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Cationic Amphiphile with Shikimic Acid Headgroup Shows More Systemic Promise Than Its Mannosyl Analogue as DNA Vaccine Carrier in Dendritic Cell Based Genetic Immunization

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Mannosylated cationic vectors have been previously used for delivering DNA vaccines to antigen presenting cells (APCs) via mannose receptors expressed on the cell surface of APCs. Here we show that cationic amphiphiles containing mannose-mimicking quinic acid and shikimic acid headgroups deliver genes to APCs via mannose receptor. Cationic amphiphile with shikimic acid headgroup was more efficacious than its mannosyl counterpart in combating mouse tumor growth by dendritic cell (the most professional APC) based genetic immunization.

Introduction

Development of vaccines is considered as one of the most remarkable triumphs of medical science. Traditionally, vaccines comprise proteins, live attenuated viruses, or killed bacteria. More recently, DNA vaccination, the administration of antigen encoded DNA, is gaining increasing attention as an emerging therapeutic approach for the treatment of many complex disorders including cancer, infectious disease, and allergies.[1a,b] DNA vaccines are capable of inducing humoral and cellular immune responses and are regarded as potentially safer than their attenuated virus counterparts.[2] To elicit an immune response, the antigen encoded DNA first needs to be captured by body's antigen presenting cells (APC) including dendritic cells (DC), macrophages, and B-lymphocytes. Transfected APCs can then process the expressed antigenic proteins through its proteosome complexes into small peptide fragments and can present these small peptide fragments to immune systems ($CD8^+$ and $CD4^+$ T-lymphocytes) in a recognizable fashion in complexation with major histocompatibility complexes (MHC) class I and class II molecules.[3a−d] However, antigen presenting cells are hard to transfect. Use of cationic microparticles,[4a,b] cationic liposomes,[4c] and cationic peptide,[4d] etc. have previously been reported for direct transfection of APCs in DNA vaccination.

An emerging approach for enhancing the efficacy of genetic immunization is based on targeting DNA vaccines to APCs via mannose receptor, a 180 kDa multidomains unique transmembrane receptors expressed on their cell surfaces.[5] For instance, use of mannan (a ligand for the mannose receptor) coated liposomes for intranasal delivery of HIV-1 DNA vaccine,[6a] mannan-coated cationic nanoparticles for topical immunization,[6b] and mannosylated cationic liposomes has been reported for delivering DNA vaccine to APCs.[6c−e] Grandjean and co-workers have demonstrated that lysine based clusters of mannose mimicking carbocyclic acids such as quinic and shikimic acid are also effective ligands for the mannose receptor of dendritic cells.[7a,b] Using a model lipopeptide vaccine, they have demonstrated that such mannose mimicking ligands hold potential for solubilizing lipopeptide vaccines (which are otherwise prone to aggregation), and the resulting mixed micelles are taken up mainly via endocytosis by DCs in vitro.[7c] However, systemic studies aimed at exploring the use of cationic amphiphiles containing such mannose-mimicking shikimic and quinic acid headgroups have not yet been undertaken. To this end, herein we show that cationic mannose-mimicking amphiphiles with quinic and shikimic acid headgroups (**1** and **2**, respectively, Figure 1) can target DNA to antigen presenting cells via mannose receptors. Importantly, we show that subcutaneous administration of DCs pretransfected with electrostatic complex of pCMV-MART1 plasmid DNA (encoding MART1 antigen of human melanoma tumor) and cationic liposomes of mannose mimicking amphiphile with shikimic acid headgroup (**2**) provides more tumor protective effect in C57BL/6 mice challenged with aggressive B16F1 melanoma tumor than subcutaneous administration of the corresponding lipoplex of mannosylated cationic glycolipid (**3**, Figure 1). Taken together, the present findings demonstrate for the first time that use of a cationic amphiphile with mannose-mimicking shikimic acid headgroup provides enhanced therapeutic benefits compared to its mannosyl analogue in the emerging field of DC-based DNA vaccination.

Results and Discussion

We synthesized two cationic amphiphiles containing mannose-mimicking quinic acid (**1**) and shikimic acid (**2**)

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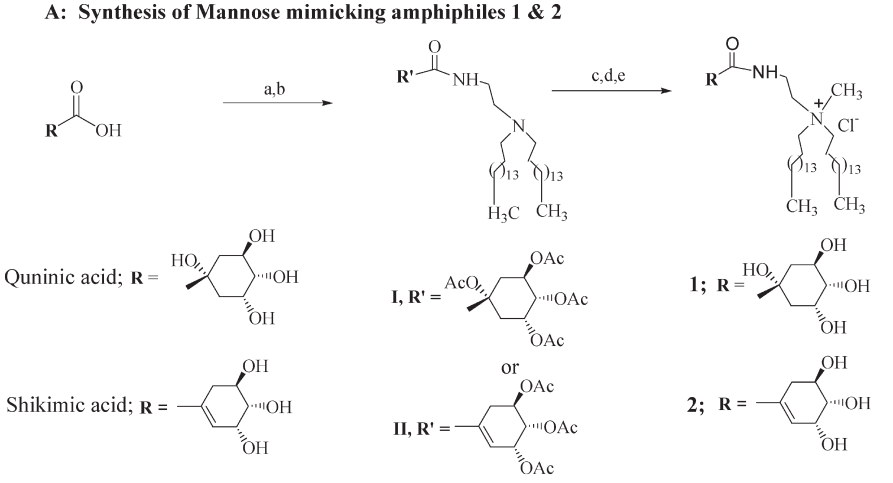

Figure 1. Syntheses of mannose-mimicking cationic ampiphiles **1** and **2** and their mannosyl analogue **3**.

headgroups as shown schematically in Figure 1A. Briefly, the quinic and shikimic acids were first converted to their tetra-*O*-acetyl and tri-*O*-acetyl derivatives, respectively. The acetyl derivatives of quinic and shikimic acids (intermediates **I** and **II**, respectively, Figure 1A) upon peptide coupling with *N*-2-aminoethyl-*N*,*N*-di-*n*-hexadecylamine followed by sequential quaternization with methyl iodide, acetyl deprotection with sodium methoxide/methanol, and chloride ion exchange over amberlyst resin afforded the target cationic mannose mimicking amphiphiles (Figure 1A).

The control mannosylated cationic glycolipid (**3**, Figure 1B) was synthesized by selective deprotection of the anomeric *O*-acetyl group of penta-*O*-acetyl-D-mannose followed by conversion of the anomeric OH group to its trichloroacetamidine derivative. The resulting trichloroacetamidine derivative upon reaction with *N*-2-hydroxyethyl-*N*,*N*-di-*n*-hexadecylamine afforded intermediate **III** (Figure 1B). The intermediate **III**, upon quaternization with methyl iodide followed by acetyl deprotection with K_2CO_3/methanol and chloride ion exchange over amberlyst resin afforded the control mannosylated cationic glycolipid (**3**, Figure 1B).

To establish that the mannose mimicking cationic amphiphiles **1** and **2** and the control mannosylated cationic amphiphile **3** target DNA to APCs via mannose receptor, first we evaluated their transfection efficiencies in RAW 264.7 cells (murine macrophage cells as a model antigen presenting cells) using luciferase reporter gene expression assay. All three amphiphiles in combination with equimolar cholesterol as colipid were transfection efficient across the entire lipid/DNA charge ratios 1:1 to 8:1 (Figure 2A), and the transfection efficacies of all the amphiphiles **1**−**3** were significantly diminished when RAW 264.7 cells were pretreated with mannan, a natural ligand of mannose receptor (Figure 2B). No such decrease of transfection efficiencies in presence of mannan was observed in NIH 3T3 cells (mouse fibroblast cells used as a control non-APCs). Rather the transfection efficacies in NIH3T3 cells were found to be somewhat enhanced presumably because of some favorable nonspecific interactions between the lipoplexes and the cell surface receptors (Figure 2C). We also carried out cellular uptake experiments using lipoplexes of lipids **1**−**3** containing fluorescently (FITC) labeled plasmid DNA and using lipoplexes prepared with fluorescently labeled (Rho-PE) cationic liposomes in RAW 264.7 cells. In both these cellular uptake experiments, the number of fluorescently labeled cells was found to be remarkably less when cells were preincubated with mannan than for untreated cells (Figures S12−S17, Supporting Information). Such findings in cellular uptake experiments and the results summarized in Figure 2 are consistent with the supposition that the mannose mimicking amphiphiles **1** and **2** and the control mannosylated cationic glycolipid **3** deliver DNA to APCs via mannose receptor.

Among the various APCs, DCs are the most professional antigen presenting cells (since they express the costimulatory molecules, e.g., CD86, necessary for efficient antigen presensation to naive T cells) and play a very critical role in antigen presentation under systemic settings.[8] The therapeutic approach based on expressing tumor associated antigen (TAA) or viral antigen in DCs is finding increasing exploitation in genetic immunization for treating cancer and infectious diseases.[4d,9a−c] Since such approach of using DCs pretransfected with expression constructs for TAA genes is effective

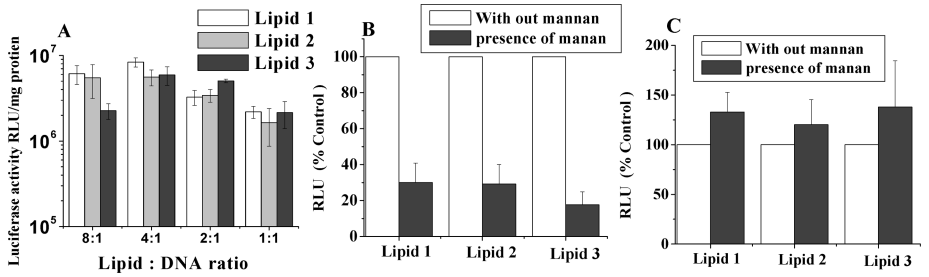

Figure 2. (A) Transfection efficiencies of **1**−**3** in RAW 264.7 cells with equimolar cholesterol as colipid across the lipid/DNA charge ratios 1:1 to 8:1. The RLU (% control) values (filled bars) refer to the average values of the RLU/mg of protein (done in triplicate) in the presence of mannan compared to the average values of the RLU/mg of protein (done in triplicate) in the absence of mannan (taken as 100, open bars) in RAW 264.7 cells (B) and in NIH3T3 cells (C) using lipid/DNA charge ratios of 8:1.

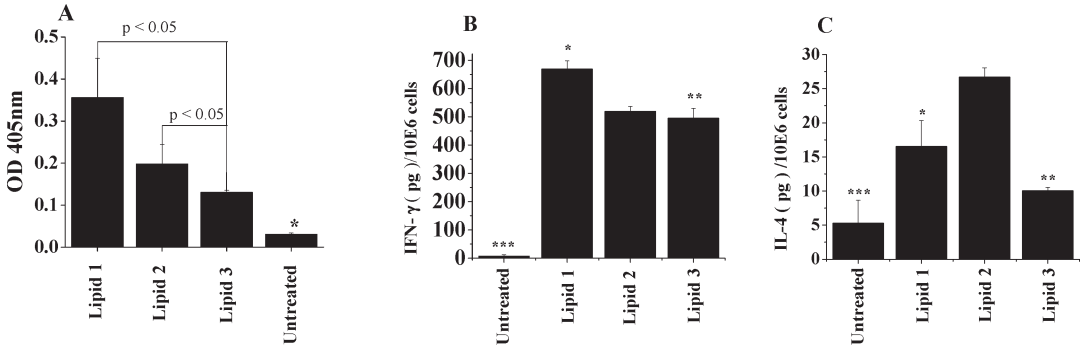

Figure 3. (A) Humoral immune responses in C57BL/6J mice upon subcutaneous administration of mbmDCs pretransfected with lipoplexes of **1**−**3** and p-CMV-β-gal as a model genetic vaccine. The 6−8 week old female C57BL/6 mice (each weighing 20−22 g, $n = 3$) were immunized subcutaneously with 5×10^5 cells of pretransfected mbmDCs (twice with a 7-day interval). Two weeks after second immunization, serum samples were collected from mice and assayed for β-gal antibodies by ELISA. The *Y*-axis represents absorbance obtained with a 1:300 dilution of serum: (∗) $P < 0.005$ for lipids **1** and **2** compared with values for untreated mbmDCs. (B, C) Humoral (Th2) and cellular (Th1) immune responses in C57BL/6J mice upon subcutaneous administration of mbmDCs pretransfected with lipoplexes of **1**−**3** and the therapeutic DNA vaccine pCMV-MART1. Subcutaneous administration of mbmDCs pretransfected with pCMV-MART1 lipoplexes of **1**−**3** elicit Th1 and Th2 immune responses. The 6−8 week old female C57BL/6 mice (each weighing 20−22 g, $n = 5$) were immunized subcutaneously with 5×10^5 mbmDCs (twice with a 7-day interval). Two weeks later, mice were sacrificed to isolate splenocytes and used immediately (without invitro restimulation) in ELISA assays for IFN-γ (B, (∗) $P < 0.01$, (∗∗) $P < 0.05$, (∗∗∗) $P < 0.0005$) and for IL-4 (C, (∗) $P < 0.05$, (∗∗) $P < 0.01$, (∗∗∗) $P < 0.01$). ∗, ∗∗, ∗∗∗ refer to comparison between mannose mimicking amphiphile **2** and **1**, **2** and **3**, and **2** and untreated mbmDCs, respectively.

and safe and because DCs, the most professional antigen presenting cells, express high levels of mannose receptors on their cell surface,[5] we next measured the abilities of **1**−**3** to transfect DCs using pα5GFP (plasmid DNA encoding green fluorescence protein). First, we confirmed the expression of major histocompatibility complex II (MHC-II, surface and intracellular) and the costimulatory molecules CD86, CD11c, CD40 and the mannose receptors (the common immature DC markers) in mouse bone marrow derived DCs (mbmDCs) isolated from the bone marrow of male C57BL/6 mice by flow cytometry (Figure S10, Supporting Information). Although low in efficiency (∼3%), flow cytometric analysis confirmed expression of GFP in mbmDCs transfected by lipids **1**−**3** (Figure S11, Supporting Information). It is worth mentioning that mbmDCs are hard to transfect and successful genetic immunization has previously been reported using mbm DCs transfected with cationic peptide, CL22, with DC transfection efficiency as low as 1%.[4d] Before probing the relative therapeutic potentials of **1**−**3** in genetic immunization, next we examined whether subcutaneous administration of the mbmDCs transfected with the lipoplexes of **1**−**3** and pCMV-SPORT-β-gal plasmid (as a model DNA vaccine) was competent in inducing antigen-specific humoral response in vivo. Importantly, mbmDCs transfected with the mannose mimicking amphiphiles **1** and **2** were found to be more efficient than the mbmDCs transfected with mannosylated lipid **3** and untreated mbmDCs in eliciting anti-β-Galactosidase antibody responses (Figure 3A).

Finally, we evaluated the therapeutic potentials of the mannose mimicking amphiphiles **1** and **2** and their mannosylated counterpart **3** under systemic settings in DC-based genetic immunization. Human MelanA/Mart1 antigen shares 68.6% amino acid sequence identity with its murine counterpart[10] and has been used in inducing immune response in mouse against the murine B16 melanoma.[11a,b] Subcutaneous administration of the pCMV-MART1 plasmid DNA encoding the human melanoma MART1 antigen in complexation with the mannose mimicking amphiphile **2** with shikimic acid headgroup was found to be effective in inhibiting the growth of the B16 melanoma in C57BL/6 mice when the latter was challenged with aggressive B16F1 melanoma tumor after genetic immunization (Figure 4). However, the corresponding lipoplexes of the mannose mimicking amphiphile with quinic acid headgroup (**1**) and its mannosyl analogue (**3**) were found to be significantly less efficient in inhibiting the B16 melanoma tumor (Figure 4). Since cytokine production and tumor regression in mice model have recently been reported to be mediated by activation of toll-like receptor 9 (TLR9) by lipoplexes,[14a,b] we also carried out the tumor challenge experiment using a control lipoplex prepared with lipid **2** and a nonspecific plasmid DNA (pCMV-β-gal). The tumor growth inhibition observed with such control lipoplex

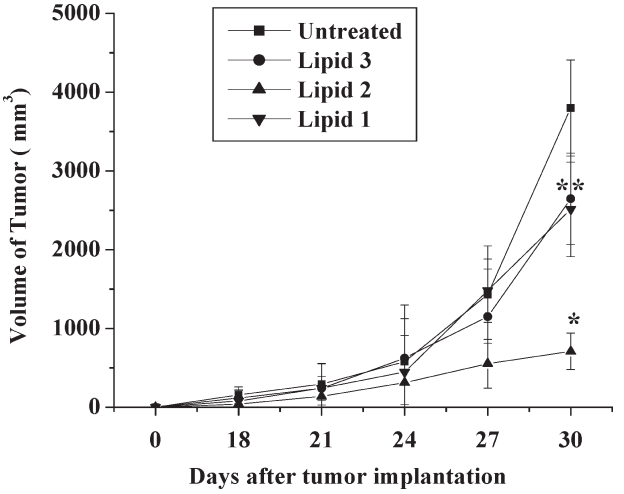

Figure 4. Tumor growth inhibition efficiencies of lipoplexes of **1**−**3** and pCMV MART1 in DC based genetic immunization. The 6−8 week old female C57BL/6 mice (each weighing 20−22 g, $n = 5$) were immunized by subcutaneous administration of mbmDCs pretransfected with lipoplexes of **1**−**3** and p-CMV-MART1, twice with a 7-day interval. Two weeks after second dose mice were challenged by subcutaneous injection of B16F1 cells. Tumor volumes ($V = {}^1/_2ab^2$ where a is maximum length of the tumor and b is minimum length of the tumor measured perpendicular to each other) were measured with a slide calipers for up to 30 days. Results represent the mean ± SD for $n = 5$ tumors: (*) $P < 0.005$ compared to the control untreated mbmDCs.

was remarkably less than that observed for pCMV-MART 1/lipid **2** lipoplex (Figure S18, Supporting Information).

The tumor growth inhibition results summarized in Figure 4 demonstrate the therapeutic promise of mannose mimicking amphiphile with shikimic acid headgroup in DC-based DNA vaccination. $CD4^+$ Th cells exhibit their helper functions through secreted cytokines. Differences in cytokine secretion patterns among the two Th cell subsets (Th1 and Th2) determine the type of immune response mounted against a particular antigenic challenge. Th1 subset is responsible for mounting cell-mediated immune response, e.g., activation of T_C cells, while Th2 subset stimulates humoral responses, e.g., activation of antibody producing B cells. Two defining cytokines secreted by Th1 and Th2 cells are interferon γ (INF-γ) and interleukin-4 (IL-4), respectively.[12] To gain insights into the contribution of each of these two subsets of Th cells in providing melanoma tumor protection, we measured the amounts of these two signature cytokines in mice spleenocytes after genetic immunization with DCs pretransfected with lipoplexes of pCMV-MART1 plasmid. While the amount of secreted INF-γ was higher for lipoplex of lipid **1** than for the lipoplexes of lipids **2** and **3** (Figure 3B), lipoplex of the mannose mimicking amphiphile **2** provided higher amount of IL-4 than lipoplexes of **1** and **3** (Figure 3C). Thus, a mixed Th1/Th2 immune response is likely to be responsible for the observed tumor protective properties of the mbmDCs transfected with pCMV-MART1/**2** lipoplex. Although IL-4 might be required for tumor rejection in some animal models, in general, INF-γ is more important than IL-4 in mediating tumor rejection. It is not obvious at this stage of investigation why IL-4 rather than INF-γ mediates tumor rejection in the present model. Clearly, further mechanistic studies need to be undertaken in the future toward gaining more insight to this end. In addition, future studies aimed at probing possible role of cytotoxic T cells and natural killer (NK) are likely to throw further mechanistic insights into the observed tumor rejection property of lipoplex **2**.

In conclusion, the present findings demonstrate that cationic amphiphiles containing mannose-mimicking quinic acid and shikimic acid headgroups target DNA vaccines to APCs via mannose receptor. Cationic amphiphile with mannose-mimicking shikimic acid headgroup provides enhanced tumor protective therapeutic benefits compared to its quinic acid and its mannosyl analogue in DC-based DNA vaccination. Measurements of the amounts of the secreted INF-γ and IL-4 from the spleenocytes of the immunized mice indicated a mixed Th1/Th2 immune response to be responsible for the observed tumor protective properties of the mbmDCs transfected with pCMV-MART1/**2** lipoplex. Future structure−activity studies are expected to further enhance the therapeutic benefits of cationic amphiphiles containing mannose-mimicking shikimic/quinic acid headgroups in DC-based genetic immunization.

Experimental Section

General Methods and Reagents. Mass spectral data were acquired by using a commercial LCQ ion trap mass spectrometer (ThermoFinnigan, SanJose, CA) equipped with an ESI source or micromass Quattro LC triple quadrapole mass spectrometer for ESI analysis. The FABMS analyses were performed on a Micromass AUTOSPEC-M mass spectrometer, Manchester, U.K. ^{1}H NMR spectra were recorded on a Varian FT 200 MHz or AV 300 MHz NMR spectrometer. Quinic and shikimic acids were purchased from Fluka (Switzerland). D-Mannose was procured from SD Fine Chemicals, Hyderabad, India. EDCI[a] and HOBt were purchased from Sigma-Aldrich. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60−120 mesh). The purities of all the target lipids **1**−**3** were confirmed to be ≥95% by reversed phase analytical HPLC analysis using two different mobile phases (A, pure methanol; B, 95:5, v/v, methanol/water).

Preparation of Liposomes. The liposomes of **1**−**3** were prepared by the conventional method. Briefly, lipid and cholesterol (at a mole ratio of 1:1) were dissolved in chloroform. The solvent was then evaporated under a thin stream of nitrogen gas, vacuum-dried for 8 h, and hydrated in deionized water overnight to give a final lipid concentration of 1 or 5 mM. Initial optimization experiments revealed that liposomes of lipids **1**−**3** containing mixed colipids, namely, equimolar mixture of DOPE, cholesterol, and DOPC, were more efficient than liposomes prepared with only equimolar cholesterol in transfecting dendritic cells (data not shown). The hydrated lipid film was first vortexed for 30 s and then sonicated until clarity using a Branson 450 sonifier at 100% duty cycle and 25 W output power. The resulting clear aqueous liposomes were used in preparing lipoplexes.

Statistical Analysis. Data are represented as the mean ± SD and were compared among different groups using the Student t test. $p < 0.05$ was considered as significant.

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[a]Abbreviations: EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenztriazole; Chol, cholesterol; DCM, dichloromethane; DMEM, Dulbecco's modifid Eagles medium; DMF, *N*,*N*-dimethylformamide; FBS, fetal bovine serum; PBS, phosphate buffered saline; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phasphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; mbm DC, mouse bone marrow derived dendritic cells.

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Supporting Information Available: Detailed descriptions for syntheses of lipids **1**−**3**, ^{1}H NMR, high resolution mass spectra, reverse phase HPLC chromatograms, and HPLC conditions in two mobile phases for the cationic amphiphiles **1**−**3**, details for transfection and cellular uptake experiments, isolation of mbm DCs, DC-based immunization of mice, ELISA assays, and FACS protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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