Long-lasting and transmission-blocking activity of antibodies to *Plasmodium falciparum* elicited in mice by protein conjugates of Pfs25

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Malaria is a leading cause of morbidity and mortality, estimated to cause >1 million childhood deaths annually. *Plasmodium falciparum* causes the most severe form of the disease. There is as yet no licensed vaccine for this disease, despite over a half century of research. In this study, we investigated a transmission-blocking vaccine candidate, the ookinete surface protein Pfs25. Antibodies against Pfs25, drawn in during a bite, can block parasite development in the mosquito midgut, preventing transmission to other individuals. Pfs25 is a low-molecular-weight protein, by itself not immunogenic. To increase its immunogenicity, we investigated several methods of conjugating Pfs25 to itself and to other proteins: recombinant *Pseudomonas aeruginosa* exotoxin A, and ovalbumin, using amide, hydrazone, or thioether linkages. All conjugates were immunogenic and induced booster responses in mice. The scheme to form amide bonds between proteins by using adipic acid dihydrizide as a linker produced the most immunogenic conjugates. Adsorption of the conjugates onto aluminum hydroxide further increased the antibody response. Remarkably, the antibody levels 3 or 7 months after the last injection were significantly higher than those 1 wk after that injection. The observed transmission-blocking activity of immune sera correlated with antibody levels measured by ELISA.

malaria | vaccine

One approach for a vaccine against malaria is to block transmission of the parasite from mosquitoes to humans. When ingested by a mosquito with the blood meal, antibodies against the sexual and the mosquito stage-specific surface antigens can block parasite development inside the vector (1). Four proteins have been identified as potential inducers of transmission-blocking antibodies (2–5). Two of these are expressed on the surface of gametes and in intracellular gameotocytes. The other two are the *Plasmodium falciparum* surface proteins with apparent molecular masses of 25 kDa (Pfs25) and 28 kDa (Pfs28), expressed exclusively on the zygote and ookinete surfaces during the mosquito stage of the infection. No antibody response to these two proteins has been shown in people infected with malaria and living in endemic countries (6). Pfs25 from different parts of the world has shown minimal variation in its amino acid sequence (7). This relative homogeneity, likely a result from not being subjected to immune pressure in the human host, makes Pfs25 an attractive candidate for a malaria transmission-blocking vaccine (8). Pfs25 is poorly immunogenic in mice and in humans, even if administered with adjuvant (9, 10). In this article, we show that Pfs25 bound onto itself or onto another protein induced high levels of transmission-blocking antibodies in mice.

Results

Characterization of Conjugates. Pfs25 was bound to itself, to *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA), or to ovalbumin (OVA) by formation of amide, hydrazone, or thioether linkages. Higher antibody levels were obtained with conjugates having a molar ratio of Pfs25 to carrier greater than 1 (Table 1). All conjugation methods increased the molecular mass of Pfs25, shown by the column elution profile and by SDS/PAGE, summarized in Table 1. Conjugates Pfs25-AH/Pfs25 (Conjugates 1 and 2), Pfs25-AH/rEPA (Conjugates 14 and 15), and Pfs25-CHO/AH-OVA (Conjugates 9 and 10) were heterogeneous in their molecular masses and were separated into two, partially overlapping fractions (F1 and F2). Conjugates that had estimated molecular masses >300 kDa were collected in only one fraction.

Derivatization of protein with adipic acid dihydrazide (ADH) was performed in two ways: (*i*) formation of amide bonds between carbodiimide activated aspartic and glutamic acid carboxyl groups of the proteins and the hydrazide of ADH (Conjugates 1, 2, and 11–15; Fig. 1*A*); (*ii*) formation of hydrazone linkages between benzaldehyde and hydrazide derivatized proteins (Conjugates 5–10; Fig. 1*B*). The longer linker, composed of an ADH molecule between two benzaldehyde molecules, was also prepared (Conjugate 7). Conjugates 3 and 4, containing thioether linkages between the two proteins, were also tested (Fig. 1*C*). All conjugates precipitated by double immunodiffusion with Pfs25 and carrier antibodies with an identity line, confirming that the antigenicity of both components was preserved.

Immunogenicity of Conjugates. The antibody levels induced by all conjugates after three injections were significantly higher than those induced by Pfs25 alone (Table 1; $P < 0.001$). The most immunogenic conjugates were Pfs25 bound to itself by ADH [geometric mean (GM) 352 μg/ml] or to rEPA (GM 284 μg/ml) in a two-step reaction. Conjugates prepared with ADH induced statistically higher antibody levels than similar conjugates prepared with thioether (352 vs. 88 μg/ml; $P = 0.02$) or hydrazone linkages (352 vs. 71 μg/ml; $P = 0.001$). The Pfs25 linked to itself by a two-step procedure was more immunogenic than by a one-step procedure ($P = 0.008$). The conjugate containing longer linker (Conjugate 7), with ADH separated from the protein by benzaldehyde rings, produced significantly lower antibody levels compared with levels induced by Conjugates 1 and 12, where only ADH served as the linker.

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Abbreviations: ADH, adipic acid dihydrazide; GM, geometric mean; OVA, ovalbumin; rEPA, P*seudomonas aeruginosa* recombinant exoprotein A.

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Table 1. Composition and serum IgG anti-Pfs25 elicited by conjugates prepared by binding Pfs25 to itself, to rEPA, and to OVA

No.	Conjugate	Molecular mass by SDS/PAGE, kDa	Pfs25/carrier, mol/mol	GM anti-Pfs25, μg/ml	
				Second injection	Third injection
	Pfs25 alone	25	na	nd	0.05
	Conjugation by amide linkages: two-step procedure				
1	Pfs25-AH/Pfs25 F1	50–250	na	nd	352
2	Pfs25-AH/Pfs25 F2	25–200	na	nd	98
11	Pfs25-AH/Pfs25 F1	50–250	na	nd	296
14	Pfs25-AH/rEPA F1	>300	1.8/1.0	nd	284
15	Pfs25-AH/rEPA F2	100–>300	2.5/1.0	nd	331
	Conjugation by amide linkages: one-step procedure				
12	Pfs25-AH–Pfs25 F1	50–200	na	nd	150
13	Pfs25-AH–Pfs25 F2	25–100	na	nd	11
	Conjugation by thioether linkages				
3	Pfs25-Br/SH-Pfs25	50–>300	na	nd	88
4	Pfs25-SH/Br-OVA	>300	0.6/1.0	0.4	24
	Conjugation by hydrazone linkages				
6	Pfs25-AH/CHO-Pfs25	>300	na	5.0	36
8	Pfs25-AH/CHO-Pfs25	>300	na	4.5	71
7	Pfs25-CHO/AH/CHO-Pfs25	>300	na	0.005	17
5	Pfs25-CHO/AH-OVA	>300	0.3/1.0	0.02	12
9	Pfs25-CHO/AH-OVA F1	>300	2,0/1.0	17	69
10	Pfs25-CHO/AH-OVA F2	>300	4.4/1.0	24	123

Five- to 6-wk-old NIH general purpose mice ($n = 10$) injected s.c. with 2.5 μg of Pfs25 as conjugate 2 wk apart and exsanguinated 7 d after the second or third injection. Statistics: 1 vs. 2, $P = 0.002$; 1 vs. 12, $P = 0.008$; 11 vs. 2, $P = 0.03$; 12 vs. 13, $P = 0.003$; 3 vs. 4, $P = 0.05$; 8 vs. 7, $P = 0.02$; 1 vs. 3, $P = 0.02$; 1 vs. 8, $P = 0.001$. na, not applicable; nd, not done.

Carrier Effect. Conjugates of Pfs25 linked to rEPA via ADH induced antibody levels similar to those induced by Pfs25 linked to itself by using the same method. Conjugates prepared by linking Pfs25 to OVA by thioether linkages induced significantly lower antibody levels than Pfs25 bound to itself by the same method (24 vs. 88 μg/ml; $P = 0.05$). Conjugate of Pfs25 bound to itself (Conjugate 3) and to OVA (Conjugate 4) by hydrazone linkages elicited similar levels of anti-Pfs25 but significantly lower than those prepared with ADH (71 vs. 352 μg/ml; $P = 0.001$). Except for fraction F2 of Pfs25-AH/rEPA, the first fraction F1 with higher average molecular masses induced statistically higher antibody levels than the second fraction F2 (352 vs. 98 μg/ml; $P < 0.005$). This difference may be at least partially attributed to the presence of free Pfs25 in the second pool.

Size Effect. The immunogenicity of the conjugates was not directly related to their sizes. To be immunogenic, conjugates of Pfs25 onto itself and onto other proteins had to have at least 2 moles of Pfs25 per carrier molecule. Conjugates prepared by the same method with molecular masses between 100 and 300 kDa (Conjugate 14) and >300 kDa (Conjugate 15) induced similar levels of antibodies (Table 1).

Dosage Effect. Two dosages of several Pfs25 conjugates were tested in mice (Table 2). There were no statistically significant differences in the antibody levels between the dosage of 2.5 and 10 μg per mouse per injection.

Duration of Pfs25 Conjugates-Induced Antibodies. Remarkably, the levels of antibodies of mice bled 3 or 7 months after the last injection were significantly higher than those obtained 1 wk after the last injection (Table 3; 96 vs. 5 μg/ml; $P = 0.0005$; 142 vs. 36 μg/ml; $P = 0.03$; 247 vs. 5 μg/ml; $P = 0.002$).

Conjugates-Induced Mouse Anti-Pfs25 Exerted Transmission-Blocking Activity. Transmission-blocking assay was performed by using pooled sera of mice injected with Conjugates 11 (Pfs25-AH/Pfs25 F1) and 14 (rEPA/AH-Pfs25 F1) formulated with Alhydrogel. Sera from naïve mice were used to calculate the percent inhibition of oocyst count. Both pools showed significant transmission-blocking activity, demonstrated by reduction of oocyst numbers in mosquito midgut (Fig. 2). This activity correlated with serum antibody levels measured by ELISA.

Adsorption onto Aluminum Hydroxide Enhances the Immunogenicity of the Conjugates. After the third injection, Conjugate 14 induced a GM of 284 μg/ml, compared with the adsorbed Conjugate 14 that induced a GM of 1,445 μg/ml ($P < 0.001$). Similarly, Conjugate 11 alone induced a GM of 296 μg/ml, and the adsorbed Conjugate 11 induced a GM of 2,125 μg/ml ($P < 0.001$).

Discussion

The *P. falciparum* surface protein Pfs25 is expressed only on the ookinetes in the midgut of the mosquito vector. It is not encountered by the human host, and therefore human antibody response to it cannot be expected, even in endemic areas. Antibodies elicited by immunization of experimental animals with Pfs25 plus adjuvant inhibit the malarial parasite development in mosquitoes (8). Alone or adsorbed onto aluminum hydroxide, Pfs25 is poorly immunogenic in experimental animals and in humans (9, 10). A simple method was devised to increase the immunogenicity of the Pfs25 by conjugation, following our approach for the capsular polysaccharide of *Haemophilus influenzae* type b (11). This method is reproducible and clinically used, including in infants. Pfs25 conjugates may therefore be considered for clinical evaluation. Increase in immunogenicity of protein–protein conjugates was obtained by others. Polymeric preparations of the cytochrome *c* were more immunogenic in animals than the corresponding monomeric proteins (12), and an influenza hemagglutinin–diphtheria toxoid conjugate vaccine was more protective against influenza infection than the hem-

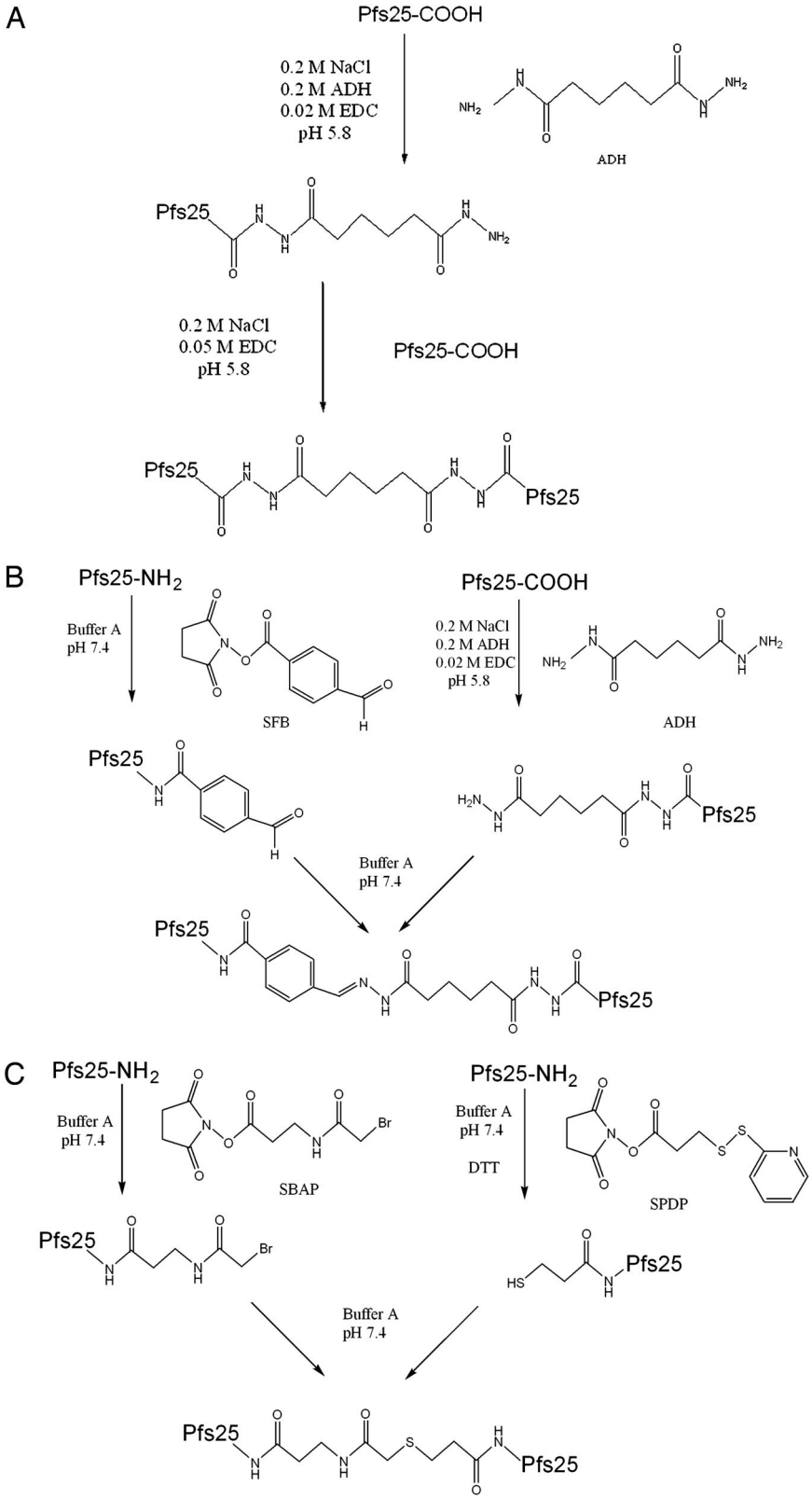

Fig. 1. Binding of Pfs25 to proteins by amide (*A*), hydrazone (*B*), and thioether linkages (*C*).

agglutinin alone (13). Consistent with our experience with polysaccharide–protein conjugates, ADH, of several other "linkers" studied, provided the most immunogenic Pfs25 conjugates (14–16).

Two variables, the conjugation method and the conjugate composition, were examined for Pfs25 conjugates prepared with the ADH linker. First, the two-step procedure, derivatization of Pfs25 with ADH, followed by binding of the Pfs25-AH to

Table 2. Dosage/immunogenicity relation of conjugates of Pfs25 bound to itself or to OVA

No.	Conjugate	Dosage, μg per mouse	GM anti-Pfs25, μg/ml	
			Second injection	Third injection
1	Pfs25-AH/Pfs25	2.5	nd	352
1	Pfs25-AH/Pfs25	10	nd	311
2	Pfs25-AH/Pfs25	2.5	nd	98
2	Pfs25-AH/Pfs25	10	nd	161
3	Pfs25-Br/SH-Pfs25	2.5	nd	88
3	Pfs25-Br/SH-Pfs25	10	nd	76
4	Pfs25-Br/SH-Pfs25	2.5	0.15	24
4	Pfs25-Br/SH-Pfs25	10	0.3	72
5	Pfs25-AH/CHO-Pfs25	2.5	0.007	12
5	Pfs25-AH/CHO-Pfs25	10	1.2	32

Five- to 6-wk-old NIH general purpose mice ($n = 10$) were injected s.c. with 2.5 or 10 μg of Pfs25 as conjugate, 2 wk apart and exsanguinated 7 d after the second or third injection. nd, not done.

nonderivatized Pfs25, resulted in more immunogenic conjugates than the one-step method. Second, the immunogenicity of conjugates prepared with rEPA or of Pfs25 linked to itself was similar. However, the simplest scheme for production and standardization renders the Pfs25 linked to itself a better choice.

Our conjugates had the following properties: conjugates of Pfs25 composed of 2–10 molecules (50–250 kDa) seemed to be best immunogens; increasing the dosage of Pfs25 conjugates from 2.5 to 10.0 μg per injection did not affect the antibody levels; adsorption of the Pfs25 conjugates onto aluminum hydroxide enhanced their immunogenicity; conjugate-induced transmission-blocking activity correlated to antibody levels measured by ELISA.

Remarkably, rather than declining, the levels of Pfs25 antibodies rose 3 and 7 months after the second and third injections, relative to levels obtained after 1 wk. What could explain this effect? The increase in molecular size of Pfs25 as a conjugate, bound to itself or to another protein, may affect its immunogenicity. Also, conjugation of Pfs25 could confer the property of a polymer, making more of the same epitopes available for interaction with lymphocytes.

Plasmodium vivax ookinete surface protein, Pvs25, an orthologue of Pfs25, was found to be safe and immunogenic and elicited transmission-blocking antibodies in adult humans when adsorbed onto aluminum hydroxide (17). The authors expressed concern that the vaccine-induced levels of anti-Pvs25H were too low for an effective vaccine and that natural boosting of these

Table 3. Serum IgG anti-Pfs25 elicited at different times after immunization by 2.5 μg of conjugate per mouse

No.	Conjugate	Time of bleeding after last injection	GM anti-Pfs25, μg/ml	
			Second injection	Third injection
6	Pfs25-AH/CHO-Pfs25	1 wk	5	36
6	Pfs25-AH/CHO-Pfs25	7 mo	96	142
12	Pfs25-AH-Pfs25 F1	1 wk	5	nd
12	Pfs25-AH-Pfs25 F1	3 mo	247	nd

Five- to 6-wk-old NIH general purpose mice ($n = 10$) were injected s.c. with 2.5 μg of Pfs25 as conjugate 2 wk apart and exsanguinated 7 d, 3 mo, or 7 mo after the second or third injection. nd, not done. Statistics: 5 vs. 96, $P = 0.0005$; 36 vs. 142, $P = 0.03$; 5 vs. 247, $P = 0.002$.

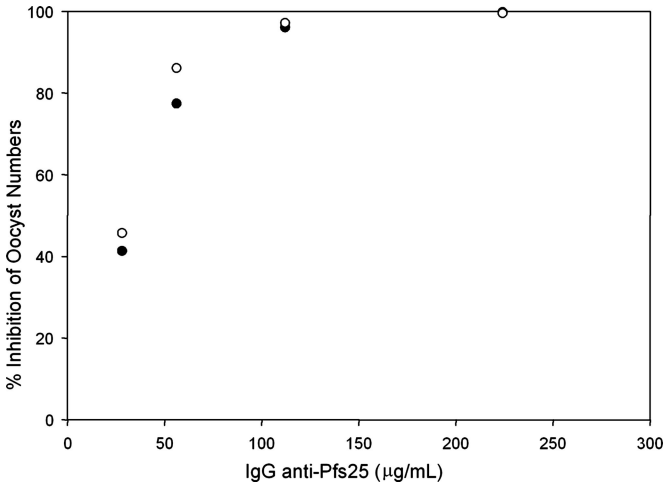

Fig. 2. Conjugates-induced transmission-blocking activity correlates with the specific IgG concentration in the assay. Two groups of mice were immunized with Pfs25-AH/Pfs25 F1 on Alhydrogel (filled circles) or rEPA/AH-Pfs25H F1 on Alhydrogel (open circles) on days 0 and 28. Antisera were obtained 2 wk after the second immunization and diluted with a naïve human serum pool to reach the desired antibody concentration. The diluted antisera and parasites were fed to mosquitoes, and the oocyst numbers in mosquito midguts were counted as an indication of the infectivity.

levels could not be expected because the antigen was not expressed on the parasite in its human host. Adaptation of our conjugation technology to Pvs25H to improve its immunogenicity should be examined.

Antibody-mediated transmission blocking has been demonstrated for another human pathogen with an insect vector. Antibodies to *Borrelia burgdorferi* induced by the OspA vaccine were active in the gut of the *Ixodes* ticks (18). The OspA protein was shown to be expressed mostly during the vector phase of the parasite, indicating that the transmission-blocking activity was induced by that vaccine.

Experimental Procedures

Analytic. Amino acid analyses were done by GLC-MS after hydrolysis with 6 N HCl at 150°C for 1 h and derivatization to heptafluorobutyryl isobutyl esters and assayed with a Hewlett-Packard apparatus (Model HP 6890; Agilent Technologies, Wilmington, DE) with a HP-5 0.32 × 30 mm glass capillary column, temperature programming at 8°C/min, from 125°C to 250°C in the electron ionization (106 eV) mode (19). Protein concentration was measured by the method of Lowry (20); ratio of Pfs25 to carrier proteins was based on amino acid analysis of the conjugate and calculated as described (21). Free ε amino groups were measured by the Fields' assay (22); thiolation was measured by the release of 2-pyridylthio groups (A_{343}) (23); incorporation of benzaldehyde groups was measured by the reaction with 2-hydrazinopyridine (24); and hydrazide was measured by the trinitrobenzesulphonic acid (TNBS) assay (11). SDS/PAGE was performed using 14% gels according to the manufacturer's instructions. Double immunodiffusion was performed in 1.0% agarose gels in PBS.

Proteins. Recombinant Pfs25 expressed in *Pichia pastoris* and rEPA from *P. aeruginosa* were prepared and characterized as described (25, 26). OVA was from Sigma, St. Louis, MO.

Immunization. Groups of 10 female NIH general purpose mice, 5- to 6-wk-old, were injected s.c. two or three times at 2-wk intervals with 2.5 μg of Pfs25 alone or as a conjugate in 0.1 ml of PBS. Mice were exsanguinated 1 wk after the last injection (27). Controls received PBS. For the transmission-blocking assay,

groups of 10 female BALB/c mice were injected s.c. two times, 4 wk apart, with 2.5 μg of Pfs25 as a conjugate formulated with aluminum hydroxide (Alhydrogel). Mice were exsanguinated 2 wk after the second injection.

Antibodies. Serum IgG antibodies were measured by ELISA (11). Ninety-six-well plates (Nunc Maxisorb, Rochester, NY) were coated with 10 μg of Pfs25/ml of PBS (determined by checkerboard titration). Plates were blocked with 0.5% BSA in PBS for 1 h at room temperature and incubated with test sera overnight, followed by phosphatase-labeled affinity-purified goat anti-mouse IgG (KPL, Gaithersburg, MD) for 4 h. A MRX reader (Dynatech, Chantilly, VA) was used to record the optical density. Antibody levels were calculated relative to a standard serum; a pool of sera with the highest antibody levels induced by Conjugate 1, containing 287 μg/ml. This concentration was assigned by comparison with affinity-purified IgG anti-Pfs25 of measured concentration. Results were computed with an ELISA data processing program provided by the Biostatistics and Information Management Branch, Centers for Disease Control, Atlanta, GA (28). A polyclonal antiserum obtained by immunizing rabbits with Pfs25 formulated with Montanide ISA720 adjuvant (SEPPIC, Paris, France) was used in the immunodiffusion assays.

Conjugation of Pfs25 to Proteins by Amide Linkages. ***Pfs25-AH/Pfs25, two-step procedure.*** Pfs25 was derivatized with ADH as described (11). The product, Pfs25-AH, contained 4.2–5.2% hydrazide. Next, equal amounts of Pfs25-AH and Pfs25 were mixed at 7.5 mg/ml each and reacted with 0.05 M 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) at pH 5.8 for 4 h at room temperature, tumbled overnight at 4–8°C, and dialyzed against PBS. This reaction mixture was then passed through a 1 × 90-cm Sepharose CL-6B column (Amersham Pharmacia, Pittsburgh, PA) in 0.2 M NaCl. Fractions reacting with anti-Pfs25 by immunodiffusion were divided into two pools according to their molecular masses, based on the column chromatography and SDS/PAGE profiles. Products: Conjugates 1 and 11, prepared by the same method at different times and containing the higher-molecular-mass fraction (F1); Conjugate 2 contained lower-molecular-mass fraction (F2) of Conjugate 1.

Pfs25-AH-Pfs25, one-step procedure. Pfs25 (15 mg/ml) was reacted with 0.006 M ADH (6% wt/wt) in the presence of 0.1 M EDC at pH 5.1 for 4 h at room temperature and tumbled overnight at 4–8°C. The reaction mixture was then treated as above. Products: Conjugates 12 and 13.

Pfs25-AH/rEPA. This conjugate was prepared in a two-step procedure as above, by using *Pfs25-A*H and rEPA at 10 mg/ml each. Products: Conjugates 14 and 15.

Conjugation of Pfs25 to Proteins by Hydrazone Linkages. ***Pfs25-AH/CHO-Pfs25.*** Pfs25 was derivatized with *N*-succinimidyl 4-formylbenzoate (Pfs25-CHO) and assayed for protein, benzaldehyde, and antigenicity as described (24). Pfs25-AH and Pfs25-CHO (10 mg each) were placed in 2 ml of Buffer A (PBS/0.1% glycerol/0.005 M EDTA, pH 7.4), reacted at pH 7.4 for 2 h, and then tumbled overnight at room temperature. The sample was then passed through a 1 × 90-cm Sepharose CL-6B column in Buffer A, and fractions reacting with anti-Pfs25 were assayed for protein concentration and molecular mass by SDS/PAGE. Products: Conjugates 6 and 8, prepared by the same method at different times.

Pfs25-CHO/AH-OVA. Derivatization of OVA to OVA-AH and Pfs25 to Pfs25-CHO and conjugation of these proteins was done as above. Products: Conjugates 5, 9, and 10.

Pfs25-AH-CHO-AH-Pfs25. Pfs25-AH was reacted with *N*-succinimidyl 4-formylbenzoate to introduce aldehyde groups onto the hydrazide groups as above for Pfs25-CHO. Pfs25-AH-CHO and Pfs25-AH (10 mg/ml each) were mixed in Buffer A at pH 7.4, tumbled overnight at 4–8°C, and passed through a Sepharose CL-6B column as above. Product: Conjugate 7.

Conjugation of Pfs25 to Proteins by Thioether Linkages. ***Pfs25-Br/SH-Pfs25.*** Pfs25 was derivatized with *N*-succinimidyl 3-(bromoacetamido) propionate and assayed for protein and antigenicity as described (Pfs25-Br) (29). Another aliquot of Pfs25 was derivatized with *N*-succinimidyl 3-(2-pyridyldithio)-propionate and assayed for protein, thiolation, and antigenicity (Pfs25-SH). Pfs25-Br and Pfs25-SH (10 mg of each) were placed in 2 ml of Buffer A and reacted at pH 7.4 for 2 h and then tumbled overnight at room temperature. The sample was then treated as above. Product: Conjugate 3.

Pfs25-SH/Br-OVA. Derivatization of OVA to OVA-Br and Pfs25 to Pfs25-SH and conjugation of these proteins was done as above. Product: Conjugate 4.

Transmission-Blocking Assay. Membrane feeding assay was performed as described (30) to evaluate the transmission-blocking activity of the conjugate-induced antisera. Briefly, sera with the highest anti-Pfs25 levels from mice immunized with Conjugates 11 (Pfs25-AH/Pfs25) and 14 (Pfs25-AH/rEPA) formulated with Alhydrogel were each pooled and heat-inactivated at 56°C for 15 min. The sera were then diluted serially with a naïve human serum pool to minimize nonspecific inhibitory effect of mouse sera. For each pool tested, a preimmune serum, diluted in parallel to the immune sera was used for baseline quantification of gametocyte. The diluted sera were mixed with a *P. falciparum* gametocyte culture (NF54 line) and fed to *Anopheles stephensi* (Nijmegen strain) through a membrane feeding apparatus. Mosquitoes were kept for 8 days to allow parasites to develop into mature oocysts. Infectivity was measured by dissecting at least 20 mosquitoes per serum sample, staining the midguts with mercurochrome (Sigma), and counting the number of oocysts per midgut. To calculate the percent inhibition, the following formula was used: $100 \times (\text{Oocyst\#}_{\text{Neg control}} - \text{Oocyst\#}_{\text{Test}})/\text{Oocyst\#}_{\text{Neg control}}$, where test and control sera were matched in serial dilution fold.

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