Proteosome-Lipopeptide Vaccines: Enhancement of Immunogenicity for Malaria CS Peptides

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Proteosomes are hydrophobic, membranous, multimolecular preparations of meningococcal outer membrane proteins that are also B cell mitogens. These characteristics suggested that proteosomes may serve as carrier proteins and adjuvants to enhance peptide immunogenicity. Although high titers of malaria circumsporozoite (CS) antibodies protect against malaria, vaccines thus far tested in humans have been insufficiently immunogenic to be clinically useful. Here it is shown that synthetic CS peptides hydrophobically complexed to proteosomes by way of lauroyl-cysteine become highly immunogenic in mice without other adjuvants. The high titers of antibodies produced and the safety of proteosomes in humans suggest that this novel system is widely applicable for the development of peptide vaccines to protect against many diseases.

THE DEVELOPMENT OF PEPTIDE SUBunit vaccines has been hampered by the inadequate immunogenicity of small peptides and the paucity of effective carrier proteins and potent adjuvants suitable for human use (*1*). Protective epitopes have been identified in peptides from several pathogenic microorganisms. One such peptide is the tandemly repeated circumsporozoite (CS) tetrapeptide NPNA of the malaria parasite *Plasmodium falciparum* (*2*). In clinical trials, humans have been protected from experimental sporozoite-induced malaria by immunization with vaccines containing these epitopes (*3, 4*), but neither of these alum-adsorbed vaccines was sufficiently immunogenic to be suitable for general use. By linking peptides to carrier proteins that provide specific T cell help, it is possible to enhance antibody responses to peptides. Such carrier proteins are most effective when conjugated at high peptide to protein molar ratios by methods that do not alter or obscure protective peptide epitopes. The carrier protein requires careful selection because prior immunization with proteins that are routinely administered alone (such as tetanus toxoid) can be paradoxically detrimental by suppressing antibody responses to the peptide (*5*).

We have described a system for making a small cyclic peptide highly immunogenic without other adjuvants by hydrophobically complexing it to purified preparations of meningococcal outer membrane protein "proteosomes" (*6*). Protein-protein hydrophobic interactions in these proteosomes induce them to form multimolecular whole or fragmented membranous vesicles 60 to 100 nm in diameter (*7*). We postulated that because of this physical structure, proteosomes, like liposomes, would provide adjuvant activity for peptides to which they were complexed. Proteosomes may also be potential adjuvants since they are B cell mitogens even in mice that are hyporesponsive to lipopolysaccharide (LPS) (*8*). Proteosomes have been given to many people as components of group B meningococcal vaccines (*9*). In these vaccines a polysaccharide is hydrophobically complexed to the protein by way of a lipid moiety (*10*) naturally attached to the polysaccharide. We reasoned that by adding a similar lipid to malaria CS peptides they would become hydrophobically complexed to the proteins by way of the lipid, leaving the peptide unaltered and exposed as required for optimal immunogenicity.

Synthetic lipopeptides (*11*) containing the amino acid repeat sequences of the CS proteins of *P. falciparum* and *P. vivax* (Table 1) were hydrophobically complexed to proteosomes by a one-step dialysis procedure (*12*). The resultant vaccines were tested for immunogenicity in outbred ICR mice without any additional adjuvants. The data presented are from mice immunized intraperitoneally, but similar results were obtained with mice injected subcutaneously and intramuscularly. The most immunogenic *P. falciparum* vaccine consisted of LCF6 complexed to proteosomes (Fig. 1); very high immunoglobulin G (IgG) titers were induced by booster immunizations. LCF6 complexed to proteosomes prepared by the direct extraction method was especially immunogenic (Fig. 1A). Immunogenicity increased as the core CS sequence was tandemly repeated from two to six times (Fig. 1B). It is possible that repeating the epitope in the synthetic immunogen more faithfully mimics the conformation of the native protein in which the epitope is repeated some 30 times. Alternatively, if longer peptides are inherently better immunogens, including tandem repeats of any epitope being used in synthetic peptide vaccines may be a useful strategy in vaccine design.

LCF4 or LCF6 alone without proteosomes (*13*) were moderately immunogenic whereas lipopeptides without cysteine (LF6 and LF4), peptides lacking the lauroyl group (CF6 and CF4), and LCF2 were ineffective (Fig. 1C). Thus, lauroyl-CYGG added to four NPNA repeats represents a minimal configuration for generating *P. falciparum* CS antibodies in saline without adjuvants. Although LCF6 was far less immunogenic alone compared to when it was complexed to proteosomes, the magnitude of the IgG response to LCF6 without proteosomes in saline was comparable to the responses elicited by an equivalent dose of the recombinant CS protein $R32tet_{32}$ administered with alum or complete Freund's adjuvant (*14*).

Lipopeptides lacking cysteines (LF2, LF4, or LF6) were not immunogenic even when complexed to proteosomes (Fig. 1B). Therefore, adding cysteine between the lauroyl and the malaria epitope is critical for immunogenicity whether or not lipopeptides are bound to proteosomes. The cysteine, which induces dimerization of the lipopeptides, may form more stable hydrophobic bonds with the proteosomes or enhance the formation of lipopeptide micelles. Other investigators have elicited antibody

Table 1. Synthetic lipopeptides representing the repeat regions of malaria CS proteins. *P. falciparum* and *P. vivax* sequences were tandemly repeated to various lengths. The lauroyl group $[CH_3(CH)_{10}CO]$ was covalently added to each peptide's amino terminus to serve as a hydrophobic anchor that enables the lipopeptide to complex with proteosomes. The tripeptide YGG was added to each peptide as a spacer and for I^{125} tracing purposes. Each construct was synthesized and purified as described (*11*) both with and without the cysteine or the lauroyl group

Name	Sequence
	Plasmodium falciparum
LCF6	Lauroyl-C-YGG-$(NPNA)_6$
LF6	Lauroyl-YGG-$(NPNA)_6$
LCF4	Lauroyl-C-YGG-$(NPNA)_4$
LF4	Lauroyl-YGG-$(NPNA)_4$
LCF2	Lauroyl-C-YGG-$(NPNA)_2$
LF2	Lauroyl-YGG-$(NPNA)_2$
	Plasmodium vivax
LCV3	Lauroyl-C-YGG-$(GDRADGQPA)_3$
LV3	Lauroyl-YGG-$(GDRADGQPA)_3$
LCV2	Lauroyl-C-YGG-$(GDRADGQPA)_2$
LV2	Lauroyl-YGG-$(GDRADGQPA)_2$

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responses against peptides that were conjugated to dipalmitoyl-lysine without adding a cysteine or a carrier protein (*15*). Because those studies used lipopeptides administered in CFA, it is difficult to determine whether the peptides they used did not require a cysteine or whether CFA was essential for the immunogenicity they observed.

The protein content is often dose-limiting in formulating vaccines for human use. We therefore tested the immunogenicity of proteosome-LCF6 vaccines using lower doses of protein. To deliver more peptide molecules per proteosome while minimizing the amount of protein present, the ratio of LCF6 to proteosome (w:w) was increased to 9:1. This ratio was still highly immunogenic when standard (50 μg) doses of peptide were used even though the amount of protein given was reduced to 6 μg (Table 2). Moreover, suboptimal (7 μg) doses of peptide were more immunogenic in a 9:1 ratio (with only 0.8 μg of proteosomes) than in a 1:1 ratio (Table 2). Thus, increasing the density of peptide molecules per proteosome is more important than the absolute amount of proteosomes present.

Table 2. The effect of increasing the ratio of peptide to proteosome on the immunogenicity of *P. falciparum* proteosome-LCF6 vaccines when suboptimal (7 μg) or standard (50 μg) doses of peptide were used. ELISA units were calculated as described in Fig. 1 and were reproducible to within 10%.

Ratio	Peptide (μg)	Proteosome (μg)	Immunization	ELISA units
1:1	50	50	Tertiary	43,900
9:1	50	6.0	Tertiary	53,400
1:1	7.0	7.0	Primary	<2
			Secondary	100
			Tertiary	3,500
9:1	7.0	0.8	Primary	32
			Secondary	1,300
			Tertiary	25,900

Proteosomes also increased the immunogenicity of *P. vivax* lipopeptides (LCV2 and LCV3), with IgG booster responses being evident after each immunization (Fig. 2A). As with *P. falciparum*, vaccines made with lipopeptides lacking cysteine were not immunogenic and the proteosome vaccine containing the longer peptide, LCV3, was most effective. In contrast to the *P. falciparum* lipopeptides LCF4 and LCF6, the *P. vivax* lipopeptides LCV2 and LCV3 were not immunogenic in the absence of proteosomes despite their similar overall length (Fig. 2A). Hence, the ability of lauroylcysteine to make peptides immunogenic without adjuvants is a function of the amino acid sequence of the peptide, perhaps because of genetic or conformational restrictions. Proteosome-LCV3 was also immunogenic when made by lyophilizing a saline mixture of the components (Fig. 2B). Although this method was less effective than the standard dialysis method, these data show that proteosome enhancement of immunogenicity is not due to the effect of residual detergent that may persist after dialysis.

The specificity of the antibodies detected in the ELISA was confirmed by showing that antibodies induced by the *P. falciparum* or *P. vivax* vaccines were specifically inhibited from binding if the sera were incubated with homologous but not heterologous antigens (*16*). In addition, antibodies induced by the proteosome vaccines recognized native malaria sporozoites in an indirect immunofluorescence assay (IFA) to a serum dilution 1:62,500, and LCF6 alone (without proteosomes) was positive to a dilution 1:2,500. Although not specifically performed in this study, in our experience, sera with similarly high IFA titers strongly inhibit sporozoite invasion of hepatocytes in vitro. The proteosome vaccines described here are also immunogenic in rabbits, although the extremely high antibody levels observed in mice are generally not seen in that system.

This study shows that proteosomes increase the immunogenicity of small linear peptides and that such peptides can maintain sufficient antigenic structure to induce antibodies that recognize recombinant protein and intact sporozoites. Since the immune responses to *P. falciparum* and *P. vivax*

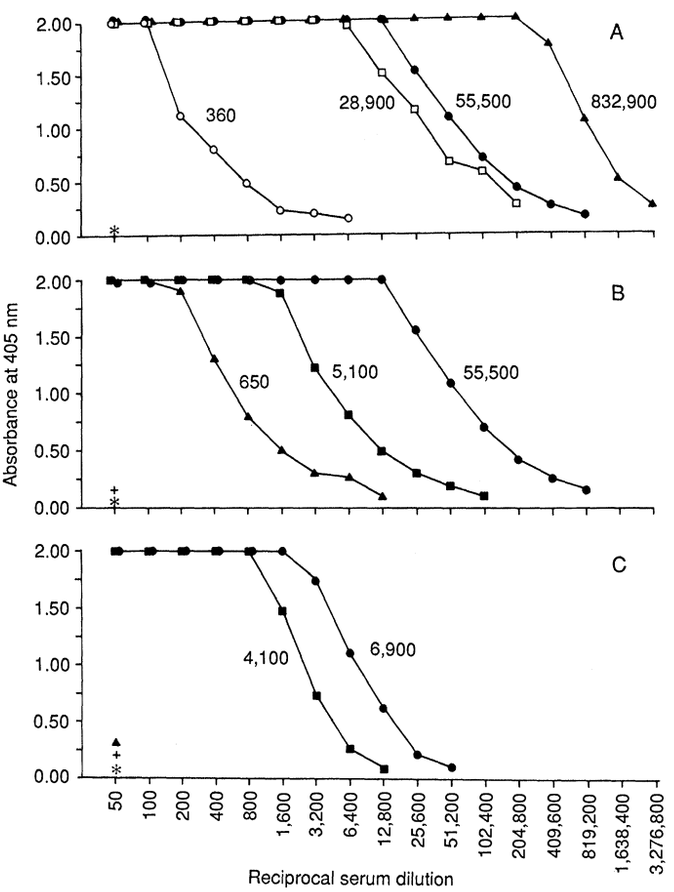

Fig. 1. Immunogenicity of *P. falciparum* vaccines. Outbred ICR mice (five per group) were immunized intraperitoneally with vaccine (50 μg of peptide per dose) on weeks 0, 3, and 7. Sera were collected 1 to 2 weeks after each immunization, pooled, and assayed for antibodies to CS proteins by ELISA (*6, 14*) with affinity-purified alkaline phosphatase–conjugated goat antibody to mouse IgG being used as the second antibody and $R32tet_{32}$ (*14*) as the detecting antigen. Titers shown next to the curves are expressed in ELISA units and were calculated by averaging the product of absorbance value at 405 nm and dilution at three points along the linear region of the titration curve and normalizing values to control sera. These calculations are reproducible to within 10%. Units represent the dilution at which the absorbance approximates 1.0; absorbance values >0.1 differed significantly from preimmunization values (which were <0.03); other investigators denote titer by this less stringent criterion (absorbance >0.1). (**A**) Proteosome-LCF6 prepared by the standard method: (○), primary response; (□), secondary response; (●), tertiary response. Proteosome-LCF6 prepared by direct extraction: (▲), tertiary response; (*), preimmunization. (**B**) Tertiary responses of *P. falciparum* proteosome vaccines containing two, four, or six tetrapeptide repeats with or without cysteines: (▲), proteosome-LCF2; (■), proteosome-LCF4; (●), proteosome-LCF6; (+), proteosome-LF6, -LF4, or -LF2; (*), preimmunization. (**C**) Tertiary responses of vaccines made without proteosomes. Lipopeptides with cysteine: (■), LCF4; (●), LCF6; (▲), LCF2; peptides without cysteine or without lauroyl: (+), LF6, LF4, CF6, CF4; (*), preimmunization.

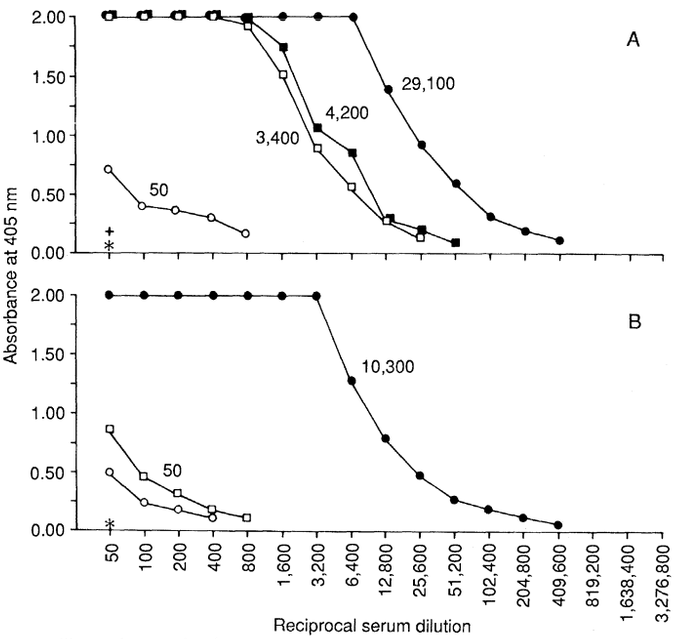

Fig. 2. Immunogenicity of *P. vivax* vaccines. ELISA were performed using $(GDRADGQPA)_2$ conjugated to casein as antigen. Data are expressed as in Fig. 1. (**A**) Proteosome-LCV3 prepared by the standard method: (○), primary response; (□), secondary response; (●), tertiary response. Proteosome-LCV2: (■), tertiary response. Proteosome-LCV3 or proteosome-LV2 (peptides without cysteines), or lipopeptides LCV3 or LCV2 alone without proteosomes: (+), tertiary responses; (*), preimmunization. (**B**) Proteosome-LCV3 prepared by lyophilization: (○), primary response; (□), secondary response; (●), tertiary response; (*), preimmunization.

CS epitopes are genetically restricted in mice (*17*), it has been proposed that vaccines should contain T helper cell epitopes from elsewhere in the CS protein to prime for antibody responses upon subsequent exposure to sporozoites (*18*). Although such synthetic T cell epitopes could also be incorporated into the proteosome system, since anamnestic responses develop over several days, it is unlikely that sporozoites-generated booster responses can be depended upon to neutralize sporozoite infectivity during the 2 to 30 minutes that elapse before mosquito-injected sporozoites leave the bloodstream and invade hepatocytes (*19*). The choice of T cell epitopes may therefore need to be based on which epitope, regardless of the source, provides high, prophylactic antibody levels that neutralize every sporozoite. Periodic booster injections may thus be required.

It has been suggested that immunity is most enhanced when a peptide is linked to both carrier protein and adjuvant in the same molecule (*20*). We speculate that proteosomes may serve as their own adjuvant since they are B cell mitogens (*8*); a positive correlation between mitogenicity and adjuvanticity has already been described for both LPS and a tripalmitoylpeptide analog of *Escherichia coli* lipoprotein (*21*). Our data, and the fact that proteosomes have been safely administered to many people (*9*) suggest that the proteosome system has potential as an important vehicle to enhance the immunogenicity of peptide vaccines in general, and of malaria vaccines in particular.

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11. Peptides were synthesized, conjugated to lauroyl, and purified by Peninsula Laboratories, Belmont, CA. The lauroyl group from lauroyl chloride was covalently conjugated to the amino terminus of the peptides in pyridine while the peptides were on the resin. Lipopeptides were purified on a C18 silica gel column and eluted as single peaks on RP-HPLC.
12. Proteosomes were prepared by extracting outer membrane vesicles of group B serotype 2b meningococci as described (*6*). LCF6 was also complexed to proteosomes prepared by a modification of the direct extract method of M. S. Blake and E. C. Gotschlich [*J. Exp. Med.* **159**, 452 (1984)]. SDS-PAGE analysis revealed three major proteins of 28, 41, and 43 kD. There was less than 1% LPS, polysaccharide, and nucleic acid contamination. Hydrophobic binding of the lipopeptides to the proteosomes was done by combining the two components in the presence of detergent and removing the detergent by dialysis (*6*). Peptide retention was 40 to 80% (determined with radiolabeled peptide), resulting in peptide to proteosome molar ratios of 5:1 to 10:1. For the experiment comparing vaccines with different protein ratios (Table 2), initial peptide to proteosome ratios were 20:1 and 2:1, which yielded vaccines with final peptide to proteosome ratios of 9:1 and 1:1 and molar ratios of 117:1 and 13:1, respectively. One preparation (LCV3 in Fig. 2B) was prepared with proteosomes precipitated and washed in ethanol, and resuspended with peptide in saline prior to lyophilization. In some experiments, preparations were sterile-filtered prior to use.
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