A bicomponent *Plasmodium falciparum* investigational vaccine composed of protein-peptide conjugates

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There is yet no licensed vaccine against malaria, a serious human disease affecting mostly children, with an annual death rate of about one million. *Plasmodia*, the malaria-causing parasites, have two obligatory hosts: mammals or birds, in which they multiply asexually, and mosquitoes with sexual multiplication. The most common and serious type of malaria is caused by *Plasmodium falciparum*. The circumsporozoite protein (CSP), a major surface antigen of sporozoites, is a protective antigen. A unique feature of *P. falciparum* CSP is its large central domain composed of over 30 tetrapeptide repeats of Asn-Ala-Asn-Pro (NANP). Several NANP peptide-protein conjugates were tested clinically but elicited a low level of CSP antibodies for a short duration. To provide a CSP-based candidate vaccine, we investigated recombinant CSP and NANP conjugates of various peptide lengths, with different N-terminal amino acids, bound at different ratios to various carrier proteins. Injected into mice, CSP alone and CSP or NANP conjugates induced antibodies with booster responses and were positive by the sporozoite imunofluorescent assay. The use of the mosquito stage *P. falciparum* ookinete surface protein, Pfs25, cross-linked onto itself as a carrier for NANP, induced in mice high levels of uniquely long-lasting antibodies to both vaccine components with secondary biological activities, that will provide immunity to liver infection by sporozoites and block transmission by mosquitoes.

malaria | circumsporozoite protein | Asn-Ala-Asn-Pro | *Plasmodium falciparum* ookinete surface protein 25

Malaria is a common serious disease causing ≈300 million cases annually, mostly in children (1, 2). *Plasmodium falciparum* causes the most severe form of the disease (3, 4). Infection begins when the malaria sporozoites are injected by a mosquito into the bloodstream of a host. Within about 20 min, the parasites localize in hepatocytes, where they multiply and differentiate into the next stage of merozoites. Immunity directed against the sporozoite has the benefit of killing the inoculum before it replicates many thousand-fold, but requires fast neutralization before the organisms reach the liver. Extensive vaccine development was directed to provide mosquito and human parasite-stage vaccines using sporozoite, pre-erythrocytic, and erythrocytic antigens (5). The circumsporozoite protein (CSP) and its central repeat region are the primary targets of protective immune responses (see ref. 6 for review). Vaccine-induced immunity, however, resulted in low-level antibodies and was of short duration in both malaria-naïve volunteers and in people living in malaria-endemic areas (7, 8). We described a mosquito transmission-blocking vaccine that induced high and long-lasting antibody levels in mice (9). A clinical lot of such vaccine will be studied soon in healthy volunteers. Now we report a candidate vaccine directed to parasite antigens expressed in mosquito and in early human stages. The synthesis, characterization, and immunologic properties of this immunogen are described.

Results

Characterization of Asn-Ala-Asn-Pro Peptide Conjugates. Synthetic tetrapeptides of four or five Asn-Ala-Asn-Pro (NANP) repeats of *P. falciparum* CSP were bound to carrier proteins: ovalbumin (Ova), tetanus toxoid (TT), BSA, recombinant circumsporozoite protein (rCSP), *P. falciparum* ookinete surface protein 25 (Pfs25), or Pfs25-AH-Pfs25 at different densities. Most conjugates used thioether linkages between the terminal cysteine on the carboxyl end of the peptides and bromoacetyl groups on derivatized proteins. One conjugate was prepared by formation of hydrazone linkages between adipic acid hydrizide at the carboxyl end of the peptide and aldehyde groups on TT derivatized with succinimidylformylbenzoate. Another peptide, containing a T-cell epitope (see *Materials and Methods*) in addition to four repeats of NANP, was bound to BSA by thioether linkages. The number of carrier-bound peptide chains was assessed by MALDI-TOF and GLC-MS, the results of which corroborated each other and are presented in Tables 1 to 5. Conjugates of TT and Pfs25-AH-Pfs25 were either too big or too heterogeneous in size to be assayed by MALDI-TOF. Therefore, for these conjugates only SDS/PAGE and amino acid analyses were used for characterization and calculation of the protein:peptide ratios.

Serum IgG Anti-CSP Induced by NANP Peptide Conjugates. All conjugates were immunogenic and induced booster responses. Several variables affected the immunogenicity of the conjugates:

Effect of carrier protein and type of linkage. One week after three injections, IgG anti-CSP levels were similar among the most immunogenic conjugates irrespective of the carrier used: Ova, BSA, TT, rCSP, or Pfs25-AH-Pfs25 (see Tables 1, 5, and 6). Peptides bound to monomeric Pfs25 did not induce anti-CSP (see Table 5). Hydrazone linkages incorporated more chains per TT than thioether linkages, but there was no statistical difference in anti-CSP induced by the two (see Table 1). Therefore, we used the simpler method of forming thioether bonds between the terminal cysteine on the peptide and the bromoacetyl groups on derivatized proteins for further studies.

Effect of peptide length. The immunogenicity of conjugates containing four or five NANP repeats was not significantly different when all other variables, like carrier, density, and end group were similar (see Tables 1 and 2; conjugates no. **2–4** vs. **10–12**). The most immunogenic conjugate of four NANP repeats induced

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Table 1. Composition and serum GM IgG anti-CSP elicited in mice by NANP conjugated to Ova and TT and BSA

Conj. no.	Conjugate	Chains/ protein (avg.)	Protein: NANP ratio (wt:wt)	IgG anti-CSP, EU
1	$NANP_4$-C/Ova	6	1:0.2	89
2	$NANP_4$-C/BSA	8	1:0.2	112
3	$NANP_4$-C/BSA	15	1:0.4	58
4	$NANP_4$-C/BSA	22	1:0.6	15
5	$NANP_4$-C/TT	30	1:0.3	132
6	$NANP_5$-AH/TT	48	1:0.7	90

Mice were injected three times, at 2-week intervals with 2.5 μg of peptide as a conjugate, and bled 1 week after the last injection: 112 vs. 58, $P = 0.05$; 112 vs. 15, $P < 0.001$.

anti-CSP of 112 ELISA units (EU), and of five NANP repeats induced 91 EU, P = not significant (NS).

Effect of peptide density. In general, about 10 chains per carrier molecule was optimal; higher and lower densities induced lower antibody levels. For example, $NANP_5$-C/BSA (C = Cys) with 12 chains induced higher antibody levels than with 19 or 8 chains and $PNAN_5$-C/BSA with 11 chains was better than with 23 or 8 chains (see Table 2).

Effect of the N-terminal amino acid. We tested four different sequences: ANPN, NANP, PNAN, and NPNA of the same length, bound to the same carrier at similar densities. The highest geometric means anti-CSP levels were elicited by peptides with Asn at their N-terminal: $NANP_5$-C/BSA with 12 chains per BSA elicited 91 EU, and $NPNA_5$-C/BSA with 14 and 8 chains per BSA elicited 188 and 90 EU, respectively (see Table 2). There was no statistical difference between NANP and NPNA conjugates. Ala at the N-teminal was the least immunogenic (8 EU).

Dosage. A dosage-response curve showed a narrow optimal range, with 2.5 μg being most immunogenic (117 EU) and half or double that dosage inducing significantly lower antibody levels (1.25 μg: 28 EU; 5 μg: 41 EU) (see Table 3).

Effect of a T-Cell Epitope. Conjugates containing the T-cell epitope sequence of CSP (10) plus four repeats of NANP had no advantage over similar conjugates without the T-cell epitope (see Table 4). The geometric means (GM) IgG anti-CSP levels measured 1 week after the third injection of conjugates containing the T-cell epitope were significantly lower than those induced by NANP-alone conjugates (71 vs. 152 EU) and of shorter duration (12 vs. 109 EU).

Table 3. Dosage/immunogenicity relation of $NPNA_5$ conjugates bound to BSA

Conj no.	Conjugate	Chains/BSA (avg.)	Dose per mouse, μg	IgG anti-CSP, EU
17	$NPNA_5$-C/BSA	8	5.0	41
17	$NPNA_5$-C/BSA	8	2.5	117
17	$NPNA_5$-C/BSA	8	1.25	28
17	$NPNA_5$-C/BSA	8	0.625	0.7

Mice were injected three times, at 2-week intervals with 2.5 μg of peptide as a conjugate, and bled 1 week after the last injection:117 vs. 41, $P = 0.04$; 117 vs. 28, $P = 0.037$; 117 vs. 0.7, $P < 0.001$; 41 vs. 0.7, $P = 0.003$; 28 vs. 0.07, $P = 0.025$.

Serum IgG Anti-CSP Induced by rCSP and Its Conjugates. Our rCSP construct contained 21 consecutive repeats of NANP, had molecular mass of 35.5 kDa by MALDI-TOF, showed a single band on SDS/PAGE in the correct mass range, and precipitated anti-CSP by immunodiffusion. IgG anti-CSP levels induced by rCSP were similar to those achieved by peptides bound to carrier proteins. Cross-linking of rCSP, binding additional NPNA peptides or conjugating it to Pfs25 or Pfs25-AH-Pfs25, did not improve its immunogenicity (see Table 6).

Long-Term Antibody Levels Elicited by $NANP_5$/Pfs25-AH-Pfs25 Conjugates. Pfs25-AH-Pfs25 was prepared using a one-step procedure (9), resulting in conjugates of 50 to 200 kDa as measured by SDS/PAGE. Pfs25-AH/Pfs25 was prepared using a two-step procedure (9), resulting in conjugates of 50 to >250 kDa; about one-third of the preparation did not enter the gel. The later conjugates were too big for binding to additional antigens, therefore only lower molecular mass conjugates of a single-step procedure were used as carriers for NANP.

Pfs25-AH/Pfs25 conjugates showed the unique property of increasing antibody levels up to at least 3 months after the last injection (Table 7). At the ninth month, antibody levels started to decline. The increase in antibody levels to Pfs25-AH-Pfs25 with time was also found for $NANP_5$-C/Pfs25-AH-Pfs25 conjugates and was directed to both vaccine components (see Table 5).

Alum adsorption also increased antibody levels to both components. The IgG anti-Pfs25 elicited by alum-adsorbed conjugates rose from 87 μg/mL at 1 week to 524 μg/mL at 3 months after the second injection, and anti-CSP rose from 53 to 120 EU. The continued antibody rise with time was not observed with other carriers like TT or BSA, nor with unconjugated Pfs25 (see Table 5).

Table 2. Composition and serum GM IgG anti-CSP elicited in mice by ANPN, NANP, PNAN, and NPNA repeats conjugated to BSA at different densities

Conj. no.	Conjugate	End amino acid	Mol. mass of conjugate, kDa	Chains/BSA (avg.)	IgG anti-CSP, EU
7	$ANPN_5$-C/BSA	Ala	118	23	8
8	$ANPN_5$-C/BSA	Ala	97	12	6
9	$ANPN_5$-C/BSA	Ala	90	8	1
10	$NANP_5$-C/BSA	Asn	112	19	34
11	$NANP_5$-C/BSA	Asn	98	12	91
12	$NANP_5$-C/BSA	Asn	90	8	56
13	$PNAN_5$-C/BSA	Pro	118	23	5
14	$PNAN_5$-C/BSA	Pro	95	11	27
15	$PNAN_5$-C/BSA	Pro	90	8	7
16	$NPNA_5$-C/BSA	Asn	101	14	188
17	$NPNA_5$-C/BSA	Asn	90	8	90

Mice were injected three times, at 2-week intervals with 2.5 μg of peptide as a conjugate, and bled 1 week after the last injection: 8 vs. 91, $P = 0.02$; 8 vs. 27, P = NS; 8 vs. 188, $P < 0.001$; 91 vs. 27, $P = 0.04$; 91 vs. 188, P = NS; 27 vs. 188 EU, $P = 0.001$.

Table 4. Effect of T-cell epitope on antibody levels iduced in mice by NANP and NANP T-cell epitope conjugates

Conj. no.	Conjugate	Chains/BSA (avg.)	Bleeding after last injection	IgG anti-CSP, EU: Second inj.	IgG anti-CSP, EU: Third inj.
4	$NANP_4$-C/BSA	8	1 week	16	152
4	$NANP_4$-C/BSA	8	3 months	14	109
18	$NANP_4$-Tcell-C/BSA	6	1 week	10	71
18	$NANP_4$-Tcell-C/BSA	6	3 months	5	12

Mice were injected two or three times, at 2-week intervals with 2.5-μg of peptide as a conjugate, and bled 1 week after the last injection: 152 vs. 71, $P = 0.01$; 109 vs. 12, $P < 0.001$; 71 vs. 12, $P = 0.001$.

Table 6. Composition and serum GM IgG anti-CSP (ELISA) elicited in mice by rCSP and rCSP-conjugates

Conj.no.	Immunogen	Dosage, μg	Bleeding after last injection	IgG anti-CSP, EU
	rCSP	2.5	1 week	99
19	rCSP-AH-CSP	2.5	1 week	72
20	$NPNA_5$-C/rCSP	2.5	1 week	116
21	rCSP/AH-Pfs25	5	1 week	39
22	rCSP-AH/Pfs-AH-Pfs	5	1 week	81
22	rCSP-AH/Pfs-AH-Pfs	5	3 months	67

Mice were injected three times, at 2-week intervals with 2.5 or 5 μg of the immunogen, and bled 1 week or 3 months after the last injection.

Sporozoite Immunofluorescence and Transmission-Blocking Assays. $NANP_5$-C/BSA conjugates induced high immunofluorescence antibody assay (IFA) titers that correlated with the antibody levels measured by ELISA. $NANP_5$-C/Pfs25-AH-Pfs25 conjugates induced both transmission-blocking and IFA-reactive antibodies (Table 8). The correlation coefficient between CSP antibody levels measured by ELISA and sporozoite IFA titers of all formulations tested was 0.87, $P < 0.0001$ and between Pfs25 ELISA measured antibody levels and oocyst reduction was 0.91, $P = 0.002$.

Discussion

P. falciparum malaria exerts considerable morbidity and mortality in Asia, Central and South America, and Africa (11, 12). There is still no licensed vaccine against this disease, although experimental vaccines continue to be studied by many laboratories (5). Our approach is to design immunogens directed to the pre-erythrocytic and the sexual parasite stages: sporozoites and ookinetes. We used synthetic peptides of four or five repeats of NANP, derived from the large central domain of CSP, a major surface antigen of sporozoites, and a recombinant CSP. We evaluated the immunogenicity of conjugates differing in their peptide lengths, N-terminal amino acid, peptide density, and carrier protein. All conjugates were immunogenic and induced booster responses. The number of peptide chains per carrier molecule, the N-terminal amino acid, and the dosage were important variables; there was no difference between peptide lengths of four or five NANP repeats and between the carriers used, assayed 1 week after the last injection. The optimal immunogen contained an average of 10 peptide chains per carrier and a terminal Asn (NANP or NPNA); the optimal dose was 2.5 μg of peptide per mouse. The addition of the CSP T-cell epitope to the NANP repeats did not enhance antibody responses or their longevity. The rCSP alone or conjugated onto itself or to NPNA induced similar antibody levels to those of the synthetic peptide conjugates. We chose synthetic peptides for further studies because of their ease of preparation and standardization.

Antibodies induced by previously described NANP conjugates, as well as our preparations of NANP bound to BSA or to TT, declined with time (6, 13). We have described a mosquito transmission blocking investigational vaccine composed of Pfs25, bound onto itself, which elicited high and uniquely long-lasting antibody levels in mice. Antibody levels continued to rise for about 7 months, and started to decline at 9 months (9). We now attached chemically the two antigens, NANP and Pfs25-AH-Pfs25, to make one immunogen and showed that the Pfs25-AH-Pfs25 retained its long-lasting immunogenicity and conferred this property to NANP. Adsorption onto alum further enhanced antibody levels. Antibodies had transmission-blocking activity and bound to sporozoites in IFA. This immunogen is simple, easy to synthesize and standardize, injected at a tenth of estimated human dose, and formulated only with alum, a safe and routinely administered adjuvant. By inducing long-lived antibodies blocking malaria transmission and preventing liver infection, it could

Table 5. Serum IgG induced by the conjugate of sporozoite peptide (NANP) bound to the surface protein conjugate of *P. falciparum* surface protein (Pfs25), BSA, and TT

Conj. no.	Conjugate	Chains/Pfs25 (avg.)	Time of bleeding after last injection	IgG anti-Pfs25, μg/mL: Second inj.	IgG anti-Pfs25, μg/mL: Third inj.	IgG anti-CSP, EU: Second inj.	IgG anti-CSP, EU: Third inj.
24	$NANP_4$-C/Pfs25-AH-Pfs25	9	1 week	0.4	4	7	74
25	$NANP_5$-C/Pfs25-AH-Pfs25	4	1 week	–	21	–	23
25	$NANP_5$-C/Pfs25-AH-$Pfs25_{alum\ ads.}$	4	1 week	6	180	7	110
26	$NANP_5$-C/Pfs25-AH-Pfs25	5	1 week	1	41	3	32
26	$NANP_5$-C/Pfs25-AH-$Pfs25_{alum\ ads.}$	5	1 week	87	321	53	165
26	$NANP_5$-C/Pfs25-AH-$Pfs25_{alum\ ads.}$	5	3 months	524	–	120	–
27	$NPNA_5$-C/$Pfs25_{alum\ ads.}$	5	1 week	5	21	0.2	0.2
27	$NPNA_5$-C/$Pfs25_{alum\ ads.}$	5	3 months	7	31	0.2	0.7
4	$NANP_4$-C/BSA	8	1 week	–	–	16	152
4	$NANP_4$-C/BSA	8	3 months	–	–	14	109
5	$NANP_4$-C/TT	30	1 week	–	–	–	132
5	$NANP_4$-C/TT	30	3 months	–	–	–	22

21 vs. 180, $P = 0.01$; 23 vs. 110, $P = 0.01$; 1 vs. 87 and 3 vs. 53, $P < 0.001$; 87 vs. 524, $P = 0.004$; 53 vs. 120, $P = 0.03$; 132 vs. 22, $P = 0.01$.

Table 7. Serum IgG anti-Pfs25 elicited by conjugate in mice by Pfs25 conjugates and bled in different times after last injection

Conj. no.	Conjugate	Time of bleeding after the last injection	GM anti-Pfs25, μg/mL	
			Second inj.	Third inj.
23	Pfs-AH/Pfs	1 week	39	439
23	Pfs-AH/Pfs	1 month	89	574
23	Pfs-AH/Pfs	3 months	314	826
23	Pfs-AH/Pfs	9 months	150	225
23	Pfs-AH/$Pfs_{alum\ ads.}$	1 week	239	–
23	Pfs-AH/$Pfs_{alum\ ads.}$	3 months	1174	–
23	Pfs-AH/$Pfs_{alum\ ads.}$	9 months	314	–

Mice were injected two or three times, at 2-week intervals with 2.5 μg of Pfs25-AH/-Pfs25 conjugate alone or alum adsorbed, and bled at different times after the last injections: 39 vs. 89, $P = 0.02$, vs. 314, $P < 0.001$, vs. 150, $P = 0.006$; 826 vs. 225, $P = 0.02$; 239 vs. 1174, $P < 0.001$; 314 vs. 1174, $P < 0.001$; 39 vs. 239, $P < 0.001$; 314 vs. 1174, $P < 0.001$; 150 vs. 314, $P = 0.04$.

provide both community and individually based protection. We plan its clinical evaluation.

Materials and Methods

Analytic. GLC-MS was used for amino acid analyses of carrier proteins or their peptide conjugates. Samples were hydrolyzed with 6 N HCl, 150°C for 1 h, followed by derivatization to heptafluorobutyryl R-(-)isobutyl esters and assayed with a Hewlett-Packard apparatus (Model HP 6890) with a HP-5 0.32 × 30-mm glass capillary column, temperature programming at 8°C/min, from 125 to 250°C in the electron ionization (106 eV) mode (14). The number of peptide chains bound to the protein was calculated by the increase in total asparagine relative to glutamine, the last being a component of the carrier protein only. Protein concentration was measured by the method of Lowry et al. (15) and incorporation of benzaldehyde groups by the reaction with 2-hydrazinopyride according to manufacture protocol (Solulink). SDS/PAGE used Tris-Glycine 14% gels according to the manufacturer's instructions. Double immunodiffusion was performed in 1% agarose gel in PBS. MALDI-TOF mass spectra were obtained with a OmniFlex MALDI-TOF instrument (Bruker Daltonics) operated in the linear mode. Samples for analysis were desalted and 1 μL mixed with 20 μL of sinnapinic acid matrix made in 30% CH_3CN and 0.1% trifluroacetic acid. Next, 1 μL of this mixture was dried on the sample stage and placed in the mass spectrometer.

Carrier Proteins. Ova and BSA were purchased from Sigma, TT was a gift from Peter Hoogerhout (NVI, Netherlands), and recombinant Pfs25 was expressed in *Pichia pastoris* and purified as described (16).

Recombinant Circumsporozoite Protein. The rCSP gene introduced into *Escherichia coli* was designed to express the mature configuration of CSP. The construct was produced using PCR amplification of plasmid pDS56-32/RBSII-CS27iVC (ATCC # MRA-272) with the N-terminal coding region reconstructed from GenBank database sequences. The GPI-anchoring domain, which interferes with immunogenicity, was deleted (17). Protein was characterized by gene sequencing, SDS/PAGE, MALDI-TOF, reaction with anti-CSP Mab 2A10 (18) in Western blotting, and precipitation with rabbit polyclonal anti-CSP in immunodiffusion. Construction, culture, and purification details will be published separately.

Peptides. Three types of malaria peptides were synthesized by AnaSpec with four or five repeats of the tetrapeptide: NANP, some with a different N-terminal amino acid. The functional group for linking to a protein was either the SH group of the C-terminal Cys or a C-terminal adipic acid hydrazide. Also tested were peptides with CSP-derived T-cell epitope, located in the C terminus of CSP, in addition to the NANP repeats (10). Their purity and authenticity were verified by GLC-MS, LC-MS, and MALDI-TOF.

Table 8. Correlation of antibody levels measured by ELISA with the IFA titers and transmission blocking measured as a percent of reduction in oocysts per mosquito

Individual serum no.	Immunogen	Chains/Pr (avg.)	Anti-CSP, EU	IFA titer	Anti-Pfs25, μg/mL	% Reduction: compared with PBS
1466B	$(NANP)_4$-C/TT	30	29	160,000	–	0
1466C	$(NANP)_4$-C/TT	30	64	160,000	–	0
1466F	$(NANP)_4$-C/TT	30	68	160,000	–	7
1766B	$(NANP)_4$-C/Pfs25-AH-Pfs25	9	204	4,000,000	7	9
1766C	$(NANP)_4$-C/Pfs25-AH-Pfs25	9	116	640,000	1	18
1766F	$(NANP)_4$-C/Pfs25-AH-Pfs25	9	196	4,000,000	76	35
1770G	$(NANP)_5$-C/Pfs25-AH-Pfs25	5	98	640,000	96	42
1791H	$(NANP)_5$-C/Pfs25-AH-$Pfs25_{alum\ ads.}$	5	123	640,000	248	80
1792F	$(NANP)_5$-C/Pfs25-AH-$Pfs25_{alum\ ads.}$	5	409	4,000,000	784	100
1764A	Pfs25-AH-Pfs25	–	–	–	316	81
1764H	Pfs25-AH-Pfs25	–	–	–	36	95
1948B	$ANPN_5$-C/BSA	23	138	4,000,000	–	–
1948C	$ANPN_5$-C/BSA	23	28	125,000	–	–
1949I	$ANPN_5$-C/BSA	12	24	250,000	–	–
1952A	$NANP_5$-C/BSA	12	91	250,000	–	–
1952B	$NANP_5$-C/BSA	12	369	4,000,000	–	–
1954E	$PNAN_5$-C/BSA	23	66	500,00	–	–
1955B	$PNAN_5$-C/BSA	11	100	500,000	–	–
1955F	$PNAN_5$-C/BSA	11	50	250,000	–	–
1957A	$NPNA_5$-C/BSA	14	479	4,000,000	–	–
1957B	$NPNA_5$-C/BSA	14	302	4,000,000	–	–
1957G	$NPNA_5$-C/BSA	14	456	2,000,000	–	–
1958A	$NPNA_5$-C/BSA	8	198	500,000	–	–
1958C	$NPNA_5$-C/BSA	8	114	1,000,000	–	–
1823B	CSP	–	65	500,000	–	–
1823H	CSP	–	31	500,000	–	–
1794A	PBS	–	0	0	0	0
1794B	PBS	–	0	0	0	0

The following abbreviations were used:

A. NAc-(Asn-Ala-Asn-Pro)$_4$-Cys-CONH$_2$ (NANP$_4$-C)
B. NAc-(Asn-Ala-Asn-Pro)$_5$-Cys-CONH$_2$ (NANP$_5$-C)
C. NAc-(Ala-Asn-Pro-Asn)$_5$-Cys-CONH$_2$ (ANPN$_5$-C)
D. NAc-(Pro-Asn-Ala-Asn-)$_5$-Cys-CONH$_2$ (PNAN$_5$-C)
E. NAc-(Asn-Pro-Asn-Ala)$_5$-Cys-CONH$_2$ (NPNA$_5$-C)
F. NAc-(Asn-Ala-Asn-Pro)$_5$- NHNH-CO-(CH$_2$)$_4$-CO-NHNH$_2$ (NANP$_5$-AH)
G. NAc-(NANP)$_4$-EYLNKIQNSLSTEWSPCSVTC-CONH$_2$ (NANP$_4$-Tcell-C)

Conjugation of Peptides A to E with TT, Ova, or BSA. To 60 mg of TT, Ova, or BSA in 1.2 mL of Buffer A (PBS, 0.1% glycerol, 5 mM EDTA, pH 7.4) 20 mg of succinimidyl 3-(bromoacetamido)propionate (SBAP) dissolved in 50 μL DMSO, was added, the pH adjusted to 7.4 with 0.2 M NaOH, and maintained at 7.4 for 2 h with mixing. The reaction mixture was passed through a Sephadex G-50 column (1 × 50 cm) in 0.2 M NaCl. Fractions containing bromoacetamidopropionyl-ε-Lys-NH-protein (Br-BSA, Br-Ova, or Br-TT) were collected and assayed for protein concentrations, antigenicity, and molecular masses.

To 5-mg Br-protein in 1.2 mL Buffer A, different amounts of peptides, dissolved in 0.2 mL of Buffer A, were added. For conjugates no. **1**, **4**, **5**, 7, **10**, **13,** and **16,** 5 mg of peptide were used; for conjugates no. **3**, **8**, **11**, **14,** and **17**, 2.5 mg of peptide was used; and for conjugates **2**, **9**, **12,** and **15,** 1.25 mg of peptide was used. Reactions were carried out at room temperature, pH 7.4 overnight. Next, the solution was passed through Sephadex G-75 column (1 × 100 cm) in 0.2 M NaCl. Fractions reacting with anticarrier and anti-CSP were pooled and assayed for peptide and protein concentrations and molecular masses.

Conjugation of Peptide F with TT. To 30-mg TT in 1.2 mL Buffer A, 7.5 mg succinimidylformylbenzoate (SFB) in 100 μL DMSO was added and reacted for 2 h at pH 7.4. The product 4-formylbenzoyl-TT (CHO-TT) was passed through a Sephadex G-50 column (1 × 50 cm) in 0.2 M NaCl, as described above. Protein-containing fractions were pooled and assayed for benzoylaldehyde contents, antigenicity, and protein concentration. Next, to 20-mg CHO-TT in 1.25 mL Buffer A, 15 mg of NANP$_5$-AH dissolved in 0.2 mL Buffer A, were added. The pH was adjusted to 7.4, the reaction mixture stirred overnight at room temperature, then passed through a Sephadex G-75 (1 × 100 cm) column, as described above. Fractions reacting with anti-TT and anti-CSP were pooled and assayed for peptide and protein concentrations and molecular mass. This conjugate was designated no. **6**.

Conjugation of Peptide G with BSA. BSA was reacted with SBAP, as described above. To 10-mg BSA-Br in 1.5 mL Buffer A, 10-mg NANP$_4$-Tcell-SH in 2 mL Buffer A were added. The pH was adjusted to 7.4 and the reaction mixture stirred overnight at room temperature. Next, the reaction-mixture was passed through a Sephadex G-75 column (1 × 100 cm), as described above, and fractions reacting with anti-BSA and anti-CSP were pooled and assayed for peptide and protein concentrations and for molecular mass. This conjugate was designated no. **18**.

Conjugation of Peptide A, B, or C with CSP, Pfs25, and Pfs25-AH-Pfs25. The rCSP-AH-rCSP and Pfs25-AH-Pfs25 conjugates were prepared as described using the one-step procedure (conjugates no. **19** and **23**) (9). To 20-mg rCSP, Pfs25, or Pfs25-AH-Pfs25 in 2 mL Buffer A, 6 mg of SBAP dissolved in 40 μL DMSO was added, pH maintained at 7.4 with 0.2 M NaOH for 2 h, and then the reaction mixture was passed through a G-50 column (1 × 50 cm) in 0.2 M NaCl. Next, to 7-mg Pfs25-AH-Pfs25-Br in 1.5 mL Buffer A, 10 mg of NANP$_4$-SH (conjugate no. **24)** or 5-mg NANP$_5$-SH in 0.1 mL Buffer A was added (conjugates no. **25** and **26**). In other experiments, to 7-mg rCSP-Br or Pfs25-Br in 1.5 mL Buffer A, 5-mg NPNA$_5$-SH in 0.1 mL Buffer A were added, (conjugates no. **20** and **27**). The pH was adjusted to 7.2 and the reaction mixture stirred overnight at room temperature, then passed through a Sephadex G-75 column as described above, and fractions reacting with anti-Pfs25 and anti-CSP were pooled and assayed, as described above.

Immunization. Five- to 6-week-old female National Institutes of Health Swiss Webster mice were injected s.c. two or three times at 2-week intervals with 2.5 μg NANP peptide as a conjugate in 0.1 mL PBS, and groups of 10 were bled at 7 days or 3 months after the second or the third injection (19). In other experiments mice were injected with 2.5 or 5 μg of rCSP alone or conjugated onto itself, to NANP peptide, to Pfs25, or to Pfs25-AH-Pfs25; the dosage was based on the total protein content (it was not possible to calculate accurately the ratio of rCSP to NANP or to Pfs25). For long-term antibody level determination, mice were injected with 2.5 μg of Pfs-AH-Pfs and assayed 1 week or up to 9 months after the last injection. For a dosage study, mice were injected with 5, 2.5, 1.25, or 0.625 μg of NANP as a conjugate. The control groups received PBS. The animal research was approved by the National Institute of Child Health and Human Development Animal Care and Use committee.

Antibodies. Serum IgG antibody levels were measured by ELISA (19). NUNC maxisorb were coated with rCSP, at 4-μg/mL PBS, or with Pfs25 at 10-μg/mL PBS. Plates were blocked with 0.5% BSA or HSA (for BSA conjugates) in PBS for 1 h at room temperature. A MRX Dynatech reader was used. Antibody levels were calculated relative to a standard; for anti-CSP, a pool of the highest level sera induced by conjugate no. **1** was assigned a value of 100 EU; for anti-Pfs25, it was a pool of sera with the highest antibody levels induced by Pfs25-AH-Pfs25 containing 287 μg/mL (8). Results were computed with an ELISA data-processing program provided by the Biostatistics and Information Management Branch, Centers for Disease Control and Prevention. Polyclonal rabbit antibodies against recombinant *P. falciparum* CSP (ATCC MRA-24 Lot #13510579) were used for immunodiffusion.

Transmission-Blocking Assay. Sera from mice injected with conjugates Pfs25-AH-Pfs25 and NANP$_{4\text{ or }5}$-C/Pfs25-AH-Pfs25 formulated with or without Alhydrogel were treated at 56 °C for 15 min. The sera were diluted 1:5.3 with naïve mouse sera. Sera from mice injected with PBS were used as control. Oocyst-inhibition analysis was performed as described (8, 20). Percent-inhibition was calculated by the following formula: %(Oocyst#$_{\text{Neg control}}$-Oocyst#$_{\text{Test}}$)/Oocyst#$_{\text{Neg control}}$.

Sporozoite IFA. *P. falciparum* sporozoite-coated slides, stored at −80C, were kept at room temperature for 20 min, fixed with formalin (20 μl per well) for 20 min, washed once with PBS, and blocked with 3% BSA in PBS for 30 min. Next, slides were incubated with sera diluted in 0.5% BSA-PBS for 1 h and washed three times with 0.5% BSA in PBS for 10 min. Next, they were incubated with secondary antibody [anti-mouse IgG-whole molecule F(ab)2 fragment-FITC, affinity isolated antibody, Sigma-F2883], diluted 1:300 in 0.5% BSA in PBS for 30 min at room temperature, and washed three times with 0.5% BSA in PBS for 10 min. Slides were mounted with the mounting medium-antifade reagent in glycerol/PBS (Molecular probe-S2828) and observed under fluorescent microscope (Nikon) (21).

Statistics. ELISA values are expressed as geometric means. Unpaired *t*-test was used to compare geometric means between different groups of mice. Spearman's rank correlation coefficient was used to describe relation between antibody levels measured by ELISA and oocyst reduction or sporozoite IFA titers.

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