Brief Definitive Report

PEPTIDES BOUND TO PROTEOSOMES VIA HYDROPHOBIC FEET BECOME HIGHLY IMMUNOGENIC WITHOUT ADJUVANTS

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Small synthetic peptides offer great potential for vaccines (1, 2). Unfortunately, such peptides are frequently not immunogenic unless they are given with adjuvants and linked to carrier proteins (1–3). There is a paucity of safe adjuvants (1, 4). In addition, peptide-carrier conjugation can be problematic when amino acids essential to the antigenic determinant are obscured or altered during conjugation (2). There is also concern that conjugation to routinely used carriers such as tetanus toxoid may result in epitope suppression of antipeptide immunity (5).

We here report a new system that makes synthetic peptides highly immunogenic without additional adjuvants by hydrophobically complexing them to purified preparations of meningococcal outer membrane proteins. These proteins are highly hydrophobic, reflecting their role as transmembrane proteins and porins (6). Due to their hydrophobic protein–protein interactions, when appropriately isolated, these proteins form multimolecular structures consisting of 60–100 nm diameter whole or fragmented membranous vesicles (7). To emphasize this physical state, we refer to these proteins as proteosomes, since like liposomes, they are hydrophobic, membranous, multimolecular structures (although proteosomes prepared as described in this report may vary in size or shape from those previously pictured [7]). This liposome-like physical state suggested to us that proteosomes may not only be good protein carriers providing T cell help for peptide immunogenicity, but may also act as adjuvants. Proteosomes were also considered potential adjuvants since meningococcal outer membranes (8) and their purified proteins (9, 10) are B cell mitogens in both conventional and LPS-hyporesponsive C3H/HeJ mice (8, 9). The idea that B cell mitogenicity and adjuvanticity are related is based upon the observation that for LPS, these functions have a striking positive correlation (11).

Proteosomes have previously been used to confer immunogenicity upon meningococcal group B polysaccharide when hydrophobically complexed to it and, in the course of development of this meningococcal vaccine, many people have been safely immunized with proteosome outer membrane protein complexes (prepared by other methods [12, 13] or by a protocol similar to that described in this report [14]). Recently, in fact, proteosomes prepared by the methods used...
here were present in a vaccine that was safely administered to 20,000 individuals (our unpublished data). Since the ability of group B polysaccharide to complex and become immunogenic is dependent upon a lipid moiety that is naturally attached to it (15), we reasoned that any hydrophilic peptide could also complex to proteosomes and thereby become immunogenic if a hydrophobic foot were added to one of its ends. While the hydrophobic foot inserted into the proteosomes, the hydrophilic peptide epitope could thereby remain both unaltered and exposed as required. We hypothesized that either a lauroyl group or the pentapeptide Phe-Leu-Leu-Ala-Val (FLLAV) added to the peptide’s NH₂ terminus could serve as the hydrophobic foot.

In this report, we show that peptide vaccines made with proteosomes and either of these hydrophobic feet induce high levels of specific IgG in both conventional (BALB/c) and LPS-hyporesponsive (C3H/HeJ) mice without any additional adjuvants. The peptides we used contain an amino acid sequence identical to a conserved region of the variable surface glycoprotein (VSG) of Trypanosoma brucei (16). Inducing immunity to a conserved VSG epitope was sought because trypanosomes repeatedly change the hypervariable region of their VSG. Since proteosomes (made by any of several methods described) are safe for human use (12–14), and since peptide epitopes that are important antibody binding sites of proteins are generally hydrophilic (17), this novel system should be applicable to a wide variety of peptides that need to be made into immunogenic vaccines.

Materials and Methods

Peptides. The peptides used contained the amino acid sequence Tyr-Gly-Gly-Gly-Cys-Thr-Gln-Ile-Thr-Glu-Pro-Thr-Cys-Asn. This sequence, peptide G, was synthesized alone and with a hydrophobic pentapeptide (FLLAV) added to its NH₂ terminus using the principles of manual solid-phase synthesis (18). The lauroyl group, CH₃(CH₃)₄CO, was covalently conjugated to the NH₂ terminus of peptide G using lauroyl chloride and pyridine while the peptide was on the resin. The peptides were cycled by ferricyanide oxidation to effect disulfide bonding of the two cysteines (19). Such cyclic constraints can be antigenically important by helping maintain a peptide’s conformation (20, 21). The peptides were purified on a C18 silica gel column, eluted as single peaks on reverse-phase HPLC, and had the expected amino acid analyses. Peptide synthesis, conjugation, and purification was done by Peninsula Laboratories, Inc., Belmont, CA. The 11 COOH-terminal amino acids represented the trypanosome epitope. Tyrosine was added for ¹²⁵I tracing and two glycines were added as spacers.

Proteosomes. Outer membrane complex vesicles were extracted from group B serotype 2b meningococci as described (12), and outer membrane proteins were isolated from the other components by solubilization in a buffer with 1% zwitterionic detergent, Empigen BB (Albright and Wilson, Whitehaven, Cumbria), followed by precipitation with (NH₄)₂SO₄ three times (500 g/liter) and dialysis against 0.1% Empigen BB buffer as described (14). PAGE in SDS showed three major protein bands with molecular weights of 43, 41, and 28 × 10³. There was <1% LPS, polysaccharide, and nucleic acid.

Complexing the Peptides to the Proteosomes. Hydrophobic binding of the peptides (with either the lauroyl or the pentapeptide hydrophobic foot) to the proteosomes was done by resuspending equal amounts (by weight) of each of the components in a 1% solution of Empigen BB, buffered to pH 8.5 with Tris, EDTA, and NaCl. The components were combined to yield a final concentration of 1 mg/ml of each of the components and the mixture was exhaustively dialyzed across a 1,000 mol wt cutoff membrane against PBS, pH 8.5, for 8–10 d. Using ¹²⁵I-labeled peptide it was found that ~75% of peptide was
FIGURE 1. Immunogenicity of peptide G complexed to proteosomes via either the lauroyl or FLLAV hydrophobic foot. (a-d) BALB/c mice; (e and f) C3H/HeJ mice; (a, c, and e) peptide G complexed to proteosomes via the lauroyl foot; (b, d, and f) peptide G complexed to proteosomes via the FLLAV pentapeptide foot; (a, b, e, and f) IgG responses; (c and d) IgM responses; (O) primary response; (Δ) secondary response; (x) tertiary response; (□) 6-mo response; (*) control groups, all responses; the control groups received peptide G in either saline or CFA or mixed with proteosomes; or peptide G linked to either the lauroyl or FLLAV hydrophobic foot without proteosomes.

Results and Discussion

Immunization of BALB/c mice with peptide G hydrophobically complexed to proteosomes via either the lauroyl (Fig. 1 a) or FLLAV (Fig. 1 b) hydrophobic foot resulted in exceedingly high serum anti-peptide G IgG titers after two or three immunizations. IgM titers were much lower (Fig. 1, c and d). None of the control vaccines (see legend) induced significant antibodies. The anamnestic IgG responses indicate that proteosomes complexed to peptides hydrophobically do provide protein carrier T cell help.
The proteosome vaccines with the lauroyl foot (Fig. 1e) and the FLLAV pentapeptide foot (Fig. 1f) were also highly immunogenic in C3H/HeJ mice, which are hyporesponsive to the adjuvant effects of LPS (11). The effect of the proteosomes was therefore not due to the <1% LPS in the proteosome preparations.

In both BALB/c and C3H/HeJ mice, anti–peptide G IgG titers were high even 6 mo after immunization (Fig. 1, a, b, e, and f). The proteosome vaccines also induced high anti–meningococcal protein IgG in both mouse strains (data not shown).

Antibody binding to peptide G was specifically inhibited by homologous but not heterologous peptides; hence, the antibodies induced were specific for the immunizing peptide (Fig. 2). Notwithstanding this specificity, conserved trypanosome VSG epitopes may not be as useful as had been hoped since this part of the VSG of live trypanosomes appears to be unavailable to antibodies (23). Indeed, in fluorescent assays, monoclonal and polyclonal antibodies to several conserved VSG epitopes (including peptide G) bound to acetone-fixed but not to live trypanosomes (Hall, T., personal communication).

Dreesman et al. (21) reported that an uncoupled cyclic peptide given with CFA or alum induced antibodies detectable in 50% of murine sera diluted 1:4. Hopp (24) and Jacob et al. (25) enhanced peptide immunogenicity by immunizing with CFA emulsions of peptides conjugated to the hydrophobic moiety dipalmitoyl-lysine. In contrast, our proteosome–hydrophobic foot peptide vaccines induced high IgG titers without any adjuvant. Furthermore, without proteosomes, our cyclic peptide was not immunogenic, even when hydrophobic feet were added or when CFA was used.

These data suggest that proteosomes may serve as an adjuvant as well as a protein carrier. Such adjuvanticity may be related to proteosomes' B cell mitogenicity (9–11). A relationship between adjuvanticity and mitogenicity has been demonstrated for LPS (11) and for a tripalmitoyl-peptide analogue of Escherichia coli lipoprotein (26). Unlike LPS, proteosomes are effective in C3H/HeJ mice in vitro (8, 9) and in vivo (Fig. 1, e and f). B cell mitogens may act as adjuvants by expanding B cell clones or by providing a second signal to stimulate antibody production. Alternatively, peptides complexed to B cell mitogens may be targeted to B cells when these cells bind the mitogen via their mitogen receptors. Enhanced
immunogenicity would occur if these B cells could then, as APCs, more efficiently present the mitogen-bound peptides to helper T cells. In this regard, it has recently been shown (27) that complexing an antigen to anti-immunoglobulin can increase immunogenicity and B cell antigen processing by targeting the antigen to B cell surface immunoglobulin antigen receptors (27).

Summary

Addition of either a lauroyl or a pentapeptide (FLLAV) hydrophobic foot to the NH\textsubscript{2} terminus of a small, synthetic peptide allowed the peptide to hydrophobically complex to meningococcal outer membrane protein proteosomes by simple dialysis. Both conventional and LPS-hyporesponsive mice immunized with these complexes without any adjuvants developed high-titered and persistent anti–peptide IgG. Since proteosomes have been safely given to many people and since important antigenic determinants are generally hydrophilic, this system should be widely applicable to the development of peptide vaccines for human use.

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