INFECTION AND IMMUNITY, Mar. 1997, p. 1109–1113 Vol. 65, No. 3
0019-9567/97/$04.00+0
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A Novel Malaria Protein, Pfs28, and Pfs25 Are Genetically Linked and Synergistic as Falciparum Malaria Transmission-Blocking Vaccines

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Received 14 August 1996/Returned for modification 30 September 1996/Accepted 11 December 1996

Antibodies to Pfs28 block *Plasmodium falciparum* transmission and when combined with antibodies to Pfs25 provide synergy in blocking transmission. Pfs28 and Pfs25 are immunogenic, have limited antigenic diversity, and are structurally similar and genetically linked on chromosome 10. Pfs28 may prove a useful addition to Pfs25 in an effective transmission-blocking vaccine.

Malaria causes suffering beyond comprehension: 300,000,000 clinical cases and 1.5 million to 3 million deaths per year, with 90% of the latter occurring among sub-Saharan African children less than 5 years of age (12). Given the paucity of effective control methods, we and others have pursued a novel malaria vaccine strategy: a transmission-blocking vaccine that induces antibodies which, when ingested with a blood meal, disrupt the development of the parasite in the mosquito vector (4). When a female mosquito draws a blood meal from a malaria-experienced donor, antiparasite antibodies are also ingested. These antibodies recognize proteins expressed by the parasite while it circulates in the vertebrate host, but not the new repertoire of antigens appearing on the surface of the parasite as it develops in the mosquito midgut. We and others have sought to develop a transmission-blocking vaccine directed against surface or secreted proteins from the sexual stages of the malaria parasite that occur in the mosquito midgut. Such vaccines have two distinct advantages: in addition to the absence of prior immune pressure to select for immunologically transparent antigens, these stages represent a developmental bottleneck, with only a few of the thousands of ingested parasites surviving to infect the mosquito. Rather than having to eliminate the thousands to billions of erythrocytic asexual-stage parasites circulating in the human host, a transmission-blocking vaccine will need to prevent the formation of perhaps fewer than 10 oocysts in a mosquito.

A prototype malaria transmission-blocking vaccine based on Pfs25 (5, 6), the predominant protein on the surface of the *Plasmodium falciparum* zygote, is currently in human clinical trials. Pfs25 was identified by a monoclonal antibody that, when fed to mosquitoes along with a parasitized blood meal, blocked infectivity (13). Sequence analysis of the gene encoding Pfs25 revealed that the predicted protein product comprised four epidermal growth factor (EFG)-like domains (7), which, owing to multiple disulfide bonds, has required eukaryotic expression systems to adequately recreate the reduction-sensitive epitopes necessary to elicit transmission-blocking antibodies (6). Yeast-expressed Pfs25 has elicited transmission-blocking antibodies in several species of laboratory animals, including nonhuman primates (1, 6).

Pfs25 and other Pfs25-like proteins from other *Plasmodium* species have similar structures composed of four tandem EGF-like domains presumably anchored to the parasite surface by glycosylphosphatidylinositol (4). We had observed, after sequencing the gene encoding Pgs28 (2) (a surface protein in sexual stages of the avian malaria parasite *P. gallinaceum*), that the family of EGF-like proteins in sexual-stage malaria parasites could be segregated into two subfamilies based on the amino acid spacing between cysteines, the truncation of the fourth EGF-like domain, and the temporal expression of these sexual-stage proteins by the parasites (4). We suspected that, like the avian malaria parasite, the evolutionarily related human malaria parasite, *P. falciparum*, would have two family members: Pfs25 and Pfs28.

Aligning the gene sequences of three known proteins, Pgs28 (2), Pfs25 (7), and Pfs25 (8), we identified two areas of nucleotide similarity (Fig. 1A) and synthesized degenerate PCR oligonucleotides [sense oligonucleotide 5′-GG(AT) T(AT)T (CT)TA AT(AT) (CG)AG ATG AG-3′ and antisense oligonucleotide 5′-ACT (AT)T(AG) CC(AT) ATA (AT)(AT)A CAT GA(AG) CA-3′] encompassing the observed nucleotide permutations of these three known proteins. What appeared to be a single 320-bp fragment from genomic DNA of *P. falciparum* (strain 7G8) was amplified and digested with restriction endonuclease *Nsp*7524I, which recognizes a site within the amplified portion of Pfs25. A novel band of 310 bp, in addition to the two fragments predicted to result from digestion of Pfs25, was isolated and sequenced (data not shown). The deduced amino acid sequence had 14 cysteines spaced in a manner typical of EGF-like domains. Unique oligonucleotide primers (sense oligonucleotide 5′-GAG GAC ACG TGT GGA AAG-3′ and antisense oligonucleotide 5′-CCA TAC TTA ACC ACA ATA-3′) were then used to amplify a fragment to be radiolabeled for screening a *P. falciparum* genomic DNA library for Pfs28. Southern blot analysis of restriction enzyme-digested genomic DNA revealed hybridization of the radiolabeled probe with a 1.8-kb *Eco*RI/*Nsi*I band. DNA fragments migrating between 1.5 and 2.0 kb were purified (Gene-Clean) and ligated into *Eco*RI/*Pst*I-digested pUC18. A library of approximately 10,000 colonies of electrotransformed *Escherichia coli* DH10B (Gibco-BRL, Gaithersburg, Md.) was screened by hybridization with the radiolabeled 270-bp PCR fragments.

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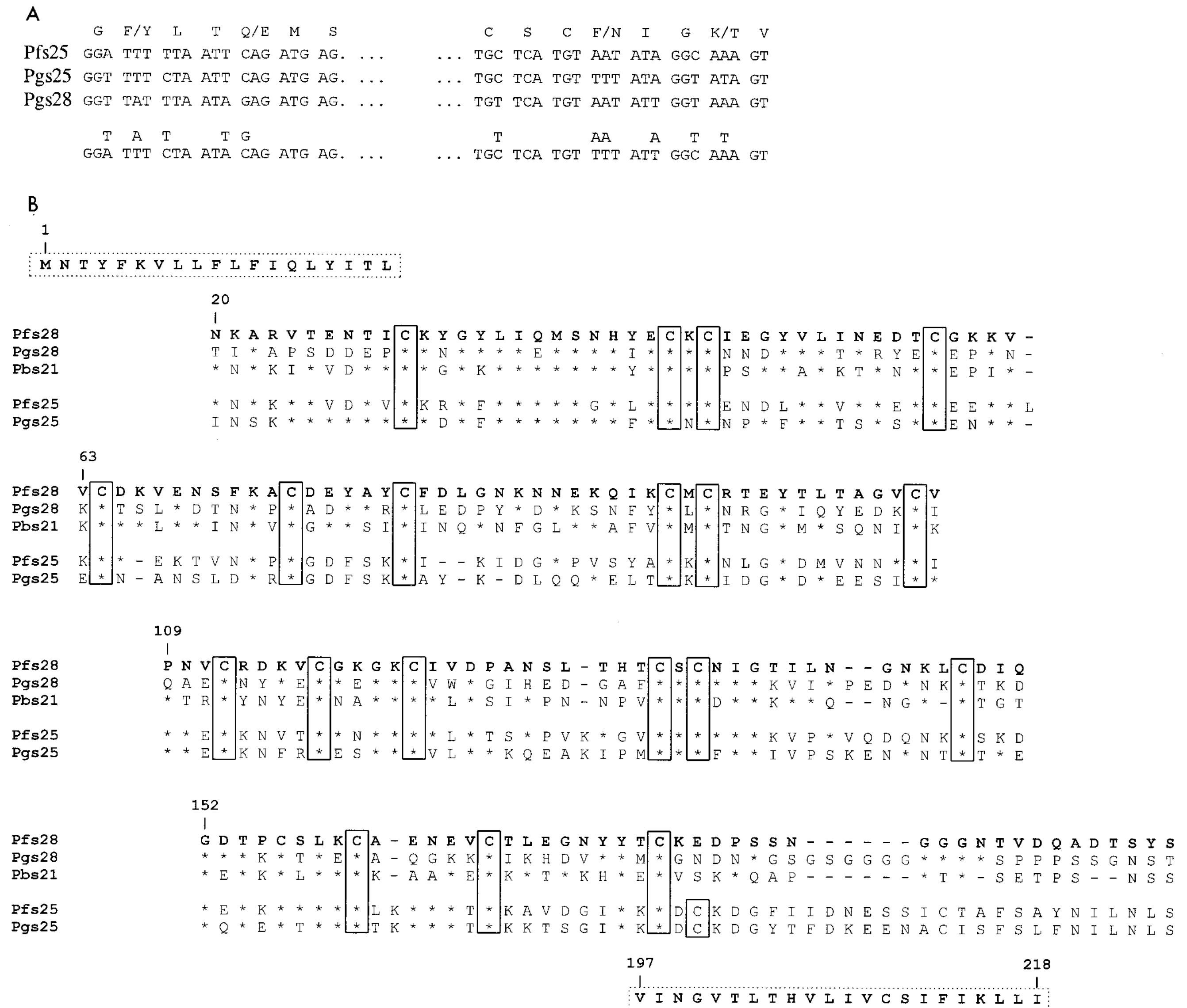

FIG. 1. (A) Nucleotide sequence alignment of Pfs25 (7), Pgs25 (8), and Pgs28 (2), used to design the degenerate oligonucleotides for amplifying Pfs28 by PCR. The numbers shown above the sequence are the nucleotide positions for Pfs25 (7). (B) Deduced amino acid sequence alignment of Pfs28 with Pfs25, Pgs25, Pbs21 (10), and Pgs28. The Pfs28 sequence (in boldface) has been arrayed in six lines, representing the signal sequence (dashed box), four EGF-like domains, and the terminal hydrophobic region (dashed box). The cysteine residues (closed boxes) that comprise the EGF-like motifs were aligned with selected segments from the amino acid sequences of other sexual-stage malaria parasite proteins that contain EGF-like domains, including Pfs25 from *P. falciparum*, Pgs25 and Pgs28 from *P. gallinaceum*, and Pbs21 from *P. berghei*. The amino acid sequence was deduced from universal codon usage and manually aligned with previously published deduced amino acid sequences.

Clone p6-1 was selected and sequenced by the dideoxynucleotide terminator method, using synthetic oligonucleotides. Analysis of the amino acid sequence (Fig. 1B) deduced from the 654-bp open reading frame of clone p6-1 (GenBank accession number L25843) revealed a presumptive secretory signal sequence of 19 amino acid residues, followed by four EGF-like domains and then a short hydrophobic region at the carboxy terminus. Thus, the structure of the presumptive Pfs28 protein was similar to those of several other sexual-stage proteins with EGF-like domains and placed it in the subfamily that includes Pgs28 and Pbs21 (10), proteins that have similar cysteine spacings, have only four cysteines in the fourth EGF-like domain, and are expressed late in sexual development.

As a preliminary study of antigenic diversity, we sequenced the Pfs28 gene from seven laboratory strains derived from three continents: Southeast Asia (Dd2, 2D11, and CAMP), West Africa (LE5 and LF4), and Central and South America (HB3 and 7G8). Fifty nanograms of genomic DNA from each of the seven strains was diluted in 25 μl of water, boiled for 2 min, and then subjected to 30 cycles of PCR amplification (94°C for 30 s, 48°C for 30 s, and 72°C for 45 s), using sense oligonucleotide Pfs28 start (5′-ATG AAT ACA TAT TTT AAG GTA CT-3′) and antisense oligonucleotide Pfs28 stop (5′-TTA TAT TAA CAA TTT AAT AAA TAT TGA-3′). The PCR products were gel purified by electrophoresis in Tris-acetate-EDTA-containing 1% agarose, ligated into pCRII, electroporated into *E. coli* DH10B, and then sequenced by the dideoxynucleotide terminator method. All contained identical nucleotide sequences for Pfs28 except for a single nucleotide substitution from A to G at nucleotide position 215. This point

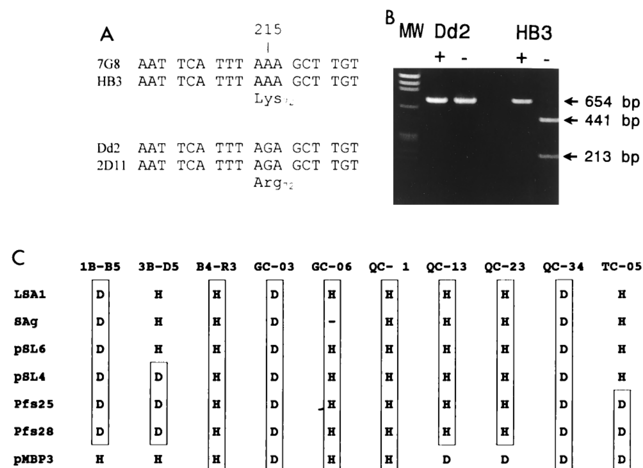

FIG. 2. Genetic analysis of Pfs28. (A) Comparison of nucleotide sequences of the Pfs28 gene PCR amplified from seven laboratory isolates revealed a single point mutation at nucleotide position 215 in *P. falciparum* Dd2 and 2D11. Shown are the Dd2 and HB3 strain sequences. No other nucleotide differences were observed, even in third base pair positions of codons. The mutation in Dd2 and 2D11 destroys a *Dra*I restriction site. (B) Ethidium bromide-stained Tris-borate-EDTA-containing 6% polyacrylamide gel of *Dra*I-digested PCR fragments amplified from genomic DNA of *P. falciparum* HB3 and Dd2. The Pfs28 gene was PCR-amplified DNA, treated (+) with *Dra*I or left untreated (−) and then subjected to gel electrophoresis. The *Dra*I-treated HB3 product migrated as two fragments, while the Dd2 product remained undigested by *Dra*I. MW, *Hae*III-digested $\phi\chi$174. (C) Genetic linkage of Pfs28 and Pfs25. The progeny of a Dd2 × HB3 cross was analyzed by *Dra*I digestion of the Pfs28 gene amplified by PCR. By restriction fragment length polymorphism analysis, the patterns of inheritance for several genes and markers on chromosome 10 (3) were compared for 10 cloned progeny. The Pfs25 and Pfs28 genes did not assort independently: each progeny inherited both genes from one parent, either Dd2 (D) or HB3 (H). None of the other markers appeared in the same linkage group as the Pfs25 and Pfs28 genes, suggesting that these two genes are genetically linked.

mutation in Dd2 and 2D11 produces a conservative amino acid change from Lys-72 to Arg-72 (Fig. 2A). The limited variability observed in the DNA sequence of Pfs28 suggests that efforts to develop it as a candidate vaccine may not require inclusion of a variety of genotypes.

Fortuitously, the nucleotide substitution also occurs at a *Dra*I endonuclease digestion site, and the parents (HB3 and Dd2) of a well-characterized genetic cross are of each genotype (Fig. 2B), offering a convenient method for rapidly genotyping the progeny of the cross. By Southern blot analysis, Pfs28, like Pfs25, mapped to chromosome 10 (data not shown). Using genomic DNA (a kind gift of Thomas Wellems, National Institute of Allergy and Infectious Diseases) from 10 progeny of the genetic cross between HB3 and Dd2, the Pfs28 gene was PCR amplified, digested with restriction endonuclease *Dra*I, size fractionated on a Tris-borate-EDTA-containing 6% polyacrylamide gel, and stained with ethidium bromide (Fig. 2C). In all 10 progeny, the genes for Pfs28 and Pfs25 were inherited from the same parent; further, none of the other chromosome 10 markers had the same inheritance pattern. The close genetic linkage of these two genes suggests that this locus may be permissive for transcription during the sexual stages of the parasite and that, in view of their amino acid homology, they have arisen as a result of gene duplication early in malaria parasite evolution, probably at or before the time the avian malaria and falciparum malaria parasites diverged from the other malaria parasite species (14).

To determine whether Pfs28 is a target of transmission-blocking antibodies, the Pfs28 gene—starting at Val-24 and ending at Pro-179 (i.e., the PCR product lacked the signal sequence, the terminal hydrophobic domain, and a portion of the fourth EGF-domain, which was PCR-amplified by using sense oligonucleotide 5′-CTT TGG ATA AAA GAG ACG CTG AAG CTT CTT TGG ATA AAA GAG TTA CTG AAA ATA CAA TAT G-3′ and antisense oligonucleotide 5′-CCA CTA GTG GTG GTG GTG GTG GTG GTG AGG ATC CTC TTT ACA TGT ATA ATA-3′ with a 3′ terminal restriction site, *Spe*I, preceded by six copies of the histidine codon)—was inserted into the *Saccharomyces cerevisiae* expression vector pIXY154 (11) (a generous gift of V. Q. Price, Immunex Corp., Seattle, Wash.) and used to transform *S. cerevisiae* 2905/6. Recombinant yPfs28 was purified from the supernatant of pH-controlled glucose-fed batch fermentation as previously described (5) and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3A).

By immunoblot analysis performed as previously described (1), sera from BALB/c mice vaccinated with this 18-kDa protein recognized a 21-kDa band in extracts of *P. falciparum* zygotes (Fig. 3B, lane b). Notably, the protein recognized as Pfs28 has a slightly greater molecular mass than that recognized by anti-Pfs25 sera (Fig. 3B, lane a), establishing that

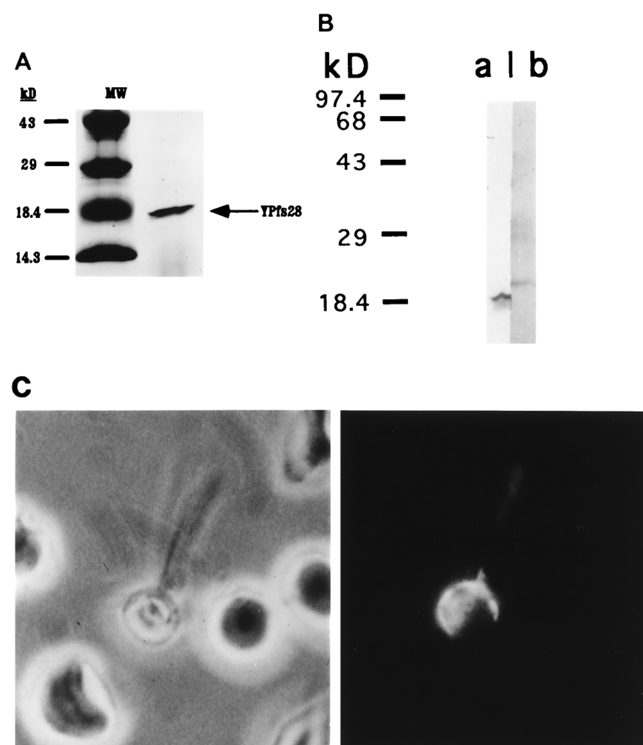

FIG. 3. Characterization of Ni-nitrilotriacetic acid-purified, yeast-secreted yPfs28 protein. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of yPfs28. In the yeast expression shuttle vector pIXY154 (11) (a generous gift of V. Q. Price), the coding sequence of Pfs28, lacking the secretory signal and anchor sequences, was fused at its 5′ end to yeast α-factor and at its 3′ end to a hexahistidine tag. The recombinant protein, yPfs28, was purified from the culture supernatant of *S. cerevisiae* by affinity chromatography using Ni-nitrilotriacetic acid (Qiagen, Chatsworth, Calif.) (5). A major single Coomassie blue-stained band that migrated with a molecular mass of 18 kDa was observed. (B) Immunoblot of *P. falciparum* gamete/zygote extract with anti-yPfs25 serum (lane a) or anti-yPfs28 serum (lane b). An immunoreactive protein migrated with a molecular mass of 21 kDa and appeared to be distinct from that of Pfs25. (C) Indirect immunofluorescence of live 24-h-old gamete/zygotes with anti-yPfs28 sera. Parasites maintained in culture for 24 h after emergence and exflagellation were assayed for surface reactivity. Shown is a retort (A, bright field; B, fluorescence microscopy).

TABLE 1. Transmission-blocking assay data

Expt	Sample[a] (adjuvant)	Sample	Dilution	Geometric mean (range)	P[b]	Infectivity (% of control)[c]	No. infected/ no. dissected[d]
A (membrane feeds with anti-yPfs28 sera)	27I94 (CFA-Ribi)	Control IID7	Neat	6.2 (2–20)			18/18
		Pfs28 IID7	Neat	0.94 (0–9)	<0.01	15	20/34
	16II94 (CFA-Ribi)	Control IIID7	Neat	0.52 (0–3)			13/28
		Pfs28 IIID7	Neat	0 (0)	<0.05	0	0/29
	1III94 (CFA-Ribi)	Control IIID7	Neat	4.1 (0–17)			26/27
		Pfs28 IIID7	Neat	0.33 (0–3)	<0.01	8.0	12/33
	24II94 (CFA-Ribi)	Control IIID7	Neat	2.1 (0–9)			19/22
		Pfs28 IIID7	Neat	0 (0)	<0.01	0	0/21
	25XI94A (CFA-Ribi)	Control	Neat	7.5 (3–24)			13/13
		Pfs28IIID174	Neat	0.15 (0–1)	<0.01	2.0	3/15
	25XI94B (alum)	Control IIID7	Neat	7.0 (2–16)			20/20
		Pfs28 IIID7	Neat	0.21 (0–2)	<0.01	3.0	4/20
B (membrane feeds with a mixture of anti-yPfs28 and anti-yPfs25 sera)	16II94 (CFA-Ribi)	Pfs25 IIID7	1:40	0.32 (0–3)		61.5	8/24
		Pfs28 IIID7	1:40	0.30 (0–2)		57.7	8/26
		Pfs25+Pfs28 IIID7	1:40 (each)	0.047 (0–1)	<0.05	9.0	2/30
	24II94 (CFA-Ribi)	Pfs25 IIID7	1:20	1.39 (0–8)		66.1	14/22
		Pfs28 IIID7	1:20	0.93 (0–5)		44.3	11/20
		Pfs25+Pfs28 IIID7	1:40 (each)	0.16 (0–3)	<0.02	7.6	4/23
	17III94 (CFA-Ribi)	Pfs25 IIID7	1:20	7.1 (0–30)		50.7	19/21
		Pfs28 IIID7	1:20	7.4 (0–17)		52.9	21/22
		Pfs25+28	1:40 (each)	3.3 (0–14)		23.6	21/24
		Normal human sera	Neat	14.0 (1–32)	<0.01		30/30

[a] Each sample is identified by vaccination number (roman numeral) and the number of days (D) postvaccination (arabic numerals). Adjuvants were complete Freund's adjuvant for the first vaccination and Ribi adjuvant for the second vaccination (CFA-Ribi) or alum for all vaccinations.
[b] Determined by Wilcoxon rank sum analysis.
[c] Calculated as the geometric mean oocyst count of the test group divided by the mean oocyst count of the control group fed at the same time × 100.
[d] Number of infected mosquito midguts (one or more oocysts)/the number of total mosquitoes dissected.

Pfs28 is expressed during the sexual stages as an antigen distinct from Pfs25.

Antiserum against yPfs28 was also used to immunostain live *P. falciparum* parasites. Twenty-four-hour-old *P. falciparum* zygotes were incubated for 30 min in mouse antiserum diluted 1:20 in phosphate-buffered saline (PBS). After being washed five times with PBS, the parasites were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse sera for 30 min, again washed five times with PBS, and examined by fluorescence microscopy. Immunofluorescence assays with control sera (Table 1) produced no detectable signal at similar dilutions (data not shown). The indirect immunofluorescent reactivity suggests that Pfs28 resides on the surface of retorts (Fig. 3C), a transitional stage between zygote and ookinete, with little or no fluorescence on gametes and untransformed zygotes and no fluorescence apparent on gametocytes and uninfected erythrocytes (data not shown).

Pooled sera after vaccination with adjuvant alone (control) or with yPfs28 (complete Freund's adjuvant used for the first vaccination and Ribi adjuvant used for subsequent vaccinations or alum used for all vaccinations) or yPfs25 were mixed with in vitro-cultured *P. falciparum* gametocytes and fed to starved female *Anopheles freeborni* mosquitoes. Mosquitoes were dissected after 6 to 8 days, stained with mercurochrome, and scored for oocysts (9). Antisera against yPfs28, collected 7 and even 174 days after the third vaccination, blocked infectivity of the parasites (Table 1, experiment A). Antisera collected 7 days after the second vaccination suppressed but did not complete block transmission (Table 1, experiment A). Alum, a delivery system suitable for use in humans, elicited nearly complete transmission-blocking activity after three vaccinations. This potent transmission-blocking activity suggested that the conformational epitopes that may have been required to elicit blocking antibodies had been re-created, at least in part, by yeast expression. To determine whether these blocking antibodies acted synergistically with blocking antibodies elicited by vaccination with yPfs25, sera against yPfs28 and yPfs25 were added—either individually as a 1:20 or 1:40 dilution or mixed together with each at a 1:40 dilution—to parasitized erythrocytes and fed to mosquitoes. In three separate feeds, the number of oocysts that developed in mosquitoes fed a mixture of antisera was significantly lower than the number in those fed either diluted antisera alone (Table 1, experiment B). These data suggest that the combination of antisera was synergistic and are consistent with our previous data that suggest that targeting multiple phases of development enhances the effectiveness of transmission-blocking vaccines (2, 6).

Pfs28 is a newly identified *P. falciparum* target antigen of transmission-blocking antibodies. The combination of antibodies to Pfs28 with those to Pfs25 substantially increases the efficacy of either alone and provides a scientific basis for combining the two antigens and perhaps others in a single transmission-blocking vaccine. An effective transmission-blocking vaccine could be administered to immunologically quarantined individuals who travel from a malaria-endemic area to a nonendemic area or from a region with drug-resistant parasites to a region not yet affected. The greater hope for this vaccine is to combine it with other modalities such as bed nets, protective vaccines, and vector control programs to control or, eventually, eradicate malaria in entire populations. Recent analyses suggest that such an intervention may be feasible even in areas with apparently intense transmission (3). The development of a high-potency transmission-blocking vaccine through the combination of antigens would be an important addition to our rapidly dwindling armamentarium against malaria parasites.

We thank D. B. Keister and O. Murtova for expert technical assistance with membrane feeding assays and D. Seeley and A. Laughinghouse for expert technical assistance with mosquito husbandry.

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Editor: J. M. Mansfield