

Macromolecular assemblage in the design of a synthetic AIDS vaccine

(synthetic peptide/liposome/cytotoxic T lymphocytes/multiple antigen peptide system)

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ABSTRACT We describe a peptide vaccine model based on the mimicry of surface coat protein of a pathogen. This model used a macromolecular assemblage approach to amplify peptide antigens in liposomes or micelles. The key components of the model consisted of an oligomeric lysine scaffolding to amplify peptide antigens covalently 4-fold and a lipophilic membrane-anchoring group to further amplify noncovalently the antigens many-fold in liposomal or micellar form. A peptide antigen derived from the third variable domain of glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1), consisting of neutralizing, T-helper, and T-cytotoxic epitopes, was used in a macromolecular assemblage model (HIV-1 linear peptide amino acid sequence 308–331 in a tetravalent multiple antigen peptide system linked to tripalmitoyl-S-glycerylcysteine). The latter complex, in liposome or micelle, was used to immunize mice and guinea pigs without any adjuvant and found to induce gp120-specific antibodies that neutralize virus infectivity *in vitro*, elicit cytokine production, and prime CD8⁺ cytotoxic T lymphocytes *in vivo*. Our results show that the macromolecular assemblage approach bears immunological mimicry of the gp120 of HIV virus and may lead to useful vaccines against HIV infection.

Most vaccine strategy developed today against human immunodeficiency virus (HIV) infection has been directed toward the humoral response of generating neutralizing antibodies. Recent advances in mapping antigens involved in immune responses have allowed detailed characterization of epitopes that confer neutralizing, T-helper and T-cytotoxic responses (1–6). These developments have led to consideration of including the T-cytotoxic response along with humoral immunity in the design of peptide-based vaccines. However, traditional methods (7, 8) for preparing peptide vaccines that present peptides as macromolecules through conjugation to protein carriers or polymerization are often unable to induce cytotoxic T lymphocytes (CTL) response *in vivo*. Use of an adjuvant in the immunizing protocol has the advantage of enhancing the humoral response (9–11) but has mixed results in priming specific CTL response. Furthermore, the most popular adjuvant used in laboratory animals, such as Freund's complete adjuvant, is too toxic and unacceptable for humans. Ideally, protection against viral infection is best provided by both humoral and cell-mediated immunities, including long-term memory and cytotoxic T cells. In this paper, we describe a rational approach to producing a synthetic peptide-based vaccine that is effective in providing both humoral and cell-mediated immunities and safe in its exclusion of Freund's complete adjuvant. Our approach uses a macromolecular assemblage principle that allows amplification of peptide antigens to a macromolecule.

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The resulting macromolecule bears the immunological mimicry of the external-surface coat protein of a pathogen.

The amplification is made possible by two synthetic components (Fig. 1). (i) A scaffolding consisting of an oligomeric branching lysine is used to amplify the peptide antigen 4-fold to give a multimeric structure. This covalent amplification, known as multiple antigen peptide system (MAPS), has been effective in inducing strong immune responses (12, 13). (ii) A lipophilic membrane-anchoring moiety at the carboxyl terminus of MAPS enables further noncovalent amplification by a liposome or micellar form. Such a macromolecular assemblage will amplify the MAP antigens many-thousandfold. Several lipophilic moieties have been studied in our laboratory, the most successful is the tripalmitoyl-S-glycerylcysteine (P3C). P3C, which is a lipoamino acid from *Escherichia coli*, is a B cell mitogen, a nontoxic adjuvant, and can induce CTL *in vivo* when covalently linked to a peptide antigen (14).

The antigen for our model is located in the third variable domain (V3 loop) of gp120, the envelope glycoprotein of HIV-1, which is the principal target for vaccine development against AIDS (4). The V3 loop of IIIB strain, amino acid sequence 291–343, contains an invariant disulfide bridge and a type-II β turn with the sequence Gly-Pro-Gly at its crest. Antibodies raised to the V3 loop neutralized the *in vitro* infectivity of HIV, and the principal neutralizing determinant has been found to be centered at the β turn. Our previous studies in mice (15) have found that a 24-residue peptide of the V3 loop, referred to as B1 (sequence 308–331), contains the minimal sequence that consists of neutralizing and T-helper epitopes. In addition, this B1 sequence also contains a T-cytotoxic epitope (sequence 315–329) (6). We show here that a vaccine model using the peptide B1 by the macromolecular assemblage approach [referred to as B1 in a tetravalent MAPS(B1M) format linked to P3C (B1M-P3C)] induces specific antibodies against gp120 that neutralize virus infectivity *in vitro* and elicits CTL *in vivo*.

MATERIALS AND METHODS

Synthesis of B1M-P3C. Synthesis was accomplished manually by a step-wise solid-phase procedure (16) on 9-fluorenylmethoxycarbonyl (Fmoc)-Ala-OCH₂-resin (17) (0.3 mmol/g of resin). After removal of the Fmoc group by 20% piperidine in dimethylformamide, the Ala-resin was coupled to a pre-made unit of Fmoc-Lys (P3C) (1.1 molar equivalent) via dicyclohexylcarbodiimide/hydroxybenzotriazole in CH₂Cl₂. The P3C was prepared as described (14, 18) and

Abbreviations: HIV, human immunodeficiency virus; B1, linear peptide amino acid sequence 308–331 of HIV type 1 strain IIIB; CTL, cytotoxic T lymphocyte(s); Fmoc, 9-fluorenylmethoxycarbonyl; MAPS, multiple antigen peptide system; P3C, tripalmitoyl-S-glycerylcysteine; B1M, B1 in a tetravalent MAPS format; B1M-P3C, B1M linked to P3C; IL-2, interleukin 2; ISCOM, immunostimulating complex; V3, third variable domain.

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contained the configuration of *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-(propyl)]-(*R*)-cysteine. Two consecutive serines as a linker were coupled to the Lys(P3C) before adding the two levels of Fmoc-Lys(Fmoc) to give a tetra-branching [Fmoc-Lys(Fmoc)]₂-Lys-Ser-Ser-Lys(P3C)-Ala resin. The protecting group scheme for the synthesis was as follows: Fmoc for the *N*^α-amino group and *tert*-butyl side-chain protecting groups for the trifunctional amino except arginine (pentamethylchromansulfonyl) and asparagine (trityl). Three equivalents of Fmoc-amino acid were used for each coupling by the dicyclohexylcarbodiimide/hydroxybenzotriazole method. Coupling was monitored by the qualitative ninhydrin test (19). Deprotection by 20% piperidine in dimethylformamide (10 min) was preceded by a 2-min wash. The completed peptide was cleaved from the resin by 95% CF₃CO₂H/2% dimethyl sulfide/2% anisole/1% ethanedithiol. The MAPS peptides were purified by precipitations in CF₃CO₂H/*tert*-butyl methyl ether. Similarly, synthesis of B1 was accomplished by the Fmoc/*tert*-butyl strategy, and the peptide was purified by reverse-phase HPLC. All peptides gave satisfactory amino acid analyses.

Preparation of Positively Charged Liposomes. Liposomes were prepared as described by Gregoriadis *et al.* (20). Briefly, 56 mg of egg lecithin, 8.4 mg of cholesterol, and 1.8 mg of stearylamine were solubilized in CHCl₃ in a 100-ml round-bottom flask. P3C (0.24 mg) was added to liposomes made with B1M. The organic solvent was removed under vacuum by using a rotary evaporator to form a thin film of lipid on the flask wall. After drying, nitrogen was kept flushing in the flask for 10 min. Two milliliters of 10 mM phosphate-buffered saline, pH 7.4, containing 2.5 mg of peptide, was added into the flask. Shaken manually for 10 min, the suspension was then allowed to stand at room temperature for 2 hr. The resulting milky solution was sonicated 45 min (Laboratory Supply, Indianapolis) until the solution became opalescent. After sonication, free B1M-P3C was separated from the liposomes on Sepharose 6B; the liposomes were then filtered on 0.45- μ m filter and kept under nitrogen.

Immunization Procedure and ELISA. Dunkin-Hartley guinea pigs (three per group) were immunized s.c. with 100 μ g of peptide on days 0 and 14 and with 50 μ g of peptide on days 30 and 45. They were bled 2 weeks after the last boosting. Control guinea pigs were immunized with the same protocol by using a noncovalent mixture of B1M, P3C, and liposome. BALB/c mice (five per group), 6–8 weeks old, were immunized i.p. four times with 1–100 μ g of B1M-P3C at 2- to 3-week intervals and bled 2 weeks after the last boosting. Control mice were immunized with a noncovalent mixture of 50 μ g of B1M, P3C, and liposome. Antisera were used without purification. ELISA was used to test antisera for their ability to react with B1 (5 μ g per well) or recombinant gp120, IIIB isolate (0.1 μ g per well) (Repligen, Cambridge, MA).

Functional Assays. Fusion-inhibition assay was done on CD4⁺ cells CEM-T4 (American Tissue Culture Collection), which were infected with either wild-type WR isolate or recombinant vaccinia virus (vPE16 recombinant vaccinia vector expressing the HIV-1 envelope glycoprotein gp160 of the IIIB isolate provided by B. Moss, obtained through the AIDS Research and Reference Reagent Program, division of AIDS, National Institute of Allergy and Infectious Diseases) at multiplicity of infection of 10. Antisera were added to the cultures 1 hr after infection, and syncytia were counted 24 hr after infection (15). For CTL assay, BALB/c mice were immunized with 100 μ g of antigen. Three to 8 weeks later, immunized spleen cells (2.5 \times 10⁶ per ml) in RPMI 1640 medium/10% fetal calf serum/2 mM glutamine/50 μ M 2-mercaptoethanol/antibiotics (GIBCO), referred to as complete culture medium in this paper, were restimulated for 6 days *in vitro* with 0.4 μ M of peptide B1 in 24-well culture plates.

The cytolytic activity of the restimulated cells was tested by using a 4-hr assay with ⁵¹Cr-labeled syngeneic cells P815 (H-2^d). The target cells were infected with vaccinia viruses v-env5 (recombinant vaccinia virus expressing the complete envelope gene of HIV-1 was provided by S.-L. Hu) at multiplicity of infection of 50 or pulsed with synthetic peptide (0.8 μ M for 2 hr at 37°C) before labeling. The percentage of specific ⁵¹Cr release was calculated as 100 \times [(experimental release – spontaneous release)/(maximum release – spontaneous release)]. 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. SEM of triplicate cultures were all <5% of the mean. Neutralization assay by inhibition of the reverse transcriptase activity was done as reported by Ho *et al.* (21) on HIV-infected H9 cells.

IL-2 Production and B-Cell Proliferation. Splenocytes of mice primed i.p. 10 days before with 50 μ g of B1M-P3C were dispensed in 24-well plates at the concentration of 4 \times 10⁶ cells per well in complete culture medium. Peptides at various concentrations were added to the cell suspension. Medium alone was added to other wells to assess the interleukin 2 (IL-2) production in the absence of antigenic re-stimulation. After 36-hr incubation at 37°C, supernatants were collected, centrifuged, and added to IL-2-dependent CTL line (CTLL-2) to determine their IL-2 activities. Briefly, 50 μ l of supernatant was added to 50 μ l of CTLL-2 suspension (8 \times 10⁴ cells per ml) in a 96-well microtiter plate. Cells were harvested after 1-day incubation. During the last 5 hr of incubation, each well was pulsed with 1 μ Ci of [³H]thymidine (1 Ci = 37 GBq). Results were expressed in units of IL-2 per ml (means of triplicates) for each group. B-cell proliferation assay was done on spleen lymphocytes grown for 72 hr in RPMI 1640 medium/3.3% fetal calf serum/glutamine/2-mercaptoethanol/antibiotics at a cell density of 3.3 \times 10⁶ cells per ml in flat-bottom microtiter plates (0.2 ml per well). Antigens were added at the beginning of culture. Before harvesting, cultures were pulsed for 24 hr by adding 1 μ Ci of [³H]thymidine to each well. The results are reported as means of triplicate determinations (SEMs were <10% of the mean) of a representative experiment.

RESULTS

Synthesis of B1M-P3C. B1M-P3C (Fig. 1) was synthesized in two parts. (i) P3C was achieved in a solution synthesis in six steps according to Wiesmuller *et al.* (18) and linked to the side-chain ϵ -amino group of Fmoc-Lys phenacyl ester. The phenacyl ester protecting group was subsequently removed to give an isopeptide, Fmoc-Lys(P3C). (ii) The synthesis of B1M that contained the B1 antigen and lysine core matrix was achieved by the solid-phase method (13, 16) with Fmoc-Ala-OCH₂-resin (17). Fmoc-Lys(P3C) as a premade unit was first attached to the Ala-OCH₂-resin, followed by a dipeptide, Ser-Ser, spacer before the synthesis of a trilycine core matrix of MAPS and the B1 sequence. The design of linking P3C to the side chain of the lysine spacer (Ser-Ser-Lys) at the carboxyl terminus of the MAPS was intended to provide flexibility for the P3C to serve as a lipid-anchoring moiety without interfering with the antigen organization at the amino terminus. Because the secondary ester bond in P3C was labile to HF, the solid-phase synthesis was done by Fmoc chemistry (22) in combination with the Wang resin (17), so that the final cleavage could be done in a mild acid, such as CF₃CO₂H. The synthesis, performed manually, was rigorously monitored for the completion of each coupling step (22) to avoid deletion peptides. B1M-P3C was obtained after trifluoroacetic acid cleavage from the resin support and was purified by repeated precipitations. The advantage of this direct approach was its simplicity. Other unambiguous routes

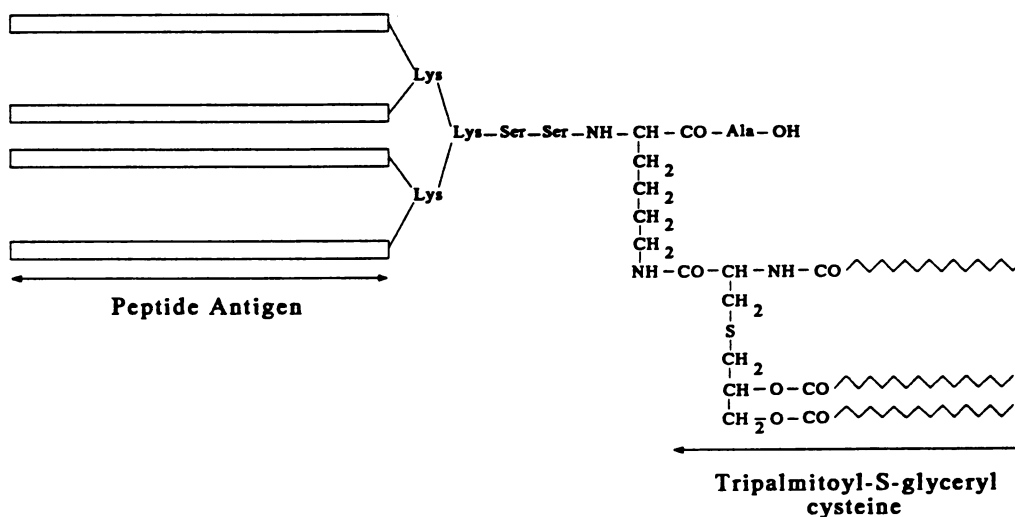


FIG. 1. Schematic representation of MAP-P3C.

for the preparation of B1M-P3C by the segment approach have also been developed (23). P3C in B1M-P3C allowed self-aggregation in aqueous solution and efficient incorporation in liposomes. About 80% of B1M-P3C was found associated with liposomes, whereas only 2% of B1M without P3C was found entrapped by liposomes. Both preparations of B1M-P3C in aggregated form in solution (B1M-P3C/free) or associated with positively charged liposomes (B1M-P3C/liposome) were tested in animals for humoral and CTL responses.

B Cell Mitogenic Activity and Humoral Response of B1M-P3C. Mouse spleen cells were used to show that the mitogenic activity of the P3C was retained in B1M-P3C (Fig. 2). The mitogenicity was dose-dependent with increased incorporation of [³H]thymidine in spleen cells with escalated concentrations of B1M-P3C. B1M without P3C did not show any mitogenic activity.

The ability of B1M-P3C/liposome or free, without any adjuvant, to induce humoral response was studied in mice and guinea pigs. High-titered antibodies were found in sera from animals immunized four times with both preparations and treated with B1 (linear peptide 308-331), B1M, or gp120 in an ELISA assay (Table 1). There was no significant difference between B1M-P3C/free or B1M-P3C/liposome, but there was a dose-response relationship of titers with increased immunizing doses. Titers from mice immunized with 100 μg of B1M-P3C/liposome were 2- and 4-fold higher

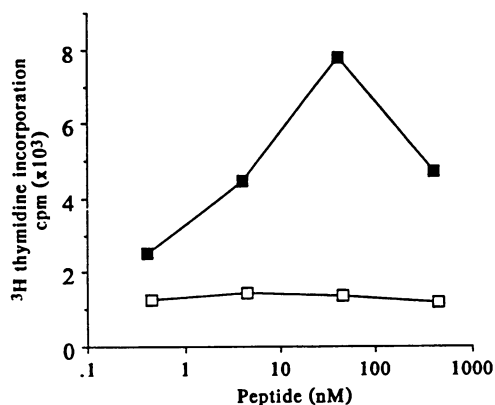


FIG. 2. Mitogenic activity of the B1M-P3C. BALB/c mouse spleen cells (3.3×10^6 cells per ml) were grown for 72 hr in the presence of B1M-P3C (■) or B1M (□). Twenty-four hours before harvesting, cells were pulsed with 1 μCi of [³H]thymidine.

than those immunized with 50 or 10 μg (data not shown). Both preparations of B1M-P3C elicited antibodies in mice and guinea pigs that showed ability to neutralize HIV, as shown by the inhibition of syncytia formation in vPE16-infected cells and the reverse transcriptase activity of HIV IIIB-infected H9 cells (Table 1). Control mice immunized with a noncovalent mixture of B1M/P3C/liposome did not develop detectable antibodies against B1 or gp120. In contrast, this noncovalent mixture elicited significant titers in guinea pigs. However, both sera of control mice and guinea pigs had no effect on the ability to inhibit the syncytia formation or the reverse transcriptase activity.

Cytokine Production and CTL Response. B1M-P3C induced CD4⁺ T-helper cell response in immunized mice (Fig. 3). IL-2 activity was found in the supernatant of spleen cells restimulated with B1. A control and unrelated peptide from the circumsporozoite protein [(Asn-Ala-Asn-Pro)₃MAP] did not show any activity. Spleen cells of mice immunized with B1M-P3C free or in liposome were assayed for their ability to lyse target cells preincubated with B1 or infected with vaccinia virus expressing gp160. Fig. 4 shows that B1M-P3C, in liposome or free, elicited CD8⁺ CTL in mice against vaccinia virus-infected cells (v-env5) and B1 peptide-coated cells. The CTL response was mediated by CD8⁺ lymphocytes (unpublished work).

Table 1. Immune response to B1M-P3C

Immunogen	Antibody titer ($\times 10^{-3}$)		Inhibition	
	Peptide	gp120	Syncytium formation [†]	RT activity [‡]
Mouse				
Control	<0.01	<0.01	0	ND
Liposome	10	4	20	8
Free	4.3	2	10	8
Guinea pig				
Control	6.8	1.2	0	0
Liposome	6.5	3.2	20	8
Free	8.2	3.3	10	8

RT, reverse transcriptase; ND, not done.

*ELISA titers represent the reciprocal of the end-point dilution (serum dilution at which OD was 0.2 unit). Mice were immunized with 100 μg of B1M-P3C, and control group was immunized with 50 μg of a noncovalent mixture of B1M/P3C/liposome.

[†]Fusion inhibition titers are dilutions reducing the number of syncytial foci by 90%.

[‡]Neutralization titers are dilutions reducing RT activity by 87%.

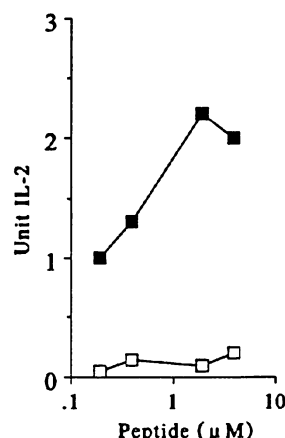


FIG. 3. IL-2 production of lymphocytes primed with B1M-P3C. Splenocytes from mice immunized with 50 µg of B1M-P3C were cultured with B1 (■) or a control peptide from the malaria circumsporozoite protein [(Asn-Ala-Asn-Pro)₃MAP] (□).

DISCUSSION

We describe here the rational design of a totally synthetic peptide-based vaccine that induces neutralizing antibody and CTL as well as a vaccine that is safer and more versatile than a whole virus or viral protein vaccine. We use a macromolecular assemblage approach to produce a multimeric form of peptide antigen, B1M-P3C, that consists of a lipophilic membrane-anchoring moiety covalently linked to a MAPS core matrix and four dendritic arms of peptide antigens derived from the V3 loop of the gp120 envelope protein of HIV-1. Mice antisera against B1M-P3C neutralize the virus infectivity as shown by the inhibition of syncytium formation and reverse transcriptase, induce T-helper response as shown by the IL-2 production, and elicit CD8⁺ CTL that lyse syngeneic cells expressing gp160 on their cell surface. Furthermore, the B1M-P3C produced long-term T-cell memory, as the CTL of the immunized mice remained undiminished 5 mo after the last boost immunization (29). These results are particularly pertinent to the development of a synthetic vaccine against AIDS because the predominant vaccine strategy has focused on neutralizing antibodies rather than cell-mediated immunity, which may be an equally effective mechanism to overcome cell-to-cell virus transmission in HIV infection. Subunit protein administered with a clinically acceptable adjuvant, such as gel of aluminium or calcium salt,

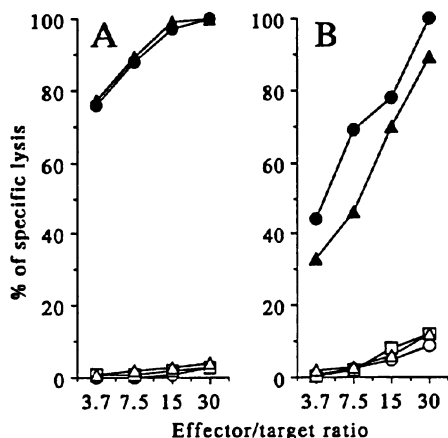


FIG. 4. Induction of HIV-1 envelope-specific CTL. Spleen cells from mice immunized with B1M-P3C in liposome (A) or free (B) were restimulated with B1. Cytotoxic activity was tested on P815 target cells alone (○), presensitized with a control peptide T1 derived from the fourth constant region of gp120, amino acids 429-443 (△), presensitized with B1 (●), infected with vaccinia virus strain WR (□), or with vaccinia virus v-env5 expressing gp160 (▲).

is usually insufficient to elicit CTL response, particularly CD8⁺ restricted, as shown by the results of Orentas *et al.* (25), who found CD4⁺-specific CTL by a gp160 subunit vaccine. Subunit vaccine expressed by live vectors, such as vaccinia virus (26), may overcome this limitation but will need further development to define various adverse reactions in humans. The ideal vaccine may eventually be derived from the inactivated whole or attenuated virus, but the risk and long latency associated with the infection have so far limited the enthusiasm for its development.

Although synthetic peptides, in their monomeric form, possess the minimal requirement to induce immune responses, they are often insufficient to mount an effective and sustained immune response. The advantage of a multimeric over a monomeric form in vaccine design has been demonstrated and exploited by many investigators. Synthetic peptides are often polymerized by different methods to enhance immune responses (8). Our laboratory has developed the MAPS using oligobranching lysines to present multiple copies of a single peptide with good success (12, 13). Clarke *et al.* (24) produced a highly immunogenic preparation by presenting multiple copies of a peptide antigen using the hepatitis B core antigen. Lipid matrices, such as liposomes (10, 11) and immunostimulating complex ISCOM (9), also allow noncovalent organization of multimeric presentation of antigens on their surface. ISCOM is a stable lipid matrix that contains cholesterol and Quil A, a plant extract that also serves as an adjuvant. ISCOMs have shown immune-enhancing property superior to that of micelle (9). Furthermore, ISCOM also has the ability to induce CTL response, as shown by Takahashi *et al.* (27), who could induce CD8⁺ CTL by immunization with purified HIV-1 envelope protein in ISCOMs. A liposome has the ability to embed the peptide antigen on its surface but is, by itself, a weak adjuvant as demonstrated in our results. B1M in complete Freund's adjuvant elicited high and protective antibodies in mice and guinea pig (15). In contrast, B1M, when mixed with liposomes, does not elicit significant antibody response in mice, although, with the same preparation, good but nonneutralizing antibody titers are obtained in guinea pigs. When the antigen contains a "hydrophobic foot", such as the B1M-P3C, neutralizing antibodies are obtained in both animals. The apparent difference that accounts for these results is the presence of P3C covalently linked to the antigen in these preparations. In our design, P3C, the lipophilic moiety, or hydrophobic foot, is used to serve both as a lipid anchor and an adjuvant. As a hydrophobic foot in B1M-P3C, it allows the incorporation into liposomes 40-fold more efficiently than B1M. Similar rationale has also been used by other investigators. Lowell *et al.* (28) use a long-chain fatty acid as a hydrophobic foot linked to a peptide antigen to allow noncovalent complex with meningococcal outer membrane to form proteosome and multimeric forms of the peptide antigen. Jung and his colleagues (14, 18) use P3C as a built-in adjuvant of peptide antigens in many successful applications. An additional role of the lipophilic moiety is its ability to prime CTL *in vivo* (14). Although peptides and proteins do not generally induce CTL response *in vivo*, peptides entrapped in lipid matrices or peptides containing lipophilic moieties have such a capacity. At this time the mechanism for the induction of CTL by the peptides with lipophilic associations remains unclear. Nevertheless, our design of macromolecular assemblage combines several of the well-tested principles in vaccine design, and our results have shown its effectiveness in small animal models.

Finally, because of the flexibility of the design of the MAPS and liposomal attachments, it is possible to incorporate, covalently and noncovalently, many distinct peptide antigens into the macromolecular assemblage constructs. Such a mixture containing a diverse range of epitopes may overcome

the variability of the HIV surface coat proteins among HIV isolates that have contributed to the difficulty in designing an effective vaccine against HIV.

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