LIPOPHILIC MULTIPLE ANTIGEN PEPTIDE SYSTEM FOR PEPTIDE IMMUNOGEN AND SYNTHETIC VACCINE

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Abstract—We describe the development and structural requirements of a new lipophilic multiple antigen peptide (IipoMAP) system for immunogens that contains a built-in lipophilic adjuvant and has the ability to elicit cytotoxic T-lymphocytes (CTLs). In addition to the peptide antigens of choice at the amino terminus, the basic IipoMAP design consists of three components: a tetravalent symmetrical core matrix containing two levels of branching β-alanyl-lysine as a building unit, a hydrophilic Ser-Ser dipeptide linker, and at the carboxyl terminus, palmitoyl lysines (PL) with alternating chirality. An 18-residue peptide from the third variable region in the gp120 of HIV-1 was used as antigen in eight models for a structure-function study. Alternating palmitoyl lysine (PL) was introduced as the lipid anchor and built-in adjuvant because D and L Lys (Pal) was found via molecular modeling to best mimic phosphatidylcholine and thus provide the most stable peptide antigens on the ordered lipid membranes. The requirements of the palmitoyl lysines and the L-Ser-L-Ser linker were crucial, since replacement with palmitoyl serines or L-Ser-D-Ser linkers led to a marked decrease in immune response. The stoichiometric ratio of PL vs MAP was also important. Multiple antigen peptide (MAP) constructs without the lipophilic PLs, those that were underlipidated and contained one PL, or those that were overlipidated containing four PLs, were ineffective. LipoMAPS containing three palmitic acids elicited significant humoral responses in oil-based emulsion and liposomes, but not in water or alum formulations. LipoMAP containing only two PLs was found best to be incorporated in liposomes and elicited a significant immune response and cytotoxic T-lymphocytes (CTLs). These models were compared favorably with a preparation using tripalmitoyl-S-glyceryl cysteine (P3C) as the lipid anchor. We also developed a modular synthesis of MAP-P3C that incorporated P3C as a premade unit containing a thiopyridine, which simplified the overall scheme and minimized oxidation during stepwise peptide synthesis. This IipoMAP model is a new addition to the design of our macromolecular assemblage approach mimicking peptide antigens on the surface of micro-organisms. It may be a potentially useful approach to the design of a synthetic vaccine for humans.

INTRODUCTION

Peptide immunogens have been used increasingly to produce site-specific antisera to probe and verify structure–functions of proteins in the laboratory and form the design of synthetic vaccines against various infectious diseases (Lerner, 1982; DiMarchi et al., 1986; Clark et al., 1987; Alving, 1987; Warren and Chedid, 1988). Because peptides generally elicit low immunogenicity, they are often conjugated to macromolecular proteins and administered to animals with adjuvants. Although the peptide–protein carrier and adjuvant mixture work well in eliciting the desired immunological responses, the chemical composition and stoichiometry of the mixture are often difficult to define. Recently, our laboratory developed methods to produce chemically-defined, totally synthetic peptide immunogens for laboratory and vaccine use.

Three major improvements will now produce better synthetic peptide immunogens. First, we introduced a small core matrix containing three to seven branching lysines as a scaffold for attaching the peptide immunogens known as the multiple antigenic peptide (MAP). The MAP system permits amplifying antigens four-to-eight-fold to attain a macromolecule and avoids the use of a protein carrier as well as attendant structural ambiguity (Tam, 1988). Second, we introduced a
lipophilic moiety to the MAP carboxyl terminus (lipoMAP) that leads to the induction of cytotoxic T-lymphocytes (CTLs). Finally, we provided a lipid matrix that allows antigens to be further amplified many fold in a liposomal form (Defoort et al., 1992). Such a synthetic peptide vaccine design is structurally unambiguous and conceptually mimics the features of surface peptide antigens of micro-organisms (Fig. 1). We have shown that immunization with MAPs conjugated to a lipophilic moiety without any adjuvant results in high-titered antibodies and induction of cytotoxic T-lymphocytes in mice and guinea pigs (Defoort et al., 1992; Nardelli et al., 1992). In our design, the lipophilic moiety plays a dual role, serving not only as an anchor in the lipid matrix, but also as a built-in adjuvant. In our previous approach, the lipophilic moiety was a tripalmitoyl derivatized cysteine, tripalmitoyl-S-glyceryl cysteine (P3C) (Fig. 2).

P3C is a B-cell mitogen found in the lipoprotein of E. coli, but its synthesis requires many elaborated steps (Deres et al., 1989; Wiesmuller et al., 1983). Furthermore, P3C contains a thiol ether that is prone to oxidation during peptide synthesis and in liposome preparation. A simple noncysteinyl derivative replacing P3C would be useful in our approach. For this purpose, we designed lipophilic amino acids, Lys or Ser, with long-chain fatty acids on their side chains. In this paper, we wish to describe the preparation of lipoMAP containing P3C with a disulfide linkage, and to compare it with a new lipoMAP system that contains a non-cysteinyl lipophilic derivative and has a symmetrical scaffold for the core matrix. The lipophilic moiety is made up of one or more lysine residues whose side chains are conjugated with palmitic acids or palmitoyl lysines (PLs). The core matrix originally consists of lysine residues as building units. Because the side chain amino group of lysine is four carbons longer than the a-amino group, the original matrix has a group of asymmetrical branches. In the new model, we use a different building unit, O-ala lysine dipeptide (Fig. 3), which produces nearly a symmetrical core matrix and possibly, a more flexible scaffold for peptide immunogens. The effect of the new core matrix and the number of palmitoyl chains on the immunogenicity of the constructs are examined. The adjustable scaffold is accessible by solid-phase peptide synthesis. It will be a convenient approach for preparing peptide immunogen with lipid moiety. Furthermore, it also provides a model to study systematically the conformation of lipids and their presentation in inducing humoral and cellular immune responses.

**MATERIALS AND METHODS**

*Synthesis of MAP–palmitoyl lysine conjugates with symmetrical core matrix (B2SM–PLn, n = 1–4)*

The B2SM–PLn were manually synthesized by solid-phase peptide synthesis on Boc-Ala-OCH3-Pam-resin (0.10 mmol/g) (Applied Biosystems, Foster City, CA) using a combination of Boc and Fmoc strategy. Removal of the Fmoc group was carried out by 50% trifluoroacetic acid (TFA) in dichloromethane followed by washing with dichloromethane and neutralization with diisopropylethylamine/dichloromethane/dimethylformamide (1:9:11, v/v/v). Couplings of amino acids (four molar equivalents) were carried out with the coupling reagent HBTU/diisopropylethylamine in dimethylformamide. The stepwise syntheses are described below.

After removal of the Boc group on the resin (Boc-Ala-OCH3-Pam-resin), one or more rounds of Fmoc-Lys(Boc) coupling were carried out sequentially to the alanyl resin. The N’-Boc groups on Lys were then removed and the palmitic acids (six molar equivalents) were coupled by the symmetrical anhydride method using dicyclohexylcarbodiimide (three molar equivalents) to form Fmoc-[Lys(Pal)]n-Ala-OCH3-Pam-resin. The Ser–Ser linker was introduced by two consecutive rounds of Boc-Ser(Bzl) coupling with the HBTU chemistry. After deblock of the Boc group on Ser(Bzl), Fmoc-Lys(Boc) was coupled. N’-Fmoc group on Lys was removed and Boc–β-Ala (0.4 mmol/g) was coupled to furnish the first level branching of the symmetrical MAP core. To produce a tetravalent MAP, the second level branching was constituted by repeating the above steps but employing 0.8 mmol/g of Fmoc-Lys(Boc) and Boc–β-Ala. The peptide antigen with the sequence KSIRIQRGGRAFVTIGK was coupled stepwise using the Boc/HBTU chemistry. The protected trifunctional amino acids were: Thr(Bzl), Ser(Bzl), Lys(CIZ) and Arg(Tos). For each coupling step, 1.6 mmol/g of Boc amino acids were used because the resin had been amplified from 0.1 mmol/g to 0.4 mmol/g. B2SM–PLs were cleaved from the resin (0.5 g) by stirring in 10 ml of HF/thiocresol/ p-cresol (90:3:7, v/v/v) mixture at 0°C for 1 hr. After extraction with 8 M urea in 0.1 M Tris–HCl buffer, pH 7.4, and dialysis against 0.1 M Tris–HCl buffer, pH 7.4, with decreasing urea concentrations to 0 M for 24 hr, reasonably pure B2SM–PLs were obtained. The amino acid analyses and laser desorption mass spectroscopy showed satisfactory results for all synthetic MAP-PLs.

![Fig. 1. Schematic structure of a lipoMAP in a lipid matrix.](image-url)
Lipophilic multiple antigen peptide system

Preparation of (P3C-Cys-OMe)2

To an ice-cold solution of tripalmitoyl-S-glyceryl cysteine (P3C; 0.91 g, 1 mmol), dimethylcystine-HCl (0.20 g, 0.6 mmol) and triethylamine (1.2 mmol) in THF (5 ml), N-hydroxybenzotriazole (HOBr; 0.14 g, 1 mmol) and dicyclohexylcarbodiimide (DCC; 0.23 g, 1.05 mmol) were added. The solution was stirred at 0°C for 1 hr and at room temperature for 4 hr. Ethyl acetate (10 ml), chloroform (50 ml) and saturated NaHCO3 were added. The organic layer was washed with 5% citric acid, NaHCO3 and water (3 ml each). After being dried over Na2SO4 and concentrated, pure product (0.70 g, 68% yield) was obtained by recrystallization from hexane m.p., 77.0-78.0°C; thin-layer chromatography (TLC): Rf = 0.90 in ethyl acetate/hexane (3:7).

Preparation of P3C-Cys-OMe

To a degassed suspension of (P3C-Cys-OMe)2 (2.46 g, 1.20 mmol) in chloroform, triethylamine (0.39 ml) and dithiothreitol (0.82 g, 5.20 mmol) were added under nitrogen. The clear solution was stirred for 2 hr, washed with 5% citric acid (3 x 25 ml) and water (2 x 25 ml), and dried over Na2SO4. The colorless solid was obtained (1.96 g, 80% yield) by recrystallization from methanol and drying over P2O5 under vacuum m.p. 75-77°C; TLC: Rf = 0.76 in ethyl acetate/hexane (3:7).

Preparation of P3C-Cys(PyS)-OMe

The solution of P3C-Cys-OMe in chloroform (10 ml) was added dropwise into the solution of 2,2'-dithiopyridine (0.95 g) and glacial acetic acid (0.15 ml) in absolute ethanol (4 ml). The mixture was stirred for 20 hr. During this period, the solution turned yellow in color due to the release of 2-pyridinethionol. The solution was concentrated to about 3 ml and passed through a silica gel column with ethyl acetate/hexane (1:1 to 2:1, v/v). The first colorless fraction was the product (1.47 g, 72.3% m.p. 68.0-69.0°C; TLC: Rf = 0.78 in ethyl acetate/hexane (2:1). 1H NMR, 350 MHz (not shown). 13C NMR, 350 MHz (not shown). Elemental anal. Calcd for C72H72N3O6S2: C, 66.55; N, 3.70; H, 10.04; S, 8.46. Found: C, 66.79; N, 3.65; H, 9.90; S, 8.45. MS: m/z 1137 (M+).

Preparation of B2M-Cys(P3C-Cys-OMe)-Ala

B2M-Cys(Acm)-Ala-Pam-resin was synthesized by Fmoc/tBu chemistry. The protecting group acetamidomethyl (Acm) on the cysteine was removed with Hg(OAc)2 on the resin as described (Liu et al., 1990). The B2M-Cys-Ala-Pam-resin (10 μmol) in chloroform/propionol (4:1, v/v, 8 ml) reacted with P3C-Cys(PyS)-OMe (45 mg, 40 μmol) in the presence of triethylamine for 74 hr. The yellow by-product, 2-pyridinethiol released quantitatively (UV = 343 nm, λ = 8080) was used to monitor the reaction. The unreacted P3C-Cys(PyS)-OMe was recovered by washing the concentrate of the reaction solution with methanol. Compound 1 (Fig. 4) was cleaved from the resin with TFA/phenol (95:5, v/v). Satisfactory results of amino acid analyses were obtained after oxidization of cysteine with performic acid. The yield was 33-54%. The product was purified through gel
filtration (Sephadex G75) or directly used for preparation of liposomes, since unreacted MAP peptide did not incorporate into liposomes and was separated by gel filtration later.

Preparation of liposome

MAP-PLs were incorporated into liposome by the detergent-dialysis method (New, 1990) with modification. In a round-bottle flask, phosphatidylethanolamine (5 mg), cholesteryl (5 mg) and octylglucoside (100 mg) were dissolved in about 1 ml of acetone and evaporated under nitrogen. The residue was re-dissolved in diethyl ether and re-evaporated to form a lipid film on the glass wall. A detergent solution (23 mg of octylglucoside in 5 ml of 0.30 M NaCl aqueous solution) containing 0.5 mg of MAP-PLs was added to the dry lipid film and dispersed with vortexing. The resulting clear liquid was dialysed for 36 hr against three 2-liter changes of phosphate-buffered saline (PBS), pH 7.4. Purification of liposome monitored at 225 nm was carried out by gel filtration on sterile G150 with PBS.

Immunization procedure

Outbred female CD-1 mice or inbred female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 50 µg of the antigen, free or in liposomes, using PBS as vehicle, three times at 2-week intervals. Sera were collected 15 days after the last boost. The antibody response was analysed by enzyme-linked immunosorbent assay (ELISA) using plates coated with B2 peptide (5 µg/well) or gp120 (1 µg/well), kindly provided by Dr. A. Profy (Repligen). Serial dilutions of the sera were added to the wells and the bound antibodies were detected using goat alkaline phosphatase-conjugate antibody (Sigma) and p-nitrophenyl phosphate substrate (Sigma).

Cytotoxicity assay

Spleen cells from the immunized BALB/c mice were cultured in vitro for 5 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 5 x 10^{-5} M 2-mercaptoethanol and antibiotics in the presence of B2 peptide (0.4 µM). The cytotoxic activity was tested in a 4 hr assay against ^51^Cr-labeled syngeneic P815 cells untreated or preincubated with B2 peptide (0.8 µM). The percentage of specific ^51^Cr release was calculated as (experimental release - spontaneous release/(maximum release - spontaneous release)) x 100. Maximum release was determined from supernatants of P815 cells lysed by the addition of 5% Triton X-100 and spontaneous release from target cells incubated alone.

RESULTS

Synthesis of MAP–PLs conjugates

The basic design of the model used for our study is shown in Fig. 1. It consisted of four components: an antigen, a core matrix, a hydrophilic linker and a lipophilic carboxyl end. The selected peptide antigen for this study was an 18-residue peptide, KSIRIQRGAPGRAFVTIGK (sequence 312–329, referred to as B2), which is derived from the third variable domain (V3 loop) of gp120, the envelope glycoprotein of HIV-1 strain IIIB (Robey et al., 1986). This 18-residue sequence includes T-helper and T-cytotoxic epitopes (Takahashi et al., 1989) and has been shown to elicit excellent antibody titers in mice using the MAP system in Freund's adjuvant (Nardelli et al., 1993) or as a covalent conjugate with P3C (Fig. 2) in liposomes (Defoort et al., 1992). The branching core matrix was made by three units of β-alanyl-lysine. Because β-alanyl-lysine contains amino groups nearly equidistant from the α-carbon of the lysine, the resulting core matrix is nearly symmetrical (Fig. 3). It may also have the advantage of greater flexibility than the conventional asymmetric core matrix consisting only of lysine. The carboxyl end of the core matrix contains a hydrophilic dipeptide linker, Ser–Ser, followed by a series of palmitoyl lysines that are used as anchors to the lipid matrix. From molecular modeling studies, these palmitoyl lysines are best positioned in alternating chirality (D or L) so that the lipid anchors are in parallel orientation as required for inserting into liposome membrane.

Because the conjugations of all parts in these MAP PLs are amide bonds, they can be synthesized stepwise as a complete unit by the Merrifield solid-phase
method using a combination of Boc and Fmoc chemistry. The lipophilic palmitoyl lysines can be built by two methods. In the first method, palmitoyl lysine is incorporated as Boc-Lys(Fmoc) and then the Fmoc group is removed so that palmitic acid is conjugated to the side chain. Alternatively, the palmitoyl lysine could be pre-made in solution chemistry and incorporated as a single unit. Next, to form the core matrix, β-Ala-Lys was incorporated sequentially as Fmoc-Lys(Boc). Removal of the Fmoc and incorporation of the Boc-β-Ala produced the desired β-Ala-Lys unit of the resins. Again, a premade dipeptide of Boc-β-Ala-Lys(Boc) could be incorporated as a single unit to reduce several repetitive steps in solid phase manipulations. Once the core matrix was completed, the antigens to be amplified four-fold were synthesized sequentially to produce the desired model. The MAP-PLs containing antigens and palmitoyl lysine were cleaved from the resin, extensively dialysed against decreasing concentrations of urea-buffer solution, and then purified by gel permeation chromatography. The products showed the expected amino acid ratio by amino acid analysis. Seven MAP-PLs were prepared to investigate the various structural contributions to immunogenicity. (Fig. 5).

**Immunogenicity of MAP–PLs in liposomes**

Because MAP–PLs were developed as a simple replacement of P3C, a lipoMAP model containing three palmitoyl lysine substitutions, B2SM–PL3, a MAP containing four B2 peptides, and three palmitoyl lysines on a symmetrical core matrix (Fig. 5C), was tested as a prototype. The humoral response elicited by B2SM–PL3 in mice was analysed by ELISA. Immunization with B2SM–PL3 in liposomes (B2SM–PL3/liposomes) elicited antibody responses, while B2SM–PL3 alone was not immunogenic (Fig. 6). Furthermore, the response of PL3 was specific, because the same construct with palmitic acids conjugated on the serine side chains, such as B2SM–PS3 (Fig. 5A), elicited significantly lower titers than B2SM–PL3 (Fig. 6).

An important question was whether the lipid side chains served solely as a depot and required a lipid matrix for correct presentation. The immunogenicity of B2SM–PL3 was then compared in different aqueous and oil-based formulations. In PBS or alum, no significant antibody response was obtained. Only in oil-emulsion or in liposome did B2SM–PL3 produce a significant antibody response (Table 1).

Fig. 5. Schematic representation of MAP conjugates containing (A) palmitoyl groups linked to the side chain of serines (B2SM–PS3); (B) L-Ser–D-Ser linker (B2SM–D–PL3); (C) zero to four palmitoyl lysines.
Ining three palmitoyl lysines was generally not as good as mice (Fig. 6).

We thought such a turn might cause the lipid portion containing the palmitoyl lysines to fold back to the antigen-core matrix, leading to a completely different presentation on MAP-PLs, we compared the linker L-Ser-L-Ser with L-Ser-D-Ser in B2SM-PL3 and B2SM-D-PL3 (Fig. 5B) models, respectively. With L-Ser-L-Ser as linker, we envisioned that this linker would allow the antigens to be extruded from the lipid matrix. L-Ser-D-Ser, however, imparts a reverse turn to the conjugate and we thought such a turn might cause the lipid portion containing the palmitoyl lysines to fold back to the antigen-core matrix, leading to a completely different antigen presentation in the liposomal matrix. Indeed, B2SM-PL3 in liposomes was immunogenic, while B2SM-D-PL3 containing the L-Ser-D-Ser linker was only weakly immunogenic after three immunizations in mice (Fig. 6).

Stoichiometric requirement of palmitoyl side chains

The immunological responses of B2SM-PL3 containing three palmitoyl lysines was generally not as good as B2M-P3C containing three palmitoyl chains on a cysteine when administered on PBS or in alum. (Table 1).

Table 1. Immune responses of lipoMAP conjugates

<table>
<thead>
<tr>
<th>Delivery system</th>
<th>Conjugate titers (10^7)</th>
<th>B2SM-PL2</th>
<th>B2SM-PL3</th>
<th>B2M-P3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Alum</td>
<td>ND*</td>
<td>&lt;0.1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Oil-emulsion</td>
<td>ND</td>
<td>20</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Liposome</td>
<td>2.0</td>
<td>1.7</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Titers were measured by ELISA with the pooled sera of five CD-1 mice by immunization in liposomes with B2SM-PL3 (●), B2SM-D-PL3 (○), B2SM-PS3 (▲), or alone B2SM-PL3 (□).

No antibody response was detected in the mice immunized with B2SM-PL3 (A), or alone B2SM-PL3 (0). B2SM-PL6 were used to immunize mice alone or incorporated in liposomes. Three of the five models containing zero, one, and four palmitoyl lysines, B2SM, B2SM-PL1 and B2SM-PL4, showed no or very low responses after four immunizations. In the presence of liposomes, nearly no incorporation was observed in the case of B2SM without PL. We found that B2SM-PL1 with a single lipid anchor was very poorly incorporated in the liposomes. It was very difficult to reach the dose of 50 μg of peptide bound to liposomes, for the immunization. The mice were therefore immunized with B2SM-PL2 (two palmitoyl lysines), B2SM-PL3 (three palmitoyl lysines) and B2SM-PL4 (four palmitoyl lysines) in liposomes or as free construct, while B2SM (without palmitoyl lysine) and B2SM-PL1 (one palmitoyl lysine) were immunized as free constructs. The best response was obtained from B2SM-PL2/liposomes. As determined by ELISA against the gp120 peptide (Fig. 7A) and the native protein (Fig. 7B), B2SM-PL2/liposomes elicited higher titers than B2SM-PL3/liposomes. B2SM-PL4/liposomes were not immunogenic. No antibody response was detected in the mice immunized with free B2SM-PLs in the same concentrations.

Induction of cytotoxic T-lymphocytes

We then turned our attention to the induction of cytotoxic T-lymphocytes (CTLs), which is an important contributor to immunity against viral infections. Recently, several groups have demonstrated in vivo CTLs priming with peptides or soluble proteins. A common feature in these preparations was the presence of lipophilic moieties attached to peptides (Deres et al., 1989) or the use of hydrophobic adjuvants, such as Freund’s adjuvant or ISCOMs (Morein, 1988; Schultz et al., 1991; Takahashi et al., 1990). To assess the induction of CTL response after immunized in our lipoMAP system, spleen cells of the syngeneic target cells were sensitized with B2M peptide. Induction of CTL response, following immunization with the MAP-PL system, was analysed in BALB/c mice (Fig. 8). Strong cytolytic activity was found in the spleen of the animals immunized with B2SM-PL2 free or in liposomes, indicating that immunodominant cytotoxic epitopes can be coupled to the palmitoyl lysines to raise CTLs in vivo.
Lipophilic multiple antigen peptide system

Reciprocal serum dilution

Fig. 7. Effect of stoichiometry of palmitoyl side chain on the immunogenicity of B2SM-PLs. The antibody response was measured by ELISA with the pooled sera of mice immunized with B2SM-PL2/liposomes (●), -PL3/liposomes (▲), -PL4/liposome (■) or with the free constructs B2SM (×), B2SM-PL1 (□), -PL2 (○), -PL3 (△), -PL4 (□). (A) antibody response against B2 peptide; (B) against gp120.

Comparison with B2M–P3C

Previously, we synthesized B2M–P3C, in which P3C was conjugated to the lysine side chain at the carboxyl terminus, and found that it elicited both a humoral response and CTLs when incorporated in liposomes (Gregoriadis, 1990; Allison and Gregoriadis, 1974).

However, such synthesis of B2M–P3C using the lysine side chain as the linkage lacked flexibility. We therefore developed a modular approach (Fig. 4) to the synthesis of B2M–P3C using the thiol side chain of cysteine by conjugating B2M to P3C through a disulfide linkage in solid phase (Fig. 9). The MAP containing a cysteinyl residue at the carboxyl terminus was conjugated to a Cys–P3C containing a thiopyridine residue while it was still attached to the resin. The advantage of conjugating in the solid phase was that all side products and excess reagents could be removed by washing. The yield of the reaction was 33–54%. However, those B2SM lacking P3C were removed either during gel permeation chromatography or the incorporation to liposomes. The immunological characteristic of the modular B2M–P3C was compared with those of the new model of B2SM–PL2 and B2SM–PL3. The results (Table 1) show that B2SM–P3C combines some of the attributes of B2SM–PL2 and PL3 in eliciting antibody responses in oil-emulsion and liposomes. However, it differs from the PL models and has the additional ability to elicit immune response in PBS and in alum.

DISCUSSION

The task of transforming a synthetic peptide antigen into a self-sufficient immunogen capable of eliciting both humoral and cell-mediated responses is challenging. Our results show that the new lipoMAP system with appending, dendritic PLs in a lipid matrix such as liposomes may provide a useful solution. The new system may also be useful to further our understanding of immunogenicity and the roles played by the adjuvants.

Adjuvants are known to induce nonspecific B- or T-cell proliferation by the induction of cytokines (Vitetta et al., 1987). They may also provide a depot for the slow release of peptide antigens (Edelman, 1980). The lipid chains on the lipoMAP appear to provide one or both of these functions. However, it remains to be determined whether the PLs in our lipoMAP play a role in inducing cytokines. Our preliminary results have shown that MAP–PLs are not B-cell mitogens (data not shown), therefore differing from P3C.
The advantages of a built-in adjuvant on a peptide antigen have been shown by Chedid and his coworkers (Audibert et al., 1985; Ho et al., 1989; Ido et al., 1989) using derivatives of the muramyl dipeptide, a component of the Freund's adjuvant. Lipid moieties have been used as covalent attachments to the amino terminus, mainly to increase the ability as a depot (Kenney et al., 1989; Brynestad et al., 1990; Finberg et al., 1978). We have found that adding lipid moiety to antigens often alters their immunogenicity. We approached this problem in a systematic manner to design the lipoMAP system. First, we examined the role of a more flexible core matrix containing a β-alanyl-lysine as a building unit that will provide less steric crowding than those using lysine alone. However, the difference between the conventional asymmetric and the new symmetrical core-matrix in improving epitope peptide presentation to enhance the antibody response does not always appear to be an important factor (Defoort et al., 1992; Huang et al., 1992). It might be dependent on the antigen sequence. The hydrophilic linker, Ser–Ser, appears to be important since using the Ser–D–Ser linker which orients the lipid differently did not produce the desired immunogenicity. Furthermore, replacing Lys(Pal) with Ser(Pal) also severely reduces its adjuvant effect.

We next tested the stoichiometric requirement of lipid side chains important for incorporating into liposomes. The optimal number appears to be two. B2SM PL2 has two parallel lipid tails, similar to phosphatidylethanolamine and elicits the highest antibody responses in this series when presented in ordered lipid structure such as liposomes. B2SM–PL3 is slightly inferior to B2SM–PC3 in liposomes. This structural similarity is important for the incorporation and rigid orientation of MAP–PLs on liposomes. The low response of B2SM–PL1 may result from its inability to anchor liposomes. B2SM–PL4 has lipid tails that cross each other, as shown by molecular simulation, and coupled with its low aqueous solubility, does not elicit significant immune response. The stereospecific requirements of lipid attachments in Lipid A have been clearly shown by Kotani and his coworkers (Kotani et al., 1983), who found that altering the number or chirality of the lipid side chains of Lipid A leads to less potent molecules. Similarly, Metzger and his coworkers (Metzger et al., 1991), have shown that the chirality as well as the orientation of the palmitoyl side chains of P3C are important for mitogenicity. These results imply that the design of the lipoMAP may require us to consider the specific conformation of lipid side chains for presenting peptide antigens on the surfaces of liposomes.

Our results reveal the role of lipid as depot appears to be important because B2SM–PL3 in alum or PBS alone did not result in any significant immunological response, while B2SM–PL3 in liposomes (Gregoriadis, 1990; Allison and Gregoriadis, 1974) or oil-emulsion (Pyle et al., 1989) induced significantly elevated antibody titers.

Finally we also focused our design on the ability of the peptide antigen to elicit CTLs. We found that the attachment of PLs with or without the aid of liposomes can induce CTLs capable of killing syngeneic cells expressing gp120 on their cell surfaces. These results showed that the processing of B- and T-cell antigens have different requirements (Vitetta et al., 1987; Abbas, 1987; Buus et al., 1987; Babbitt et al., 1985). More importantly, it showed the versatility of the lipoMAP in eliciting both humoral and cell-mediated responses. Due to its versatility and simplicity of design, lipoMAP may be a useful tool for many mechanistic investigations.

In summary, covalent modification by lipids on MAPs or to other peptidyl or protein immunogens may require careful structural and stoichiometric considerations. Underrapiditation leads to poor incorporation in liposomes (e.g. B2SM–PL1) and overlipidation leads to poor solubility in water (e.g. B2SM PL4). However, when optimal lipidation occurs, such as with B2SM–PL2 and B2SM–PL3, their incorporation into either liposomes or oil-emulsion provides remarkably high immunogenicity which is not observed when they are administered alone. In addition, those that mimic lipid-membrane components such as B2SM–PL2 have the advantage to be inserted into ordered structure such as liposomes.

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REFERENCES


