Antibody Response to Polyhistidine-Tagged Peptide and Protein Antigens Attached to Liposomes via Lipid-Linked Nitrilotriacetic Acid in Mice

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Particulate delivery systems enhance antibody responses to subunit antigens. However, covalent attachment of protein antigens can disrupt protein structure and mask critical epitopes, altering the antibody response to the antigen. In this report, we evaluate noncovalent metal chelation via nitrilotriacetic acid (NTA) as a nondestructive method to attach peptide and protein antigens to liposomes. Two model antigens, ovalbumin (OVA) and a peptide derived from the membrane-proximal region of HIV-1 gp41 (N-MPR), were polyhistidinylated and attached to liposomes via monovalent NTA (mono-NTA; \( K_D \) [equilibrium dissociation constant], \( \sim 10 \mu M \)), trivalent NTA (tris-NTA; \( K_D \sim 1 \mathrm{nM} \)), or a covalent linkage. Attachment of N-MPR, but not OVA, to liposomes via an NTA lipid elicited stronger antibody responses in BALB/c mice than a formulation in which unassociated antigen was simply admixed with control liposomes lacking NTA. However, the tris-NTA linkage did not increase antibody responses to either N-MPR or OVA compared to the level for the mono-NTA linkage, despite the greater liposomal association of the antigen. For both antigens, covalently attaching them to a lipid elicited significantly stronger antibody responses than NTA-anchored antigens (OVA titer, \( 3.4 \times 10^6 \) versus \( 1.4 \times 10^6 \) to \( 1.6 \times 10^6 \) \( P < 0.001 \)); N-MPR titer, \( 4.4 \times 10^5 \) versus \( 5.5 \times 10^5 \) to \( 7.6 \times 10^5 \) \( P < 0.003 \)). The data indicate that NTA linkages may increase antibody titers to weak antigens such as N-MPR, but NTA-mediated attachment remains inferior to covalent conjugation. Moreover, enhancements in antigen-liposome affinity do not result in increased antibody titers. Thus, additional improvements of NTA-mediated conjugation technology are necessary to achieve an effective, nondestructive method for increasing the humoral response to antigens in particulate vaccines.

Protein and carbohydrate subunit vaccines are attractive alternatives to traditional killed or inactivated vaccine preparations because their compositions can be precisely controlled and they offer superior safety profiles (1, 36). Currently available vaccines against hepatitis B virus and human papillomavirus are two examples of successful protein subunit vaccines (26, 30). However, subunit preparations elicit weak antibody and T lymphocyte responses when administered without adjuvants and generally must be formulated in a particulate delivery system to elicit a robust immune response (19).

Particulates, including emulsions, gels, liposomes, and microparticles, facilitate delivery to antigen-presenting cells, provide prolonged antigen presentation through a “depot effect,” and in some cases generate proinflammatory “danger” signals (1, 29, 32). In these systems, robust immune responses generally require that the subunit antigen be chemically or physically associated with the particulate (1). Precipitation or adsorption onto aluminum salts is the traditional approach, and alum remains the only vaccine adjuvant approved for use in the United States (16, 18). Alternatively, proteins can be associated with lipidic or polymeric particulates via encapsulation or chemical conjugation (5, 8, 28, 43). However, these strategies present significant challenges. For example, encapsulation techniques can result in protein denaturation through exposure to harsh emulsification processes or organic solvents (42). Covalent conjugation relies on chemical modification of the protein surface and can alter or destroy critical epitopes (10, 49). Adsorption to solid particles, such as poly(lactide-co-glycolide) (PLG) microparticles, represents an improvement over these methods but does not allow precise control of antigen orientation and display (17, 25).

Noncovalent chemical attachment methodologies have been proposed to address these issues. One promising approach to noncovalent antigen conjugation involves metal chelation, in which polyhistidine-tagged proteins are attached to nitrilotriacetic acid (NTA)-containing liposomes or microparticles with micromolar affinity (9, 13, 44). Since NTA-Ni(II)-His binding is site specific, the physical orientation of the antigen on the particulate surface can be controlled. This is of particular importance for delivery of membrane protein antigens such as HIV-1 gp41 and other viral envelope glycoproteins, where...
plasma membrane vesicles to which polyhistidinylated dendritic cell-targeting moieties and costimulatory molecules were engraved via trivalent NTA elicited functional antitumor immunity upon administration to mice (44). However, the retention of His-tagged proteins in trivalent NTA-containing formulations in vitro remains unclear; we recently found that despite stable association of His-tagged proteins with tris-NTA-containing liposomes in serum in vitro, the proteins dissociate within minutes upon intravenous administration (37). Thus, more work is needed to clarify the relationship between NTA-Ni(II)-His affinity in vitro and biological activity in vivo.

In this study, we evaluated metal chelation via nitrilotriacetic acid as a noncovalent conjugation method for liposomal delivery of two model antigens, a short peptide derived from the membrane-proximal region (MPR) of HIV-1 gp41 (N-MPR) and a traditional strong antigen, ovalbumin (OVA). Liposomes were an appropriate delivery system for this study because they deliver associated antigens efficiently to antigen-presenting cells (12) and stimulate potent immune responses when adjuvanted with monophosphoryl lipid A (MPL) (5), and lipid-anchored NTA molecules can be readily incorporated into the formulation (9, 22). Ovalbumin was selected because it is widely used as a model antigen for assessment of humoral and cellular responses (7, 50). The N-terminal peptide of the membrane-proximal region of HIV-1 gp41 (N-MPR) was selected because this sequence is a key target for development of vaccines that elicit neutralizing antibodies (24). A site-specific NTA-mediated tether could allow presentation of the MPR in its native orientation within a lipid bilayer environment, which may be required for elicitation of MPR-targeted neutralizing antibodies (2). Moreover, since the N-MPR peptide is smaller than OVA and contains fewer antigenic determinants, its inclusion may provide insight into the use of NTA linkages for delivery of less potent antigens.

The goals of our study were 2-fold. First, we sought to determine if noncovalent attachment of OVA and N-MPR to liposomes via NTA is an effective method for eliciting antibody responses in mice compared to covalent attachment. Second, we tested the hypothesis that increased affinity of the noncovalent antigen-particulate interaction would result in enhanced antibody responses. Collectively, the data indicate that NTA linkages between antigens and liposomes are useful for increasing antibody titers to weak antigens (e.g., N-MPR), but further improvements are required to achieve titers elicited by covalent attachment of the antigen to a lipid anchor.

**MATERIALS AND METHODS**

**Materials.** Amino acid building blocks, resins, and coupling agents were obtained from Novabiochem (Darmstadt, Germany), AnaSpec (San Jose, CA), or ChemPep (Miami, FL). Cholesterol, dimyristoylphosphatidylglycerol (DMPC), dimyristoylphosphatidylcholine (DMPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-[N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]; DOD-tris-NTA, dioctadecyl-tris(trinitrilotriacetic acid); N-MPR-CHEMS, N-MPR peptide with cholesteryl hemisuccinate conjugated at the C terminus; N-MPR–His, N-MPR peptide with hexahistidine conjugated at the C terminus; OVA–His, ovalbumin with decahistidine conjugated to thiolated amines.

**FIG. 1.** Structures of NTA lipids and polyhistidine-tagged antigens. DOGS–mono-NTA, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]; DOD-tris-NTA, dioctadecyl-tris(trinitrilotriacetic acid); N-MPR-CHEMS, N-MPR peptide with cholesteryl hemisuccinate conjugated at the C terminus; N-MPR–His, N-MPR peptide with hexahistidine conjugated at the C terminus; OVA–His, ovalbumin with decahistidine conjugated to thiolated amines.
Synthesis of peptides and lipids. Peptides were synthesized on resin amide 4-methylbenzhydrylimine (MBHA) or NovaPEG resin in an automated solid-phase synthesizer (ABI 433A; Applied Biosystems, Foster City, CA) with standard fluorenylmethyloxycarbonyl, o-benzotriazol-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate, n-hydroxybenzotriazole (FMOC-HBTU-HOBT) protocols. Peptides containing N-MPR were synthesized on Novapeg resin with an orthogonally protected lysine (Fmoc-Lys(4-(4-dimethylamino-2-di(oxy-cyclohexyliden))-3-methyl-butylo)-OH [Fmoc-Lys(OAwDk)-OH]) incorporated in anhydrous DMF-DCM (DCM as needed for lipid solubilization) for 30 min at room temperature, each of HBTU, HOBT, and diisopropylethylamine (DIEA) in anhydrous DMF-preparative C4 column (214TP510; Grace Vydac, Deerfield, IL) until the unconjugated peptide was no longer detectable by matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS). Lipopeptide fractions were identified by resolution as described above. The peptide was cleaved and purified as described above, except the cleavage cocktail did not contain ethanedithiol. The molecular weights of all peptides were confirmed by MALDI-MS in a dihydroxybenzoic acid matrix. The peptide concentrations of stock solutions were confirmed by determining the A280 values of tryptophan residues, with the extinction coefficient calculated by the method of Pace et al. (34). The nomenclature, sequences, and molecular weights of the peptides used in this study are summarized in Table 1.

Preparation of polyhistidylated ovalbumin. Endotoxin contamination of ovalbumin was reduced by centrifugal filtration of a 10-mg/ml solution in phosphate-buffered saline (PBS) through a 100-kDa membrane (Amicon Ultra; Milipore, Billerica, MA) twice to remove aggregates, followed by passage down a polymyxin B endotoxin removal column (Detoxi-Gel; Pierce, Rockford, IL). Protein prepared in this manner contained less than 0.15 endotoxin units (EU)/mg endotoxin as determined by an endotoxin chromatographic LAL assay (QCL-100; Lonza, Allendale, NJ). Prior to polyhistidinylation, ovalbumin (73 nmol) was thilated by treatment with 0.6 mM 2-iminothiolane (1.6 μmol) in sodium phosphate buffer (0.1 M NaPO4, 50 mM NaCl, pH 7.5) for 1.5 h at room temperature. Thiolated ovalbumin (OVA-SH) was then allowed to react with 2×10−4 M His10-maleimide (300 nmol) in sodium phosphate buffer containing 20 mM imidazole, and OVA-His10 was eluted in sodium phosphate buffer containing 500 mM imidazole. To remove imidazole and excess His10, the solution was dialyzed at a sample to a dialysate volume ratio of 1:150 overnight in sterile PBS with 3 buffer changes (Slide-A-Lyzer; 10,000 molecular weight cutoff [MWCO]; Pierce). Protein stability and extent of modification were monitored by native sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the Bradford method, and preparations were stored at 4°C until use.

Preparation of mKate. A far-red fluorescent protein (monomeric Katushka; mKate) (40) was used as a surrogate protein for characterization of polyhistidine-tagged protein binding to NTA liposomes. The gene was codon optimized for E. coli K-12 and synthesized by overlap PCR (39) with GoTaq DNA polymerase (Promega, Madison, WI). The synthesized gene was ligated into pET15b (Novagen, Madison, WI) and transformed into a 1-mL Ni²⁺-NTA column (HisTrapFF; GE Healthcare). The column was washed with 10 ml sodium phosphate buffer containing 20 mM imidazole, and OVA-His10 was eluted in sodium phosphate buffer containing 500 mM imidazole. To remove imidazole and excess His10, the solution was dialyzed at a sample to a dialysate volume ratio of 1:150 overnight in sterile PBS with 3 buffer changes (Slide-A-Lyzer; 10,000 molecular weight cutoff [MWCO]; Pierce). Protein stability and extent of modification were monitored by native sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the Bradford method.

Table 1. Peptide sequences and molecular weights of peptide antigens

| Name       | Peptide sequence (N–C) | Expected MW   | Observed MW |
|------------|------------------------|---------------|-------------|
| N-MPR-CHEMS| NEQELDKWASLWNGK-CHEMS | 2,698.3       | 2,718.2 (Na⁺) |
| N-MPR-His  | NEQELDKWASLWNGKGGHHHHHHH | 3,166.5 | 3,163.0 |
| N-MPR-biotin| NEQELDKWASLWNGK-biotin | 2,455.6       | 2,455.0 |
| His10-maleimide | Maleimide-WGGHHHHHHHHHH | 1,896.9     | 1,901.0 |
| His10-biotin| Biotin-WGGHHHHHHHHHH | 1,972.1       | 1,973.7 |
Liposome preparation. Peptide and protein antigens were formulated in liposomes composed of 15:2:3:0.3 DMPC-DMPG-cholesterol-MPL (15, 47). NTA-containing and maleimide-containing liposomes composed of DMPC-DMPG-cholesterol-MPL and either DOOD-tris-NTA, DOGS-NTA, or MPB-PE were formed at a molar ratio of 15:2:3:0.3:0.3. Prior to use, glassware was rinsed with MeOH and CHCl₃, and dried for at least 90 min at 150°C to destroy pyrogens. Lipid solutions were combined in borosilicate glass tubes and dried to a thin film by rotary evaporation under reduced pressure. For liposomes containing N-MPR–CHEMS, lipopeptide in MeOH was added to the lipid solution prior to drying of the films. Films were further dried under a high-level vacuum overnight. Lipids were hydrated in endotoxin-free PBS (UCSF Cell Culture Facility) by intermittent vortexing, and liposomes were prepared by bath sonication for 10 min at room temperature under argon. Liposomes prepared in this manner contained endotoxin levels of less than 0.15 EU/ml when disrupted with 1.5% (vol/vol) C₂₃E₆₉ detergent (20) and assayed for endotoxin activity by an endpoint chromogenic LAL assay (QCL-1000; Lonza, Walkersville, MD).

N-MPR–His₁₀ and OVA–His₁₀ were associated with NTA-containing liposomes for 1 h at room temperature immediately prior to injection. Covalent attachment of OVA to liposomes was accomplished by addition of OVA–SH to liposomes containing MPB–PE immediately after liposome formation and continued reaction overnight at 4°C. The final formulations contained 0.1 mg/ml OVA derivative or 0.5 mg/ml N-MPR derivative and 0.5 mg/ml monophosphoryl lipid A in 20 mM carrier lipid. Vessel size was characterized by dynamic light scattering (Zetasizer 3000; Malvern, New Bedford, MA). Liposomes were stored at 4°C under argon until use. As a control, OVA–His₁₀ was adsorbed onto alum according to the manufacturer’s instructions.

Liposome-antigen association in vitro. Association of OVA–His₁₀ with NTA-containing liposomes was characterized by size exclusion chromatography. From the liposomes prepared for animal injections, an aliquot of each preparation was passed down a 1- by 20 cm Sepharose 4B–CL column under gravity flow. Liposomes eluted in the void volume and were assayed for the presence of OVA by the Bradford method. Controls included liposomes containing covalently bound OVA, liposomes lacking NTA, and liposomes lacking protein.

The dissociation of polyhistidine-tagged protein from liposomes in the presence of serum was monitored using a surrogates 27-kDa fluorescent protein, mKate. Dissociation from liposomes was measured by challenging preassociated mKate with refiltered fetal calf serum (FCS; UCSF Cell Culture Facility). Liposomes composed of 60:40 POPC-cholesterol and 1 mol% NTA lipid (where indicated) were prepared as described above. mKate was added to liposomes at a 1/2 molar ratio of protein to NTA lipid and incubated for 1 h at room temperature. Liposomal mKate was mixed with FCS at a 1:1 volume ratio and incubated at 37°C for the indicated time. After incubation, liposomes containing 2 μmol total lipid per sample were passed down a 1- by 20-cm Sepharose 4B–CL column under gravity flow to separate free mKate from liposome-associated mKate. Liposomes eluted in the void volume and were assayed for the presence of mKate by fluorescence (excitation wavelength/emission wavelength [ex/em], 544/590; Fluostar 403; BMG Lab Technologies GmbH, Durham, NC). Free mKate not associated with lipid vesicles was also monitored for fluorescence decay with time, and fluorescence intensity was unchanged after 24 h (data not shown).

Animal immunizations. All animal procedures were conducted in accordance with the policies and approval of the UCSF Institutional Animal Care and Use Committee. Ten-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were housed in a UCSF-specific pathogen-free barrier facility. Animals (4 mice per group) received subcutaneous immunizations in alternating hind hocks on days 0 and 14 as described previously (25). Each injection contained 5 μg OVA derivative or 25 μg N-MPR derivative, 25 μg MPL, and 1 μmol lipid vehicle in 50 μl sterile phosphate-buffered saline. OVA-alum injections contained 5 μg OVA adsorbed onto 325 μg alum (6.5 mg/ml) per the manufacturer’s instructions. On day 28, blood was collected from the submandibular vein for characterization of antibody responses. Cells and clotted material were removed by centrifugation at 14,000 rpm for 15 min (5415C; Eppendorf, Westbury, NY), and sera were stored at −80°C until use.

ELISA. ELISAs were developed to quantitate binding of immune sera to N-MPR, His₁₀, or OVA. Peptide ELISAs were conducted using N-MPR or His₁₀ biotinylated as described above and captured on 96-well streptavidin-coated plates (15120; Pierce, Rockford, IL). Assays were performed according to the manufacturer’s instructions, with modifications. Biotinylated peptide was added to wells in PBS containing 0.1% Tween 20 (PBS-T) and incubated for 2 h at 37°C. Following a wash step, sera were serially diluted in PBS containing 0.1% casein (C7078; Sigma-Aldrich) (PBS-C), and wells were washed 6 times with PBS-T between each step. The titer was defined as the reciprocal dilution of immune sera yielding an optical density twice that of 1:200 preimmune sera after subtraction of the level for background wells lacking serum. All samples were assayed in duplicate.

OVA ELISAs were performed as follows. Ovalbumin was diluted from a 5-μg/ml PBS stock solution to 0.1 mg/ml in carbonate-bicarbonate coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). Ten microliters of OVA (100 μl) per well was added to flat-bottomed high-capacity immunoassay plates (Costar). Plates were sealed with Parafilm and incubated at 4°C overnight. Plates were blocked with 0.5% casein for 2 h. After a wash step, immune sera were serially diluted in PBS-C and incubated in wells for 30 min. Wells were washed, and peroxidase-conjugated anti-mouse IgG was diluted 1:1,000 in PBS-C and added to wells for 30 min. Following a wash step, plates were then developed and read as indicated above. All incubations were done in 100-μl volumes at 37°C, and wells were washed 6 times with PBS-T between each step. The titer was defined as described above, and all samples were assayed in duplicate.

Statistical analysis. Statistical significance was assessed by analysis of variance and the two-tailed Student t test. For comparisons between groups with unequal variances, as determined by the Levene median test, statistical significance was assessed by the Mann-Whitney rank sum test. Differences were considered significant if they exhibited P values of <0.05. Data analyses were performed using Microsoft Excel and SigmaPlot.

RESULTS

Preparation of antigens and liposomes. Polyhistidine-modified N-MPR and ovalbumin antigens were prepared through attachment of synthetic oligohistidine sequences on solid support (N-MPR) or in solution (ovalbumin). A hexahistidine-tagged N-MPR peptide in which the polyhistidine tag was attached at the C terminus via an orthogonal protecting group was synthesized (Fig. 1). This was done for two reasons. First, it oriented the peptide in a manner that mimics the native sequence, wherein the C terminus of the sequence is tethered to the membrane and the N terminus extends outward (31). Second, attachment of N-MPR peptide to liposomes via an ε-lysyl amine permitted the structure to most closely resemble that of a previously synthesized lipid-anchored control peptide found to elicit high-antiprotein titers when administered in liposomal formulations to BALB/c mice (47, 48).

Ovalbumin was modified with decahistidine in a two-step reaction in which free amines were first modified by 2-iminothioliane to generate free thiol groups (11). These groups were then reacted with a maleimide-functionalized decahistidine peptide to generate the final conjugate. The conjugate was ensured to be free of unmodified OVA because it was purified using an NTA-Ni(II) affinity column. SDS-PAGE analysis revealed a slight increase in molecular weight upon polyhistidinylation, corresponding to 1 or 2 decahistidine peptides per protein molecule (Fig. 2). Liposomes prepared with OVA–His₁₀ exhibited vesicle diameters consistently in the 130- to 200-nm range regardless of introduction of NTA- or maleimide-functionalized lipids into the formulation (Table 2). Moreover, vesicle sizes were qualitatively consistent over time, and no visible aggregation was observed following the addition of protein.

Liposome-antigen association. The association of polyhistidineylated OVA to NTA liposomes or control liposomes in PBS was determined by size exclusion chromatography with...
protein quantitation by the Bradford method. In formulations containing DOD–tris-NTA, the protein was entirely retained in the liposome fraction (Fig. 3). Mono-NTA- and maleimide-functionalized liposomes exhibited an intermediate level of protein retention, whereas control liposomes (labeled “OVA-His10 + admixed-lipo” in the figure) did not exhibit any level of protein binding above that of the background signal of empty liposomes. When the background contribution was subtracted and peak areas were integrated, tris-NTA liposomes bound 77.7 to 83.0% of the added OVA, whereas mono-NTA- and maleimide-functionalized liposomes bound 34.4 to 38.6% of the protein (Table 3). These results underscore the differences in protein binding between mono-NTA- and tris-NTA-containing formulations, while also revealing a considerable amount of unconjugated protein in the maleimide-functionalized liposomes. This could be caused by saturation of available thiols on the protein or steric restraints at the liposome surface.

Interest in multivalent NTA adaptors arose in part due to concerns that the micromolar affinity of monovalent NTA-Ni(II)-His may be too unstable for in vivo applications (22, 45). To address this question, we determined the effect of serum on the stability of binding between liposomes containing DOGS-NTA ($K_D \sim 10 \mu M$) or DOD–tris-NTA ($K_D \sim 1 \text{ nM}$) and a surrogate hexahistidine-tagged fluorescent protein, mKate. In this experiment, protein was added to preformed NTA liposomes and allowed to associate for 1 h prior to addition of fetal calf serum. At the indicated time, liposome-associated protein was separated from free protein by size exclusion chromatography. Under the conditions studied, both mono-NTA and tris-NTA liposomes initially bound 100% of the protein added (Fig. 4). However, over time, protein dissociated from mono-NTA liposomes more quickly, with less than 50% of the protein remaining associated after 4 h, whereas at the same time point approximately 80% remained associated with tris-NTA liposomes.

**Humoral immune responses in mice.** Antibody responses to liposome-associated OVA and N-MPR in BALB/c mice were assessed. Liposomes containing N-MPR–His6 attached via either a mono-NTA or a tris-NTA linkage elicited antipeptide IgG in sera of 4 of 4 mice in each group. However, N-MPR–

![FIG. 2. SDS-PAGE gel of polyhistidinylated ovalbumin. OVA-His10 ran slightly larger than OVA-SH or unmodified OVA, corresponding to the addition of 1 or 2 decahistidine tags to the total molecular weight.](http://cvi.asm.org/)

![FIG. 3. Association of ovalbumin with liposomes containing NTA lipids. Association of OVA-His10 with NTA-containing liposomes was characterized by size exclusion chromatography. Liposomes containing tris-NTA were found to completely retain OVA-His10 under the conditions studied, whereas mono-NTA liposomes and maleimide liposomes exhibited intermediate levels of protein retention. Control liposomes lacking NTAs did not exhibit levels of protein-liposome binding greater than the background level. The results are representative of two independent liposome preparations.](http://cvi.asm.org/)

**TABLE 2. Vesicle sizes of ovalbumin-containing liposome preparations**

| Formulation                  | Vesicle diam (nm) | SD (nm) |
|------------------------------|-------------------|---------|
| OVA-His10 + admixed liposomes| 131.5             | 1.3     |
| OVA-SH + covalently attached liposomes| 157.4             | 3.1     |
| OVA-His10 + mono-NTA–liposomes| 135.5             | 0.7     |
| OVA-His10 + tris-NTA–liposomes| 168.2             | 14.8    |
| OVA-His10 + tris-NTA–liposomes (no MPL) | 194.3             | 1.6     |

* Vesicle sizes were measured by dynamic light scattering. Values represent the means of results from two independent preparations, and errors are expressed as standard deviations.

**TABLE 3. Association of ovalbumin with liposomes containing nitrilotriacetic acid-conjugated lipids**

| Formulation                  | Liposome-associated protein level (% of total) |
|------------------------------|-----------------------------------------------|
| OVA-His10 + admixed liposomes| 0.0                                           |
| OVA-SH + covalently attached liposomes| 34.4                                  |
| OVA-His10 + mono-NTA–liposomes| 38.6                                          |
| OVA-His10 + tris-NTA–liposomes| 77.7                                          |
| OVA-His10 + tris-NTA–liposomes (no MPL) | 83.0                                      |

* The association of polyhistidinylated OVA to NTA liposomes or control liposomes in PBS was determined by a size exclusion chromatography method with detection by the Bradford assay. Integrated peak areas were calculated following subtraction of the background contribution of liposomes lacking protein. Percent association was calculated as $\frac{\text{value for associated content} + \text{value for free content}}{\text{value for associated content}} \times 100$. Results are representative of two independent preparations.
His6 admixed with control liposomes lacking NTA failed to elicit a detectable anti-N-MPR antibody response. This is consistent with previous studies by our group in which liposomal N-MPR peptide required a lipid anchor to generate a substantial antibody response in BALB/c mice (47). No significant difference was observed when antibody responses elicited by mono-NTA and tris-NTA linkages were compared ($P = 0.686$). Importantly, covalent attachment of N-MPR to liposomes via a cholesteryl hemisuccinate anchor was superior to NTA-mediated conjugation for elicitation of antibody responses to N-MPR (geometric mean titer [GMT], $4.4 \times 10^4$ versus $7.6 \times 10^5$ [$P = 0.002$ for comparison with mono-NTA; $P = 0.002$ for comparison with tris-NTA]) (Fig. 5).

When OVA-His10 was the immunizing antigen, DOGS-NTA-mediated attachment elicited significantly greater antibody responses than adsorption on aluminum hydroxide gel ($GMT, 1.6 \times 10^4$ versus $4.4 \times 10^5$ [$P = 0.0002$]) (Fig. 6), as reported by Patel and coworkers in the case of p24 attached to wax nanoparticles (35). Omission of MPL from the tris-NTA formulation resulted in a decrease of an order of magnitude in anti-OVA titers ($GMT, 1.6 \times 10^5$ [$P = 0.003$]). As seen in the case of N-MPR–His6, attachment via tris-NTA did not confer any advantage over the mono-NTA linkage ($P = 0.53$). Moreover, both mono-NTA and tris-NTA formulations were inferior to covalently conjugated OVA ($GMT, 1.4 \times 10^6$ to $3.4 \times 10^6$ [$P < 0.001$]). Surprisingly, control liposomes in which the protein was unattached also elicited significantly greater anti-OVA titers than NTA liposomes ($GMT, 2.6 \times 10^6$ [$P = 0.006$ for comparison with mono-NTA; $P = 0.01$ for comparison with tris-NTA]). Statistical comparisons between groups, as determined by the two-tailed Student $t$ test and the Mann-Whitney rank sum test, are summarized in Table 4. Titer values are expressed as geometric means, and error bars represent standard deviations.

**FIG. 5.** Effect of serum on association of a fluorescent hexahistidine-tagged protein with liposomes containing NTA lipids. The dissociation of polyhistidine-tagged protein from liposomes in the presence of serum was monitored using a surrogate fluorescent protein, mKate, by incubating liposome-associated mKate with refiltered fetal calf serum at 37°C for the indicated times. After incubation, liposome-associated mKate was separated from free protein by size exclusion chromatography. Liposomes containing tris-NTA retained nearly double the protein of mono-NTA liposomes 4 h after incubation in serum.

**FIG. 6.** Effect of ovalbumin-liposome linkage on anti-ovalbumin IgG response in mice. Antibody responses to liposome-associated OVA in BALB/c mice were assessed. DOGS-NTA-mediated attachment elicited significantly greater antibody responses than adsorption on aluminum hydroxide gel ($P = 0.0002$). However, both mono-NTA and tris-NTA formulations were inferior to covalently conjugated OVA ($*, P < 0.001$). Control liposomes in which the protein was unattached also elicited significantly greater anti-OVA titers than NTA liposomes ($P = 0.006$ for comparison with mono-NTA; $P = 0.01$ for comparison with tris-NTA). No responses were observed in control groups (“No Injection” and “Empty Liposomes). Statistical comparisons between groups, as determined by the two-tailed Student $t$ test and the Mann-Whitney rank sum test, are summarized in Table 4. Titer values are expressed as geometric means, and error bars represent standard deviations.
Anti-His10 IgG antibody titers were measured to address the possibility that immunization with polyhistidine-modified antigens may generate an antibody response directed against the polyhistidine sequence. Groups of animals immunized with formulations containing OVA-His10 generated modest anti-polyhistidine sequence. Differences were considered significant if the P values were less than 0.05. All comparisons were significant except that of mono-NTA–liposomes versus tris-NTA–liposomes (indicated in bold).

In summary, NTA-mediated attachment was more effective than simply admixing antigen with liposomes lacking NTA for elicitation of serum IgG to N-MPR–His6, but not for elicitation of serum IgG to OVA-His10. NTA-mediated attachment was also more effective than adsorption on alum for elicitation of serum anti-OVA IgG responses. However, the tris-NTA anchor did not provide any enhancement compared to the mono-NTA anchor for induction of antibody to either OVA or N-MPR.

**DISCUSSION**

This study sought to characterize the importance of NTA-Ni(II)-His affinity in promoting antibody responses to polyhistidine-tagged antigens formulated with particulate carriers. Although metal chelation via NTA-Ni(II)-His has been suggested as a site-specific, nondestructive approach to particulate delivery of polyhistidine-tagged antigens, concerns that the micromolar affinity of monovalent NTA for hexahistidine may be too weak for in vivo applications have arisen (22, 27, 37, 45).

We hypothesized that the increased affinity of trivalent NTA for polyhistidine (K_D, ~1 nM) would translate to increased serum antibody responses and enhanced antibody titers compared to a monovalent NTA linkage (K_D, ~10 μM). Antibody responses to liposomal preparations of two model proteins wherein the antigen was attached via a trivalent NTA lipid anchor, a commercially available monovalent NTA anchor, or a covalent linkage in BALB/c mice were assessed.

The key findings of the study are 3-fold. First, attachment of N-MPR but not OVA to liposomes via an NTA lipid elicited stronger antibody responses in mice than a formulation in which the antigen was simply admixed with control liposomes lacking NTA (Fig. 5 and 6). N-MPR required association with the carrier, via either NTA or a covalent linkage, to elicit a response, suggesting an in vivo role for the NTA linkage in that case. No such requirement was observed for OVA; this difference may arise from the greater antigenic diversity of OVA, a large protein with many B and T cell epitopes, than of N-MPR, a peptide that contains only a few epitopes. Patel and coworkers previously reported that His-tagged HIV-1 p24 Gag, a 24-kDa protein, attached to NTA-Ni(II)-containing wax nanoparticles elicited significantly greater antibody responses than a

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**TABLE 4. Statistical significance of differences in antiovalbumin IgG titers among groups of mice immunized with ovalbumin-containing liposome formulations**

| Formulation | OVA-SH + covalently attached liposomes | OVA-His10 + mono-NTA–liposomes | OVA-His10 + tris-NTA–liposomes (no MPL) | OVA-His10 + alun |
|-------------|--------------------------------------|-------------------------------|--------------------------------------|----------------|
| OVA-His10 + admixed liposomes | 3.08E–02 | 5.61E–03 | 1.02E–02 | 2.90E–02 |
| OVA-SH + covalently attached liposomes | 2.87E–04 | 8.53E–04 | 5.28E–01 |
| OVA-His10 + mono-NTA–liposomes | | 4.11E–06 | 2.90E–04 |
| OVA-His10 + tris-NTA–liposomes | | 2.90E–02 | |
| OVA-His10 + tris-NTA–liposomes (no MPL) | | 9.33E–03 | |

**TABLE 5. Anti-His10 IgG in sera of mice immunized with OVA- and N-MPR-containing liposome preparations**

| Group | Anti-His10 IgG GMT SD |
|-------|-----------------------|
| No injection | ND ND |
| OVA-His10 + alun | 920 1,060 |
| Empty liposomes | ND ND |
| OVA-His10 + tris-NTA–liposomes (no MPL) | 350 720 |
| OVA-His10 + mono-NTA–liposomes | 1,210 2,550 |
| OVA-His10 + tris-NTA–liposomes | 800 29,580 |
| OVA-His10 + admixed liposomes | 810 1,230 |
| OVA-SH + covalently attached liposomes | ND ND |
| N-MPR-His6 + admixed liposomes | ND ND |
| N-MPR-His6 + mono-NTA–liposomes | ND ND |
| N-MPR-His6 + tris-NTA–liposomes | ND ND |
| N-MPR-CHEMS + covalently attached liposomes | ND ND |

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*a Analyses were performed using the two-tailed Student t test for comparisons with equal variances or the Mann-Whitney rank sum test for comparisons with unequal variances. Differences were considered significant if the P values were less than 0.05. All comparisons were significant except that of mono-NTA–liposomes versus tris-NTA–liposomes (indicated in bold)."
formulation lacking Ni(II) in which antigen was unassociated (35). In contrast, when Chikh and coworkers attached a short decahistidine-tagged peptide containing a cytotoxic T lymphocyte epitope to mono-NTA liposomes, they did not observe significantly increased peptide-specific splenocyte interferon gamma secretion compared to that obtained with a formulation lacking NTA following immunization of mice (9). Notably, the peptide used by Chikh et al. was found to associate with liposomes in the absence of NTA, presumably due to its hydrophobic nature, whereas N-MPR remains completely unassociated in the absence of an anchor (48). Thus, further studies will be needed to determine how the size, charge, and hydrophobicity of a particular antigen affects the utility of NTA-liposome delivery for promoting antibody responses to that antigen.

The second key finding of the study was that antigen attachment via multivalent NTA linkages with greater affinity did not result in enhanced antibody responses compared to those obtained with monovalent NTA linkages, despite association of a greater fraction of the antigen with tris-NTA liposomes (Fig. 5 and 6). This result is surprising since liposomal antigen persists at the injection site for days following subcutaneous administration (33), and differences in antigen binding to monovalent NTA and trivalent NTA liposomes would manifest over this time scale. Although tris-NTA liposomes bound more than twice the protein of mono-NTA liposomes in vitro (Fig. 3), no enhancement of antibody responses was observed. The extent of binding may be substantially altered in vivo, but similar differences in binding of OVA-His10 to mono-NTA and tris-NTA were also observed in the presence of serum (Fig. 4). It is possible that the nickel may be removed from the NTA by biological chelators such as thio compounds or zinc binding proteins such as metallothionein that are present in vivo (6). This would result in dissociation of the histidine-tagged antigen from the liposome. Alternatively, the His-tagged antigen may be exchanged with other polyhistidine-containing proteins in vivo; we recently observed that His-tagged proteins attached to liposomes via tris-NTA lipids are readily exchanged with other His-tagged proteins in the bulk solution (37). Thus, although the fate of the NTA-Ni(II)-His interaction in vivo is unclear, increased affinity up to the low nanomolar range does not translate to greater antibody responses in our system.

One interpretation of the lack of an observed correlation between antigen-NTA-liposome affinity and the magnitude of the antibody response is that an NTA-polyhistidine affinity “threshold” above which stable in vivo associations occur exists. The NTA chelator lipids tested in this study may not have reached this affinity threshold. Thus, antibody responses to NTA-anchored antigens may be improved through further modifications of the NTA moiety to increase the affinity and stability of the NTA-polyhistidine interaction. Potential modifications include further increasing NTA valency (27), modifying the flexibility and length of the spacer between the monovalent NTA and the scaffold (21), and incorporating hydrophobic shielding groups that impede removal of the chelated metal. These approaches are discussed in greater detail in recent publications from our laboratory (21, 37). We are also investigating branched and multivalent polyhistidine architectures as novel adaptors for NTA-Ni(II) (unpublished results). Although these structures could not be genetically carried in recombinant proteins, they may be useful for delivery of synthetic antigens or as bioconjugation adaptors for multicomponent vaccine constructs.

A third important observation was that NTA linkages were inferior to covalent conjugation for elicitation of antibody responses to liposomal formulations of OVA and N-MPR (Fig. 5 and 6). In the case of OVA, antibody titers did not correspond with extent of protein-liposome binding, as essentially double the amount of OVA associated with the tris-NTA formulation compared to the amount obtained with the covalently attached formulation in vitro (Fig. 4 and Table 3). Thus, extent of antigen-particulate association is not a clear correlate of antibody induction in this system. This effect may be antigen specific, since N-MPR required lipid attachment, via either an NTA linkage or a covalent conjugation, to elicit an antibody response. The greater serum antibody titers induced by covalent antigen attachment could be explained by a benefit in antigen processing downstream of particulate association and internalization or by stimulation of innate pattern recognition receptors (38, 46). Indeed, we have found that the structure of the lipid anchor dramatically affected the serum IgG response to liposomal N-MPR lipopeptidies despite complete retention of all antigens in the liposome formulation (47).

Control liposomes in which OVA was completely unassociated elicited significantly greater anti-OVA responses than liposomes containing NTA-anchored OVA (Fig. 6). These findings appear to conflict with the work of Patel and coworkers showing that NTA-Ni(II) wax nanoparticles elicited greater responses to polyhistidine-tagged p24 antigen than control nanoparticles lacking Ni(II) (35). However, this discrepancy may arise from the greater immunostimulatory capacity of the MPL-adjuvanted liposomes than of the nanoparticles used in the study by Patel and coworkers (4). In the current study, anti-OVA IgG serum titers elicited by liposomes containing NTA-anchored OVA declined by an order of magnitude when MPL was removed from the formulation, confirming that MPL plays a role in the response. In addition, OVA admixed with MPL-adjuvanted liposomes elicited significantly greater antibody responses than OVA alone in PBS (GMT, $2.6 \times 10^6$ versus $3.1 \times 10^4 \; [P = 0.0001]$) despite a lack of any detectable binding of OVA-His10 to the admixed formulation (Fig. 3 and Table 3). Thiolation of OVA also could have altered the response; however, thiolyzed bovine serum albumin was not found to elicit antibody responses when admixed with control liposomes in A/J mice in a similar experiment (41). Moreover, analogous modifications of other proteins generally reduce, rather than increase, immune responses (14). In addition, the immunogenicity of the polyhistidine tag did not appear to contribute to differences in anti-OVA antibody responses (Table 5).

In summary, these studies highlight the limitations of current nitrotriacetic acid lipid technology for particulate delivery of subunit vaccine antigens. Association with an MPL-adjuvanted liposomal carrier was required for elicitation of antibody responses to a weak antigen (N-MPR) but not for elicitation of antibody responses to a potent antigen (OVA). However, enhancements in NTA-Ni(II)-His affinity ($K_{D} \approx 10\mu M$ to $\sim 1\mu M$) did not result in increased antibody responses; there were no differences in antibody titers when the antigens were attached via the monovalent or the trivalent NTA link-
ages. Additionally, covalent attachment to the carrier was superior to NTA-mediated attachment for elicitation of antibody responses. Thus, further improvements of the NTA-mediated conjugation strategy are required before it will be an effective, nondestructive method for attaching antigens to particulate vaccine carriers and eliciting high-titer antibody responses.

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