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Controlled Lipidation and Encapsulation of Peptides as a Useful Approach to Mucosal Immunizations[1]

Ana L. Mora and James P. Tam[2]

To generate a useful strategy for mucosal immunization, we have developed an approach of lipidating a multiple Ag peptide (MAP) containing part of the V3 loop from HIV-1 $gp120_{IIIB}$. In this work, we compare two delivery systems, lipidated MAP in PBS and encapsulation in poly(DL-lactide-co-glycolide) microparticles. Subcutaneous immunization, followed by intragastric administration of MAP peptide entrapped or not entrapped in microparticles, induced mucosal and systemic immune responses at local and distant sites, including mucosal IgA in saliva, vaginal secretions and feces, and IgG in blood. However, lipidated Ag delivered in microparticles induced higher levels of mucosal Abs, particularly of intestinal IgA, and generated CTL responses. In contrast, lipidated MAP delivered by nasal route microparticles was less effective in inducing CTL responses. These results demonstrate the feasibility of using a lipidated multimeric peptide for mucosal immunization to stimulate both systemic and mucosal immune systems, including the genital tract, irrespective of the route or method of delivery and without requiring the use of a carrier or an extraneous adjuvant. ***The Journal of Immunology*****, 1998, 161: 3616–3623.**

Worldwide, approximately 80% of HIV infections are acquired by heterosexual contact through unimpaired mucosal surfaces of the genital mucosa (1–3). Because experimental studies with the SIV in monkeys have demonstrated that systemic immunizations confer protection against systemic but not mucosal challenge with the live virus (4), an effective strategy to prevent HIV transmission should involve both mucosal and systemic immunities. A strategy using virus-neutralizing Abs at mucosal surfaces could constitute a primary defense barrier (5, 6), while local cell-mediated immunity would be a secondary immune barrier preventing dissemination and latency of the virus in the draining lymph nodes. Finally, serum Abs and splenic CTLs may be a third level of immunity against further dissemination of the virus (7, 8).

Most peptide or protein Ags are ineffective for mucosal immunization when administered orally. To minimize proteolytic degradation, they must be modified and usually need to be conjugated to adhesive Ags to permit and enhance their uptake by the intestine-associated lymphoid tissues (9). For these reasons, three general mucosal vaccination strategies have been developed.

The first approach involves the use of a live vector to express the desired heterologous Ags, usually as an attenuated, avirulent form of the virus or bacteria that is capable of infecting or colonizing mucosal surfaces. These include poliovirus (10), adenovirus (11), salmonellae (12), *Escherichia coli* (13), mycobacterias (14), *Shigella* (15), and *Streptococcus* (16). The most advanced tests, which have already been employed in oral vaccine clinical trials, have used salmonellae (17). The second approach uses entrapment or adjuvant to facilitate uptake and prevent proteolytic degradation. Two examples are biodegradable microparticles using a copolymer such as poly(DL-lactide-co-glycolide), and liposomes, which are taken up by the M cells of Peyer's patches and then efficiently transported into the effector sites of the mucosal immune system (18, 19). The third approach involves the use of a covalently modified peptide or protein to facilitate specific or nonspecific uptake. Specific receptor-mediated uptake in this category includes cholera toxin subunit B and lectins, which bind to surface receptors on the mucosa (20). Nonspecific uptake includes lipidation. However, controlled lipidation of protein at specific sites with a predetermined stoichiometric number of lipid chains is difficult to achieve. Earlier work by Lustig et al. (21) showed that random lipidation of albumin with many lipid chains could elicit cell-mediated immunity. Such uncontrolled lipidation on several sites of a protein usually renders the protein insoluble and can also alter the immunogenicity of the parent molecule. On the other hand, lipidation with a single lipid chain is often insufficient to impart a lipophilic character to a protein Ag. Thus, controlled lipidation with a cluster of lipid chains at a specific site may provide a solution to the covalent modification of synthetic peptides for mucosal immunization.

Using synthetic peptides, our laboratory recently developed an approach that allows controlled lipidation by chemical synthesis to test this mucosal immunization strategy. The synthetic peptide is modified in two ways. First, to attain a macromolecularity mimicking that of proteins and to achieve a branched structure, the peptide Ag is multimerized as a cascade peptide dendrimer known as multiple Ag peptide (MAP)[3] (22, 23). Multimerization of the peptide Ag also minimizes proteolytic degradation. Second, the multimer is lipidated using a cluster of lipid chains at one end of the molecule to facilitate uptake by mucosal surfaces. For our purpose, we used tripalmitoyl *S*-glycerine cysteine (24) to form a MAP-P_3C (Fig. 1).

The objectives of the current work were to extend initial studies of mucosal immunization using a lipidated MAP administered by intragastric and nasal routes and to develop additional delivery systems to induce systemic and mucosal immune responses. We tested oral administration of a MAP-P_3C containing four copies of the peptide residues 308–331 of the V3 loop of $gp120_{IIIB}$, with the

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[3] Abbreviations used in this paper: MAP, multiple antigen peptide; DCM, dichloromethane; P_3C, tripalmitoyl *S*-glycerine cysteine.

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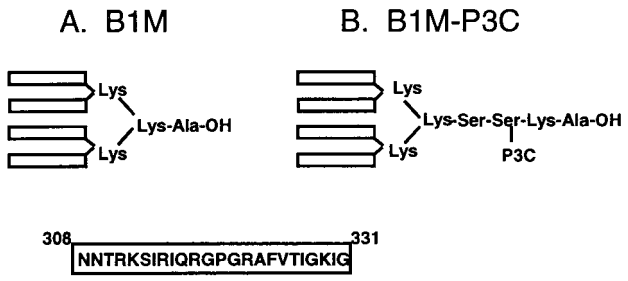

FIGURE 1. A schematic representation of *A*, B1M, peptide amino acids 308–331 from $gp120_{IIIB}$ synthesized in a MAP format consisting of four identical linear peptides. *B*, $B1MP_3C$, the multimeric peptide B1M covalently linked to the P_3C component.

minimal sequence of neutralizing, Th, T cytotoxic epitopes designated B1M, which was covalently linked to the P_3C component ($B1MP_3C$) (25, 26). Because microencapsulation could protect the MAP against inactivation by gastric acids, enhance uptake of the Ag by inductive mucosal sites in the gastrointestinal and respiratory tracts, and permit a continued release of the entrapped Ag, we evaluated the results of combining lipidated MAP with microparticle delivery. We also investigated immunization by oral and nasal routes, with or without systemic priming. Our results show that microencapsulation dramatically enhances the ability of lipidated MAPs to induce intestinal IgA, while the ability to elicit local, regional, and systemic immunities was preserved. Our results show that the combination of peptide lipidation and microencapsulation is a novel approach to mucosal immunization by the gastric route without the necessity of using adhesion molecules, extraneous adjuvants, or microparticles.

Materials and Methods

MAP peptide

B1M peptide (Fig. 1) from HIV-1 $gp120_{IIIB}$ was prepared by stepwise solid-phase synthesis on Fmoc-Ala-Wang resin. It was covalently linked to the premade P_3C using two serine residues as spacer, as previously described (27). Briefly, B1M-P_3C was synthesized in two parts. 1) P_3C was linked in a solution synthesis to the side-chain ϵ-amino group of Fmoc-Lys as an isopeptide, Fmoc-Lys (P_3C). 2) The synthesis of B1M that contained the B1 Ag and lysine core matrix was achieved by the solid-phase method with Fmoc-Ala-OCH_2 resin. Fmoc-Lys (P_3C) as a premade unit was first attached to the Ala-OCH_2 resin, followed by a dipeptide spacer, Ser-Ser, before the synthesis of a trilysine core matrix and the B1 sequence. Linking P_3C to the side chain of the lysine spacer (Ser-Ser-Lys) at the carboxyl terminus of the MAP was intended to provide flexibility for the P_3C to serve as a lipid-anchoring moiety without interfering with the Ag organization at the amino terminus. Because the secondary ester bond in P_3C was labile to HF, the solid-phase synthesis was done by Fmoc chemistry in combination with the Wang resin, so that the final cleavage could be done in a mild acid, such as CF_3CO_2H. The manually performed synthesis was monitored rigorously for the completion of each coupling step to avoid deletion peptides. $B1MP_3C$ was obtained after trifluoroacetic acid cleavage from the resin support and was purified by repeated precipitation. This direct approach had the advantage of simplicity.

Microparticle preparation

Controlled-release microparticles with entrapped B1M-P_3C were prepared with a poly(DL-lactide-co-glycolide) polymer (Resomers RG504; Boehringer Ingelheim, Ingelheim, Germany) using the solvent evaporation method according to Jeffery et al. (28). The polymers had a 50:50 ratio of lactide:glycolide and an inherent viscosity of 0.47 dl/g. A 4% solution of $B1MP_3C$ in DMSO (Sigma, St. Louis, MO) with a 6% solution of polymer in dichloromethane (DCM) (EM Sciences, Gibbstown, NJ) was mixed by vortexing. The resulting solution was emulsified at high speed with an 8% polyvinyl alcohol solution (Aldrich, Milwaukee, WI). The oil/water emulsion was then stirred magnetically overnight at room temperature to allow solvent evaporation and microparticle formation. The microparticles were isolated by centrifugation, washed three times in water, and freeze dried.

Table I. *Groups and schedules of immunization*[a]

Protocol	Regimen (n = 5)	Route	Immunogen	Dose (μg)	Vehicle
P1	A[b]	G[c]	$B1MP_3C$	100	PBS
P2	A	G	$B1MP_3C$	100	MP
P3	A	S-G	$B1MP_3C$	100	MP
P4	A	S-G	$B1MP_3C$	300	MP
B5	B[d]	S	$B1MP_3C$	100	MP
P6	B	S-G	$B1MP_3C$	100	PBS
B7	B	S-G	B1M	100	MP
P8	B	S-G	$B1MP_3C$	100	MP
P9	B	N	$B1MP_3C$	60	PBS
P10	B	N	$B1MP_3C$	60	MP

[a] Regimen A, sequential immunization days 1, 15, and 30. Regimen B, subcutaneous primary day 1, repetitive intragastric instillations days 2 and 3, and intragastric boost day 50.
[b] NIH Swiss mice.
[c] G, intragastric; S-G, subcutaneous and intragastric; N, nasal; MP, microparticles.
[d] BALB/c mice.

The final product was stored in a desiccator below 20°C. Microparticles with entrapped B1M were prepared using a water-in-oil-in-water solvent evaporation technique. A 4% solution of B1M in water was emulsified with a 6% solution of polymer in 87 to 89% DCM (Aldrich). This emulsion was added to a larger volume of an aqueous solution of 8% polyvinyl alcohol and homogenized to produce a stable water/oil/water double emulsion. The double emulsion was then processed as previously for $B1MP_3C$ microspheres.

The peptide content in microparticles was determined by placing 10 mg microparticles in 200 μl of DCM and extracting the peptide twice with 400 μl of Tris-HCl buffer (50 mM) at pH 7.2, as described by Almeida et al. (29). The extraction was assayed in triplicate samples using a BCA (bicinchoninic acid) Total Protein Assay (Pierce, Rockford, IL). The peptide content was calculated from the weight of the initial microparticles, and the amount of peptide was incorporated. The microparticles prepared with $B1MP_3C$ and B1M contained 0.85% w/w and 1% w/w of entrapped peptide, respectively, corresponding to >90% entrapment efficiency.

Morphology, size, and distribution

Samples of microparticles suspended in distilled water were placed on glass coverslips previously treated with poly-L-lysine. Microparticles were allowed to adhere for 30 to 60 min in a humidified chamber, rinsed in water, dipped in 95% ethanol, and allowed to air dry. The coverslips were attached to aluminum stubs, coated with gold in a sputter coater (Technics, Alexandria, VA), and examined in a Hitachi S-500 scanning electron microscope operated at 5 to 20 kv. Photos were taken, and the microsphere size distribution was determined according to a reference scale. The average diameter for the microparticles was 2.7 μm with $B1MP_3C$ and 1.05 μm with B1M.

In vitro peptide release

Triplicate samples (10 mg) were placed in 1.5-ml conical microcentrifuge tubes containing 1.5 ml of 0.1 M HCl. After incubation at 37°C and shaking orbitally at 70 rpm for 2 h, HCl was removed and replaced with pH 7.2 PBS. At predetermined time intervals, 100 μl of the suspension were removed, 90 μl of supernatant were obtained by centrifugation (12,000 rpm, 5 min), and the concentration of the supernatant was determined using a BCA Total Protein Assay (Pierce) (30).

Confirmation of the structural integrity of B1M-P_3C

$B1MP_3C$ and B1M were extracted from microparticles, as described above, and analyzed by SDS-PAGE under reducing conditions, with $B1MP_3C$ and B1M as controls. Subsequently, the samples were transferred to nitrocellulose membrane (Amersham, Arlington Heights, IL), and the integrity of the MAP peptide was confirmed using total mouse serum raised against $B1MP_3C$ after parenteral immunization. Detection of the anti-peptide Ab was performed using rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma).

Immunization

We used 10 different immunization protocols (protocols 1 to 10, Table I) in two regimens to assess the effect of lipidation on B1M administered by

intragastic or nasal routes with or without encapsulation by microparticles. In regimen A, National Institutes of Health Swiss mice (The Jackson Laboratory, Bar Harbor, ME) were selected to evaluate the immune response to the MAP peptide in outbred mice without a genetic restriction of MHC molecules. To facilitate the evaluation of cytotoxic responses after immunization, we used BALB/c mice (The Jackson Laboratory) in regimen B. In regimen A, two groups of mice received three intragastric administrations on days 1, 15, and 30, and two other groups were primed once s.c. and boosted twice intragastrically to determine the effect of systemic priming. In regimen B, three groups were given by s.c. injections and intragastric instillation on day 1, and three intragastric instillations on days 2, 3, and 50. Two other groups received only four nasal administrations, and a control group received only s.c. administration.

Regimen A is the conventional strategy used primarily for parenteral and mucosal immunization, and was the control for regimen B, an immunization schedule designed to improve mucosal responses by increasing the dose and frequency of Ag exposure. Previously we have shown that unlipidated B1M is ineffective for inducing mucosal immunity (25); therefore, we used $B1MP_3C$ as immunogen in 9 of 10 protocols. The primary effect of encapsulation also was studied. In 3 of the 10 protocols, immunogens were delivered in PBS, while the rest were delivered by microparticles.

Anesthetized mice (Metofane; Pitman-Moore, Mundelein, IL) were immunized using the required dose of microparticles or MAP peptide freshly resuspended in PBS (200 μl). A feeding needle was used to administer the MAP peptide intragastrically. Following light anesthesia (Metofane; Pitman-Moore), 50 μl (10 μl at a time) of PBS containing the required dose of microparticles or MAP peptide were slowly placed via micropipette in one or both of the nares for nasal immunization.

Collection of sera and secretions

Samples of sera and saliva were obtained before the first dose, as were vaginal washings (31, 32). They were also taken at predetermined intervals after each dose, according to Taylor-Robinson and Furr (33). Animals were anesthetized before each procedure by inhalation of methoxyflurane (Metofane; Pitman-Moore). Blood samples were collected from the retroorbital plexus using a capillary tube. Salivation was induced by i.p. injection of 1 μg/g mouse pilocarpine hydrochloride (Sigma) in 100 μl of PBS (21). Vaginal washings were obtained by pipetting 30 μl of PBS in and out of the vagina several times. Particulate matter was removed by centrifugation, and the supernatant was stored at −20°C (33).

Collection and extraction of feces

Before collection of secretions, mice from each group were placed in cages without shavings, and fecal pellets were collected after 30 min. Pellets were analyzed according to Haneberg et al. (31) and de Vos and Dick (32). Samples were weighed, placed into 1.5-ml microcentrifuge tubes, and added to 1 ml of PBS containing 5% nonfat milk and a fresh mixture of protease inhibitors (10 mM leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF) (Sigma). This mixture was incubated at room temperature for 15 min. The samples were vortexed, left to settle for 15 min, and revortexed until all material was suspended. After centrifugation at 13,000 rpm for 10 min, the supernatants were removed and stored at −70°C.

Detection of anti-B1M and anti-gp120 Abs by ELISA

Microtiter plates were coated with 5 μg/well B1M peptide or 0.1 μg/well recombinant gp120 $HIV\text{-}1_{IIIB}$ isolate (Advanced Biotechnology, Columbia, MD) and incubated for 1 h at 37°C, overnight at 4°C, and 1 h at 37°C. Plates were washed four times with PBS and incubated for 1 h with PBS plus 2.5% low fat milk to block nonspecific binding. After four washes with PBS, plates were treated with pooled sera; secretions and feces from BALB/c mice were diluted in PBS/2.5% lowfat milk or individual sera; and vaginal washings, saliva, and fecal extracts from National Institutes of Health Swiss mice were diluted in PBS/2.5% lowfat milk 1/100, 1/20, 1/10, and 1/10, respectively, and left overnight at 4°C. Plates were washed four times with PBS and peroxidase-conjugate secondary Abs (anti-IgG, anti-IgA; Sigma) and incubated for an additional 2 h at room temperature. Substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to the plates, and the reaction was stopped 10 min later with 1 M phosphoric acid. Absorbances were read at 450 nm.

Preparation of cell suspensions from spleen, lymph nodes, and Peyer's patches

Single cell suspensions were obtained from spleen, lymph node, and Peyer's patches 5 to 6 wk after the last immunization of regimens A and B. Peyer's patches were prepared according to Kiyono et al. (34). They were excised carefully from the intestinal wall and dissociated using the neutral protease enzyme Dispase, 1.5 mg/ml (Life Technologies, Gaithersburg, MD), in Joklik-modified medium (Life Technologies) at 37°C by stirring for 1 h. Single cell preparations resulted. Dissociated cells were washed and resuspended to the appropriate concentration in RPMI/10% FCS medium (Life Technologies). Splenocytes and lymphocytes from lymph nodes were obtained by crushing, followed by RBC lysis with ACK buffer (0.1 mM Na_2EDTA, 1 mM $KHCO_3$, and 0.15 M NH_4Cl, pH 7.2).

T cell proliferation

A total of 2×10^3 cells obtained from spleen, Peyer's patches, and genital lymph nodes was cultured in flat-bottom 96-well microculture plates in the presence of $B1MP_3C$ peptide and irrelevant control peptide at concentrations of 700, 70, and 7 nM. The control peptide consisted of MAP containing four copies of NANP peptide, an amino acid sequence derived from the malarial circumsporozoite protein (35). After 5 days, these cells were pulsed with 1 μCi of [^{3}H]thymidine/well (ICN, Costa Mesa, CA). Cells were harvested after 18 h, and the thymidine incorporation was determined by standard liquid scintillation counting. A stimulation index was calculated as the means of triplicate determinations (mean cpm stimulated [^{3}H]thymidine incorporation divided by the arithmetic mean cpm unstimulated [^{3}H]thymidine incorporation).

T cell cytotoxicity assay

Cytotoxicity assays were performed as described previously (25). Briefly, spleen cells were cultured at a density of 5×10^6 cells/ml in RPMI/10% FCS medium for 5 days in the presence of the B1 peptide (1 μg/ml) and IL-2 (10 U/ml) (Sigma). The cytotoxic activity of the restimulated cells was tested by a standard assay with ^{51}Cr-labeled syngeneic P815 cells. To permit the presentation of the peptide on the cell surface, target cells were treated overnight with the B1 peptide (1 μg/ml), control peptide (NANP), or medium alone. The next day, target cells were washed and labeled (5×10^6/ml) with 100 μCi of ^{51}Cr for 40 min at 37°C. They were then washed extensively with cold RPMI/5% FCS and resuspended in complete medium at a concentration of 4×10^6/ml. Effector cells were resuspended in appropriate concentrations to achieve different ratios (20:1, 10:1, 5:1, 2.5:1). The target/effector cells were incubated for 4 h in 96-well round-bottom plates and centrifuged. The supernatant was counted in a gamma counter, and the percentage of specific release was calculated by the formula percent specific release = [(cpm experimental release − cpm background release)/(cpm maximum release − cpm background release)] × 100.

Alternatively, before labeling, P815 cells were infected with recombinant vaccinia virus v-env5 LAV-expressing gp160 gene products or with the wild-type vaccinia virus. (The recombinant vaccinia virus v-env5 LAV was kindly provided by Dr. S.-L. Hu, Bristol-Myers Squibb.) Briefly, P815 cells were resuspended at 5×10^6/ml and incubated with 50 plaque-forming units/cell of the recombinant or wild-type vaccinia virus for 2 h at 37°C. The infected cells were washed and incubated overnight in RPMI/10% FCS. These target cells were labeled with ^{51}Cr, as was described previously.

Statistical analysis

Statistical analyses were performed using ANOVA with Bonferroni method for adjusting multiple comparisons. Differences were considered significant at the $p < 0.05$ level.

Results

Preparation and characterization of MAP and MAP-P_3C entrapped in microparticles

Studies were performed to determine whether lipidation of MAP entrapped in microparticles would influence the morphology and the rate of peptide release. When microparticles entrapped with MAPs were exposed to conditions simulating the gastric environment by incubation with 0.1 N HCl at 37°C for 2 h (30), 18% of the unlipidated MAP was released from the microparticles, compared with 17% of the lipidated MAP. Prolonged incubation of the microparticles in PBS at 37°C showed differences in the release of the lipidated (50%) and unlipidated (32%) peptide from the microparticles. However, we found that the rates of release could differ two- to threefold depending on the m.w. of the polymer used for preparing the microparticles (data not shown). The difference in the release of lipidated vs unlipidated MAPs may be explained

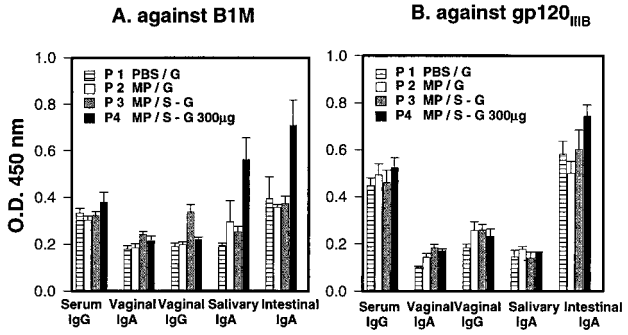

FIGURE 2. Ab response induced by B1MP$_3$C with regimen A. Samples from sera and mucosal secretions of immunized mice according to protocols 1 to 4 (days 1, 15, and 30) were collected 4 wk after the last boost. Level of Abs was measured by ELISA, against B1M in *A* and against gp120$_{IIIB}$ in *B*. Results represent the mean values (±SEM) from five mice in each group. Preimmune values were ≤0.1 absorbance unit for sera, vaginal washing, and saliva; and ≤0.2 absorbance unit for feces. MP, microparticles; G, intragastric immunization; S-G, s.c.-intragastric immunization.

by the surface appearance of microparticles entrapped with lipidated and unlipidated MAP. Scanning electron-microscope analysis showed that although both had a spherical morphology, the surface of the microparticles with entrapped lipidated MAP was smooth, while some of the surfaces of the unlipidated MAP microparticles showed small holes (data not shown). These holes may accelerate MAP release in PBS under acidic conditions. Western blotting analysis by B1M-specific antisera showed that both MAPs retained their structural integrity (molecular mass 10 kDa) and antigenicity during the microparticle preparation process (data not shown).

Effect of lipidation of MAP on IgA and IgG responses

To assess the effect of lipidation on the B1M peptide administered intragastrically with or without microparticles encapsulation, we used two basic immunization regimens (Table I). Regimen A consisted of three doses at 2-wk intervals, and regimen B had an immunization schedule with frequent and repetitive intragastric immunogen doses to improve uptake in the intestine. Previously, we have shown the oral administration of the unlipidated B1M-MAP without covalent linkage to P$_3$C does not elicit mucosal IgA (25). To determine the immune response generated after immunization with lipidated B1MP$_3$C using regimens A and B, we analyzed the level of specific Abs in sera, saliva, vaginal washings, and feces. Samples collected 4 wk after the last boost in both regimens showed the most dramatic differences between the different protocols. In regimen A, immunization with the lipidated B1M peptide either alone (protocol 1) or entrapped in microparticles (protocols 2, 3, and 4) induced enhancement of mucosal IgA and systemic IgG Ab response against the peptide B1M containing four copies of a V3 peptide, and the native gp120$_{IIIB}$ protein from which the V3 loop peptide sequence was derived (Fig. 2, *A* and *B*). Importantly, mucosal IgA Ab responses were detected in vagina, saliva, and gastrointestinal tract after immunization by the intragastric route. Statistically significant differences were observed in vaginal IgG against B1M peptide ($p < 0.0006$) in mice that were primed s.c. with B1MP$_3$C and boosted intragastrically (protocol 3) compared with immunization by the intragastric route alone (protocols 1 and 2). In the generation of intestinal IgA, the increase in the dose of the immunogen (protocol 4) produced a higher level of Abs that was statistically significant ($p < 0.01$) compared with protocols without systemic priming (protocols 1 and 2) or immunizations with systemic priming, but lower doses of the B1MP$_3$C (protocol 3) (Fig. 2*A*). When the levels of Abs generated against the gp120$_{IIIB}$ protein were compared, statistically significant differences were again observed favoring the s.c. priming used in protocols 3 and 4. These differences were evident specifically for vaginal IgA ($p < 0.0049$) (Fig. 2*B*). In regimen B, we investigated whether the encapsulation in microparticles of the unlipidated MAP, B1M (protocol 7) might improve mucosal immunogenicity. As shown in Figure 3*A*, lipidification of B1MP$_3$C (protocol 8) dramatically increased the levels of intestinal IgA ($p < 0.0024$) compared with administration of B1M-MAP alone (protocol 7). To determine the contribution of intragastric immunization in generating this humoral immune response at different sites of the mucosal immune system, mice in protocol 5 received only the s.c. priming with the lipidated peptide (B1MP$_3$C). No induction of mucosal IgA in vagina, saliva, or feces was observed, even though serum and vaginal IgG were induced by s.c. immunization, as previously reported (protocol 5, data not shown) (36).

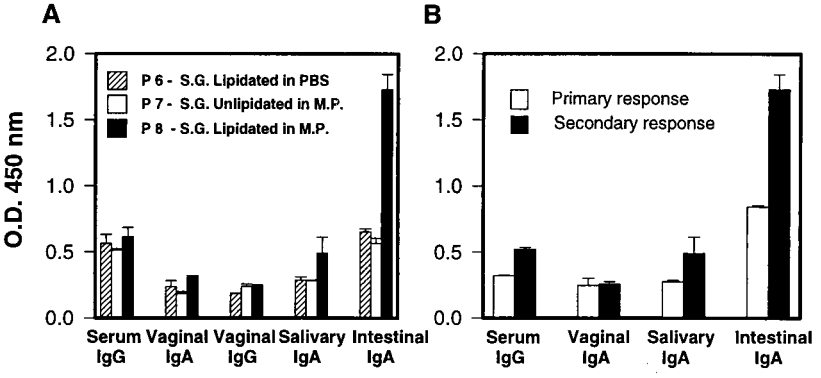

FIGURE 3. Ab response induced by B1MP$_3$C with regimen B. *A*, Sera and mucosal secretions of mice immunized according to protocols 6 to 8 (s.c. immunization on day 1; repetitive intragastric immunization on days 2 and 3; and intragastric boost on day 50) were collected 4 wk after the last boost. Ab levels were measured by ELISA against B1M. Results are presented as the mean values (±SD) of a representative assay of pooled sera and secretions from each protocol. Values obtained with preimmune samples were ≤0.1 absorbance units for sera, saliva, and vaginal washing, and ≤0.2 absorbance units for feces. *B*, Secondary response obtained after intragastric boost with B1MP$_3$C delivered in microparticles. Samples from sera and mucosal secretions were collected from mice immunized with protocol 8, on day 50 before the administration of the last boost, and on day 60, 10 days after the boost. Results are presented as the mean values (±SD) of a representative assay of pooled samples. Values obtained with preimmune samples were ≤0.1 absorbance unit for sera, vaginal washing, and saliva; and ≤0.2 absorbance units for feces. MP, microparticles; S-G, s.c.-intragastric immunization.

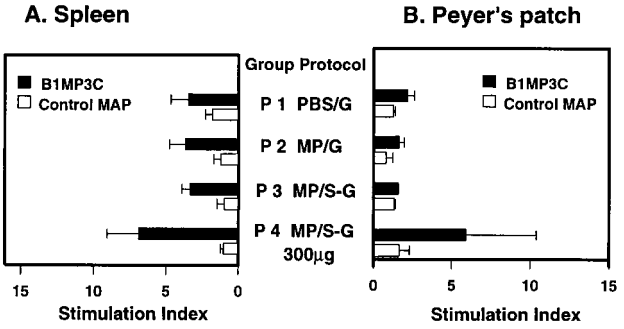

FIGURE 4. T cell proliferative response with regimen A. Lymphocytes from mice immunized with protocols 1 to 4 (days 1, 15, and 30) were obtained from spleen (*A*) and Peyer's patch (*B*) and restimulated with $B1MP_3C$ or an irrelevant MAP peptide ($(NANP)_3$-MAP_4) as control. Cell preparations were obtained 5 to 6 wk after the last boost. Results are presented as the mean (±SEM) of the stimulation index from five mice. Mice immunized with protocols 1 to 3 received 100 μg of $B1MP_3C$ peptide per dose, whereas mice immunized with protocol 4, 300 μg. MP, microparticles; G, intragastric immunization; S-G, s.c.-intragastric immunization.

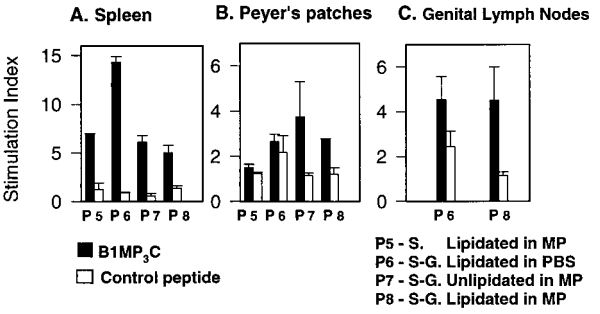

FIGURE 5. T cell proliferative response in regimen B. Lymphocytes from mice immunized with protocols 5 to 8 (days 1, 2, 3, and 50) were obtained from spleen (*A*), Peyer's patches (*B*), and genital lymph node (*C*), and restimulated with $B1MP_3C$ or an irrelevant MAP peptide ($(NANP)_3$-MAP_4) as control. Results are presented as the mean (±SEM) stimulation index from three mice in each group. Cell preparations were obtained 6 wk after the last boost. MP, microparticles; S-G, s.c.-intragastric immunization; S, s.c. immunization.

We conclude that the immunization protocols with s.c. immunization, followed by intragastric boosting with lipidated MAP entrapped in microparticles (protocols 4 and 8), could induce specific mucosal IgA responses. These immunizations were particularly efficient in the induction of intestinal IgA. Moreover, these Abs were capable of recognizing the native protein gp120, as measured in protocol 4.

Comparison of lipidated MAP-P_3C delivery in microparticle encapsulation

Previous work has suggested that the microparticle encapsulation of unlipidated Ags improves their ability to stimulate the immune system (37). Three comparative studies on the lipidated Ag were designed in regimen A to test the effect of encapsulation (protocols 1 and 2), systemic priming (protocols 2 and 3), and dosage (protocols 3 and 4). As shown in Figure 2, *A* and *B*, a limited improvement of Ab response occurred after encapsulation or systemic priming. This difference from earlier studies may be attributed to the lipidated nature of Ags. However, an effect of higher dosage was clearly observed, and a statistically significant increase was found in the levels of intestinal IgA. The dose-dependent humoral immune response was correlated with an improvement in the specific proliferative responses of lymphocytes derived from spleen and Peyer's patches from mice immunized with higher doses of $B1MP_3C$ (Fig. 4).

Other studies indicate that the frequency of exposure to an immunogen may be an important factor for generating adequate stimulation of the mucosal immune system (37, 38). Thus, the effect of encapsulation was again studied in protocols 6 and 8 using regimen B, which included systemic priming and repetitive intragastric immunizations. This approach resulted in a statistically significant ($p < 0.0024$) difference between induction of intestinal IgA using encapsulated peptide (protocol 8) and delivery in PBS alone (protocol 6, Fig. 3*A*). To measure the secondary immune response stimulated after systemic priming and initial intragastric instillations (days 2 and 3 of the immunization schedule, protocol 8), samples of sera and secretions were evaluated 1 wk before and after an intragastric boost in week 5. Statistically significant increases in the levels of serum IgG, saliva, and intestinal IgA were found (Fig. 3*B*), suggesting that this immunization schedule stimulated systemic and mucosal recall responses. Moreover, as shown in Figure 3*A*, Ab levels stayed stable for at least 30 days after the boost, corresponding to the long lasting responses obtained with Ags delivered in microparticles.

Systemic and regional cellular responses were compared in mice immunized with $B1MP_3C$ administered intragastrically or after s.c. priming in regimen A. As shown in Figure 4, the low doses of lipidated MAP used in protocols 1 to 3 induced similar proliferative responses. Systemic priming increased cellular responses by the spleen (Fig. 4*A*) and Peyer's patches (Fig. 4*B*) only when the dose of $B1MP_3C$ (protocol 4) was tripled. To evaluate systemic and regional cellular responses induced by $B1MP_3C$ delivered in microparticles via regimen B, specific lymphoproliferative responses were measured in cells derived from spleen, Peyer's patches, and the lymphoid tissue draining the genital tract. Splenocytes derived from mice immunized with protocols 5 through 8 proliferated after in vitro stimulation with $B1MP_3C$, depending on the type of dose received (Fig. 5). $B1MP_3C$ administered in PBS (protocol 6) resulted in a stronger proliferative response probably because of the intrinsic characteristic of gradual release of the Ag from the microparticles. Immunization with unlipidated or lipidated B1M-MAP entrapped in microparticles was effective in stimulating lymphocytes localized to Peyer's patches, as shown by the stimulation index of the proliferative response in protocols 7 and 8 (Fig. 5). These results suggest that microparticles were delivered to, and the B1M-MAP Ag released at this inductive site of the mucosal immune system. Moreover, lipidated B1M-MAP entrapped or not entrapped in microparticles was able to stimulate lymphocytes from the draining lymph nodes of the genital tract. Taken together, these findings show the efficacy of the lipidation in stimulating lymphocytes without requiring extraneous carriers or vehicles.

CTL responses

Previous work indicates that gastric administration of lipopeptide can prime virus-specific CTLs (25). To determine the effect of encapsulation coupled with lipidation on induction of lasting CTL activity, we compared s.c.-intragastric immunization of two groups of mice by regimen B using unlipidated and lipidated MAPs delivered in microparticles (protocols 7 and 8). Cytotoxic responses against the B1 peptide showed that although both lipidated and unlipidated peptide entrapped in microparticles stimulated CTL responses, an enhanced response was obtained from the latter (Fig. 6*A*). Importantly, substantial and comparable cytotoxic responses against target cells infected with vaccinia virus expressing gp120

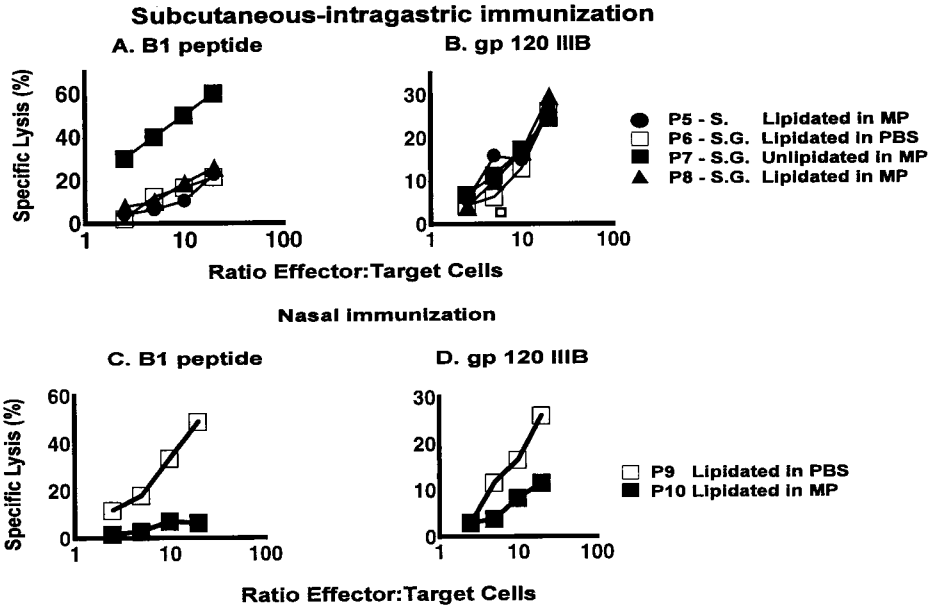

FIGURE 6. Long-lived CTL response against gp120. Spleen cells from mice immunized with protocols 5 to 8 (*A* and *B*) and protocols 9 and 10 (*C* and *D*) were measured for cytolytic activity using syngenic ^{51}Cr-labeled target cells presenting the B1 peptide or NANP peptide as control; or expressing recombinant gp120. Effector cells were obtained 6 wk after the last boost and restimulated for 5 days with B1 peptide. Results of a representative experiment from a pooled cell preparation of two to three animals are presented. This experiment was repeated twice with essentially the same result. MP, microparticles; S, s.c. immunization; S-G, s.c.-intragastric immunization.

protein were found (Fig. 6*B*). These observations indicate that microencapsulation can enhance certain CTL responses independent of lipidation. However, only immunization with a microencapsulated lipidated peptide was able to elicit simultaneously cytolytic activity against target cells expressing gp120 protein and specific lymphoproliferative responses in spleen and Peyer's patches associated with the production of specific intestinal IgA.

Nasal immunization

Nasal immunization offers a potential alternative route to oral immunization for stimulating systemic, local, and distant mucosal sites and systemic immunity. Nasal immunization with B1MP$_3$C in PBS was compared with Ag entrapment in microparticles in regimen B. In contrast to the oral route, nasal immunization was not effective in inducing intestinal IgA, showing the compartmentalization of the mucosal immune system. A small increase of IgA in saliva was observed when microparticles were used ($p < 0.01$) (Fig. 7). Systemic cellular immunity evaluated in T cell proliferative assays in splenocytes showed similar positive responses in both groups (Fig. 8). Furthermore, in vitro assay of T cell proliferation performed in Peyer's patches and genital lymph nodes as well as cytotoxic responses in splenocytes were positive when the lipidated B1M-MAP was administered in PBS (Fig. 6, *C* and *D*). These results indicate that nasal immunization by lipidated MAP alone was effective in stimulating both the systemic and mucosal immune compartments without the necessity of microparticle encapsulation.

Discussion

The development of novel strategies to induce protective immunity at the mucosal level is a priority in the design of effective vaccines against infectious diseases localized or introduced by mucosal surfaces. Systemic administration of a peptide Ag without accompanying adjuvant can create T cell tolerance rather than activation (39). We demonstrate in this work that the utility of lipidating multimeric peptide Ags for mucosal immunization can be enhanced by microencapsulation. Modified peptides, in the form of an oligomeric MAP containing a controlled cluster of lipids, such as lipidated amino acid P$_3$C, were immunogenic by the oral or nasal route. Such preparations induced humoral and cell-mediated

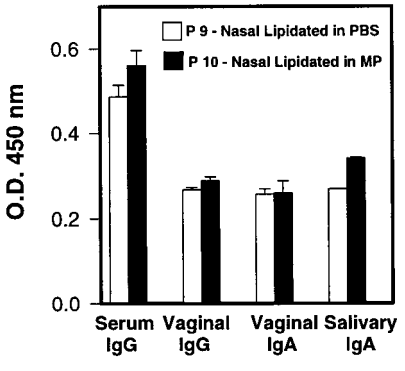

FIGURE 7. Humoral immune response induced by B1MP$_3$C by nasal immunization. Samples from sera and mucosal secretions of immunized mice according to protocols 9 and 10 (days 1, 2, 3, and 50) were collected 4 wk after the last boost. Level of Abs was measured by ELISA against B1M peptide. Results are presented as the mean values (±SD) of a representative assay of pooled samples. Values obtained with preimmune samples were ≤0.1 absorbance units for sera, vaginal washing, and saliva. MP, microparticles.

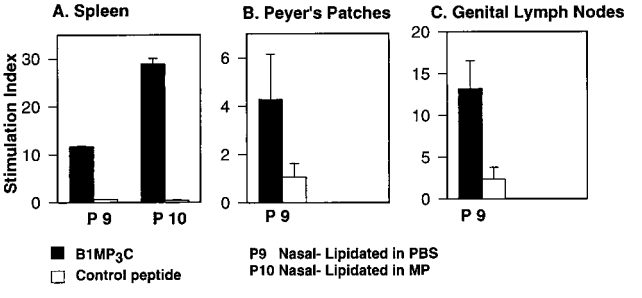

FIGURE 8. T cell proliferative response after nasal immunization with B1MP$_3$C. Lymphocytes from mice immunized with protocols 9 and 10 (days 1, 2, 3, and 50) were obtained from spleen (*A*), Peyer's patch (*B*), and genital lymph node (*C*), and restimulated with B1MP$_3$C or an irrelevant MAP peptide ((NANP)$_3$-MAP$_4$) as control. Results are presented as the mean (±SEM) of the stimulation index from three mice in each group. Cell preparations were obtained 6 wk after the last boost. MP, microparticles.

Ab responses at local, regional, and systemic levels. Abs that recognize both the immunogen, and the native protein gp120 from which it is derived, were detected in serum, saliva, vaginal secretions, and fecal extraction, despite the lack of an extraneous adjuvant. Thus, the role of lipidation can be perceived as a modification that enhances uptake by mucosal surfaces in addition to being a built-in adjuvant.

Encapsulated protein Ags have been known to elicit systemic and secretory immune responses with parenteral/oral immunization due to the uptake of microparticles by M cells (40). It is not clear, however, whether lipidated MAP delivered in PBS would be processed in a similar pathway. Thus, we can conclude that our results demonstrate the uptake of microparticles containing either lipidated or nonlipidated MAPs as well as lipidated MAPs in PBS shares a similar pathway at the mucosal inductive site. The induction of higher levels of intestinal IgA and the proliferative responses of lymphocytes derived from Peyer's patches, after intragastric immunization with B1MP3C entrapped in microparticles shows that local immune responses were enhanced by the use of microparticles as a delivery vehicle of the lipidated B1M-MAP. In addition, encapsulation of unlipidated B1M induced CTL responses that previously have been observed exclusively after immunization with lipidated MAPs.

Importantly, although systemic immunization alone without an oral boost does not elicit secretory Abs, systemic priming appears to be a powerful strategy for enhancing mucosal responses. These results extend our previous findings (25), as well as others (41), suggesting that the interactions favoring elevation of local IgA occur only when systemic and mucosal immunizations are used together. In addition, we have demonstrated a dose-dependent response after s.c.-intragastric immunization with the lipidated MAP delivered in microparticles. While some aspects of immune responses do not benefit from encapsulation, this microparticle delivery system did cause a significant increase of IgA found in fecal extractions and provided comparable efficacy in the induction of anti-gp120 CTL activity and circulating IgG.

Nasal delivery of microparticle MAP-P_3C shows no significant improvement over that in PBS alone. The development of a nasal spray for immunization, using a soluble form of Ag such as a lipidated MAP, appears to be more advantageous than the particulate microparticle form. Indeed, our results show that nasal immunization of a lipidated MAP in PBS is more effective and provides an alternative route to oral immunization by stimulating both the systemic and mucosal immune systems. An interesting finding is that a lower dose of MAP-P_3C can be used in nasal immunization to elicit a similar level of the serum IgG compared with the parenteral/intragastric route. A similar strategy has been developed by Orr et al. (42). They found that nasal immunization with a proteosome-LPS vehicle is better than the oral route for enhancing immune responses, especially at the level of the mucosa of the respiratory tract. Such compartmentalization of the mucosal immune system is evident in the analysis of secretory responses with nasal or intragastric immunization. After intragastric immunization, the IgA response is strong, although there is an absence of stimulation in intestinal secretions. In nasal immunization, however, there is a positive response in saliva and vaginal washings. These results are consistent with findings that there is a selective migration of stimulated lymphocytes to specific effector sites of the mucosal immune system (31). Mucosal immunization to produce vaginal IgA is usually difficult to achieve. For example, a herpes simplex virus synthetic vaccine administered by the nasal route resulted in perceptible levels of IgG Abs in vaginal washings, but secretory IgA was not detected (43). A more recent study using a liposome-supplemented influenza virus subunit vaccine demonstrated a secretory IgA response in the female genital tract when administered to the lower respiratory tract, but nasal and upper respiratory tract immunizations failed to demonstrate this response (44). Thus, it is interesting to note that our results obtained from nasal immunization show that a lipidated peptide could stimulate specific humoral and cellular immune responses in the genital tract. When the levels of systemic and mucosal Abs were compared in the groups primed s.c. and boosted intragastrically or nasally, a significant increase of the level of IgG Abs elicited by nasal immunization with or without microparticles was found in vaginal washings ($p < 0.0035$). These mucosal and systemic stimulations were obtained with lipidated MAP without extraneous adjuvants, delivery systems, or carriers. Our delivery system departs from conventional approaches of chemically or genetically coupling Ags to cholera toxin B or A2/B subunits, to streptococcal protein, or encasing the Ag in liposomes to elicit mucosal immunity in the genital tract (16, 45).

The necessity of stimulating antiviral immunity in the genital tract as well as in systemic lymphoid tissues is an objective for the design of an effective HIV vaccine (46). Reports on vaginal or urethral immunization with recombinant proteins indicate the possibility of stimulating local Abs and lymphoproliferative responses in genital lymph nodes and blood (47). Additionally, studies with attenuated SIV vaccine (48) administered by vaginal submucosal immunization have shown the efficacy of eliciting systemic and secretory Abs in the vagina and a virus-specific cytotoxic response in peripheral blood. The work described in this report on the stimulation of systemic and vaginal Abs, T cell proliferative response in genital lymph nodes, and a systemic cytotoxic response by a lipidated multimeric peptide provides another design relevant to vaccines against HIV. The significance of this type of design is that controlled lipidation of a synthetic vaccine can evoke a broad range of immune responses without the use of adjuvants, or adhesion Ags such as cholera toxin, or microparticles for nasal immunization. Taken together, our studies provide firm evidence for the use of lipidation on MAPs as a new method for the rational design of a high efficacy, synthetic peptide-based vaccine for mucosally acquired infections. Moreover, the data suggest that a strategy using systemic priming followed by a mucosal immunization with a combination of free and microencapsulated lipidated MAP peptide may invoke an optimal response of both CTLs and mucosal IgA.

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