially diluted 1:10 in blocking solution in the rest of the wells of the column. Each test serum was assayed in triplicate. After 1 h at room temperature, the plates were washed with PBS. One hundred microliters of peroxidase-labeled goat anti-mouse or goat anti-monkey antibodies diluted 1:1,000 in blocking solution was then placed in each well. After 1 h, the plates were washed with PBS, and 100 μl of horseradish peroxidase substrate (TMB Microwell Peroxidase Substrate System; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added. The color development was stopped by the addition of 50 μl of 10% sodium dodecyl sulfate. Titers were calculated at 0.2 absorbance units, using a V_{max} microtiter plate reader (Molecular Devices Corp., Palo Alto, Calif.) programmed to read at 650 nm with subtraction of the 490-nm reading.

Transmission-blocking assays. Murine or monkey sera were assayed for transmission-blocking activity as described previously (6). Briefly, sera were mixed in a 1:2 ratio with in vitro-cultured *P. falciparum* gametocytes (7G8 or 3D7 strains), and the mixtures were fed to *Anopheles freeborni* mosquitoes by means of a membrane feeding apparatus. Six to eight days after engorgement on the blood meal, mosquitoes were dissected and the midguts were stained with 1% mercurochrome and scored for *P. falciparum* oocysts. The following nomenclature has been adopted to express the quantitative results from transmission-blocking assays: suppression of oocyst development refers to the ability of the test reagent to suppress the mean number of oocysts per gut to less than 50% of the control value but not to reduce the ratio of uninfected mosquitoes to total mosquitoes dissected to below 0.5; reduction of transmission refers to the ability of the test reagent to reduce the ratio of uninfected mosquitoes to total mosquitoes dissected to below 0.5 but not below 0.1; and complete transmission-blocking activity refers to reduction of the ratio of uninfected mosquitoes to total mosquitoes dissected to below 0.1.

Transformation efficiency in vivo. Mosquitoes were given an infectious blood meal containing 40 μl of test serum or anti-Pfs25 transmission-blocking monoclonal antibody (MAb) 4B7 (1), supplemented with 80 μl of normal human serum, in a total volume of 270 μl of gametocyte-infected erythrocyte suspension by membrane feeding with a minifeeder as described previously (11). In control experiments 40 μl of normal human serum was used instead of test serum. After the feeding, unfed and partially fed mosquitoes were removed. At 22 h after feeding of the infectious meal, five mosquitoes per group were carefully dissected in a drop of PBS, leaving the fully fed midguts unruptured. The midguts were transferred to an Eppendorf tube and disrupted in 25 μl of PBS by repeated pippetting. An equal volume of appropriately diluted fluorscein isothiocyanate (FITC)-labelled antibody in 0.025% Evan's blue was added and incubated for 20 min. For the control serum (which was not expected to bind to the surface of parasites, and thus parasites would not fluoresce with anti-mouse antibody), a mixture of fluorescein isothiocyanate-labelled anti-Pfs25 MAbs 32F81 (blocking MAb) and 32F71 (nonblocking MAb) was used to visualize the zygotes, retorts, and ookinetes present in the blood meal. For test sera, a fluorescein isothiocyanate-labelled goat anti-mouse conjugate (Nordic) was added to visualize parasites in the blood meal. After the incubation period, the contents of the Eppendorf tube were diluted with PBS and centrifuged at 10,000 rpm in a microcentrifuge for 3 min. The pellet was resuspended in 25 μl of PBS, and the parasites were counted in a Bürker-Türk hemacytometer with an incident light fluorescence microscope. Sporogonic stages could easily be recognized and quantified.

RESULTS

Immunogenicity in mice of yeast and bacterial recombinant Pfs25 adsorbed to alum. Previously, yeast recombinant Pfs25 (Pfs25-B), emulsified in Freund's adjuvant or in the muramyl tripeptide adjuvant MF59, was found to elicit complete transmission-blocking activity in experimental animals (1). To examine whether any adjuvant was required in order to induce transmission-blocking antibodies and, if so, whether one approved for use in humans could induce transmission-blocking antibodies, CAF-1 mice were immunized with 50 μg of Pfs25-B suspended in PBS or adsorbed to alum. Optimal adsorption of Pfs25-B to alum occurred within 30 min in phosphate buffer at neutral pH (7.0) and physiological salt concentration (150 mM NaCl) (data not shown). Transmission-blocking activity (Table 1, experiment 1) (measured by a standard membrane feeding assay) and antibody response (determined by ELISA) were examined in sera collected from the mice 1 week after the third immunization. The antibody response in mice immunized with Pfs25-B without adjuvant was almost 100-fold lower than that in mice immunized with Pfs25-B/alum (1:118,000 versus 1:8,262,592). The group of two mice receiving Pfs25-B/alum developed complete transmission-blocking immunity, whereas those that received Pfs25 without any adjuvant did not even suppress oocyst development in the experiment shown in Table 1.

In addition to reproducing the results of the first experiment by using a different lot of Pfs25-B/alum in a second experiment, the efficacy of recombinant Pfs25 (TrpE-Pfs25) expressed in *E. coli* in induction of transmission-blocking antibodies was also tested. Pfs25-B has previously been shown to partially recreate the native structure (1), as assayed by immunoblot with conformation-dependent MAbs, whereas TrpE-Pfs25 is not recognized by these MAbs (data not shown). Sera from mice immunized with TrpE-Pfs25/alum recognized live *P. falciparum* gametes as determined by indirect immunofluorescence (data not shown) and suppressed oocyst development but did not block transmission of malaria parasites (Table 1, experiment 2). In contrast, the yeast-produced material, Pfs25-B/alum, again induced complete transmission-blocking immunity in mice.

TABLE 1. Transmission-blocking activity of sera from immunized mice

Expt	Serum sample[a]	Mean no. of oocysts (range)	Infectivity (% of prebleed level)	No. of mosquitoes infected/no. dissected
1	Pfs25-B (prebleed, 0D0)	4.2 (1–7)	100	16/16
	Pfs25-B (IIID7)	3.8 (0–7)	90	11/14
	Pfs25-B/alum (prebleed, 0D0)	4.1 (0–10)	100	7/8
	Pfs25-B/alum (IIID7)	0 (0)	0	0/14
2	TrpE/alum (prebleed, 0D0)	9.3 (0–29)	100	28/32
	TrpE/alum (IIID7)	15.0 (0–34)	163	17/20
	TrpE-Pfs25/alum (prebleed, 0D0)	6.3 (0–21)	100	21/25
	TrpE-Pfs25/alum (IIID7)	3.3 (0–19)	52	15/23
	Pfs25-B/alum (prebleed, 0D0)	9.6 (0–24)	100	23/26
	Pfs25-B/alum (IIID7)	0 (0)	0	0/14

[a] Each sample is identified by immunization number (roman numerals) and the number of days (D) postimmunization (arabic numerals); e.g., IIID7 denotes 7 days after the third immunization. 0D0 indicates sera collected on or before the day of the first immunization.

Immunogenicity in primates of yeast recombinant Pfs25 adsorbed to alum. Pfs25-B/alum also elicited transmission-blocking antibodies in primates. Of the three *A. vociferans* monkeys (monkeys 225, T308, and 927) immunized three times with 50 μg of Pfs25/alum, one (monkey T308) developed transmission-blocking activity after the second immunization and one (monkey 225) developed this activity within a week after the third immunization (Table 2, experiments 1 and 2). The third monkey (monkey 927) never developed complete transmission-blocking activity, even after the third immunization (Table 2, experiments 1, 2, and 3). Three other *A. vociferans* monkeys (control group monkeys 1180, 1164, and 931) immunized three times with alum alone did not develop transmission-blocking antibodies (Table 2, experiment 2).

TABLE 2. Transmission-blocking activity of sera from immunized monkeys

Expt	Serum sample[a]	Mean no. of oocysts (range)	No. of mosquitoes infected/no. dissected
1	Normal human	53.1 (2–109)	17/17
	Test monkey 225 (IID14)	6.9 (0–7)	22/23
	Test monkey T308 (IID14)	0.5 (0–4)	7/24
	Test monkey 927 (IID14)	34.5 (0–111)	22/24
	Test monkey 225 (IIID14)	0.1 (0–1)	3/29
	Test monkey T308 (IIID14)	0 (0)	0/25
	Test monkey 927 (IIID14)	1.5 (0–7)	15/22
2	Normal human	47.3 (21–71)	13/13
	Test monkey 225 (IIID7)	0 (0)	0/22
	Test monkey T308 (IIID7)	0 (0)	0/14
	Test monkey 927 (IIID7)	3.8 (0–14)	13/16
	Control monkey 1180 (IIID7)	6.1 (1–12)	10/10
	Control monkey 1164 (IIID7)	9.9 (1–22)	11/11
	Control monkey 931 (IIID7)	1.4 (0–5)	9/14
3	Normal human	30.9 (11–53)	25/25
	Test monkey 225 (IIID61)	2.1 (0–7)	9/12
	Test monkey T308 (IIID61)	0 (0)	0/21
	Test monkey 927 (IIID61)	12.8 (0–25)	19/20
	Control monkey 1164 (IIID61)	13.0 (1–37)	23/23
4	Normal human	20.8 (7–47)	16/16
	Test monkey 225 (IIID89)	28.2 (11–48)	16/16
	Test monkey T308 (IIID89)	21.8 (1–62)	21/21
	Test monkey 927 (IIID89)	19.9 (7–37)	13/13

[a] Each sample is identified by immunization number (roman numerals) and the number of days (D) postimmunization (arabic numerals); e.g., IIID7 denotes 7 days after the third immunization.

Pfs25-specific antibodies in both groups of monkeys were measured by ELISA (Fig. 1). The primary antibody responses to Pfs25 in the both groups were very low, but with subsequent immunizations, the test group developed significantly higher levels of anti-Pfs25 antibodies. Sera collected after the second immunization had ELISA titers of 1:13,600, 1:89,100, and 1:213,700 but did not completely block transmission, whereas sera collected 1 week after the third immunization, with ELISA titers of 1:45,100 and 1:76,100, completely blocked transmission. Serum from monkey 927, which had an ELISA titer of 1:108,200 1 week after the third immunization, did not block transmission. Thus, transmission-blocking activities did not correlate with ELISA titers. The duration of complete transmission-blocking activity was rather short. Serum from monkey T308 had complete transmission-blocking activity 5 weeks after the third immunization, while that from monkey 225 only suppressed transmission and that from monkey 927 had no effect on transmission. The sera collected 12 weeks after the third immunizations had essentially no transmission-blocking or suppressive activity (Table 2, experiment 4).

Effect of polyclonal and monoclonal antibodies to Pfs25 on normal in vivo development of zygotes to ookinetes. Clues to the function of Pfs25 in parasite development might come from better understanding of the mechanism by which antibodies to Pfs25 block transmission. Previously we had observed that polyclonal antisera blocked transmission qualitatively better than monoclonal antibodies (7). To determine when and where parasite development is arrested in the presence of polyclonal anti-Pfs25-B sera, mosquito midgut contents were examined 22 h after an infectious blood meal and were scored for the presence of round forms (presumed unfertilized female gametes), partial transformants (retorts), or complete transformants (ookinetes). Polyclonal sera from mice immunized twice with Pfs25/alum did not appear to interfere with transformation of zygotes into ookinetes (Table 3). After the third immunization, however, polyclonal sera from these same mice reduced the total number of parasites present in the blood meal after 22 h and completely blocked the transformation of retorts into ookinetes (Table 3).

DISCUSSION

The ability of recombinant Pfs25 to induce Pfs25-specific transmission-blocking antibodies in mice requires both an adjuvant and at least partial native conformation. TrpE-Pfs25,

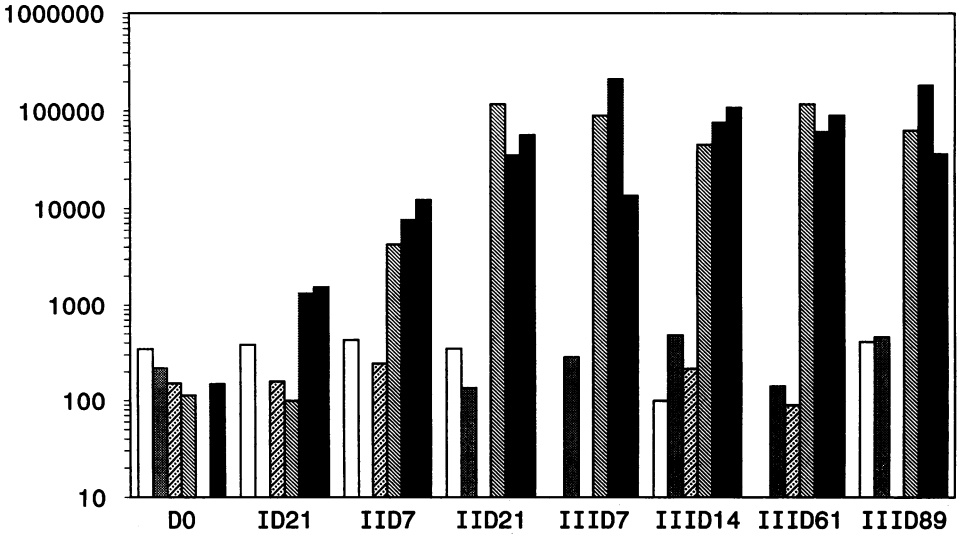

FIG. 1. Antibody titers in *Aotus* monkeys immunized with Pfs25-B/alum. Monkeys were injected intramuscularly with three 50-μg doses of Pfs25-B/alum at three weekly intervals. Titers were measured by ELISA on the days indicated (samples are identified by immunization number [roman numeral] and the number of days [D] postimmunization [arabic numerals]). Selected serum samples were used for mosquito feeding experiments (Table 2). Samples were from control monkeys 931 (□), 1164 (▨), and 1180 ( ) and test monkeys 225 (▧), T308 (▩), and 927 (■).

a recombinant Pfs25-TrpE fusion protein synthesized in *E. coli*, does not recreate the known conformational epitope(s) that is the target of transmission-blocking monoclonal antibodies to Pfs25 (5a), nor does TrpE-Pfs25 induce complete transmission-blocking antibodies in mice immunized three times with the fusion protein adsorbed to alum. Thus, although at least one nonconformational (i.e., linear) epitope is present in Pfs25, it does not seem to be the target of transmission-blocking antibodies. Pfs25-B, a recombinant analog of Pfs25 synthesized by secretion from *S. cerevisiae*, reacts with conformation-dependent, transmission-blocking monoclonal antibodies (1); however, in the absence of adjuvant, Pfs25-B does not induce transmission-blocking antibodies in mice. When Pfs25-B is adsorbed to alum or, as shown previously, when it is emulsified in Freund's adjuvant or a muramyl tripeptide adjuvant (1, 6), complete transmission-blocking activity can be elicited in mice and monkeys after the two or three immunizations. Unfortunately, the *Aotus* data, if indicative of the human response to alum-adjuvanted Pfs25, suggest that alternative delivery systems may be required to elicit long-lasting immunity. Nevertheless, human trials with Pfs25 formulated on alum may allow "proof of principle" that anti-sexual stage vaccines can induce antibodies that block transmission in vivo.

With regard to the mechanism of transmission-blocking antibodies, transmission-blocking MAbs to Pfs25 (MAb 4B7 in this study) and to the analogous protein in *Plasmodium gallinaceum*, Pgs25 (4), do not block zygote-to-ookinete transformation; rather, these antibodies interfere with development that occurs later, sometime between the ookinete's penetration of the peritrophic matrix and midgut epithelium and the parasite's formation of an oocyst beneath the basal lamina of the midgut (12). Recently Duffy et al. observed that polyclonal sera against parasite-produced Pgs28, a *P. gallinaceum* late ookinete surface antigen that is structurally related to P25 (Pfs25 in *P. falciparum* and Pgs25 in *P. gallinaceum*) and to other P28 proteins (Pfs28 in *P. falciparum* [2a] and Pbs21 in *Plasmodium berghei* [10]), blocked transmission by interfering with in vitro transformation of zygotes to ookinetes and by inhibiting in vivo development of ookinetes to oocysts (3). Here we report that polyclonal sera against recombinant yeast-produced Pfs25 that block transmission also appear to reduce the total number of parasites in the midgut and block transformation of zygotes to ookinetes. Whether combining the structurally related P25 and P28 antigens, which appear to have similar mechanisms of blocking parasite infectivity to mosquitoes, will produce a more potent vaccine needs to be determined.

TABLE 3. Inhibition of ookinete development by sera from immunized animals

Sample	10^2 forms/gut (%)		
	Zygotes	Retorts	Ookinetes
MAb 4B7	3 (17)	3 (17)	12 (66)
Pfs25-B/alum, bleed 3	1 (5)	7 (39)	10 (56)
Pfs25-B/alum, bleed 6	0 (0)	3 (100)	0 (0)

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