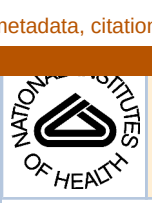


Published in final edited form as:

Int J Pharm. 2009 February 23; 368(1-2): 56–62. doi:10.1016/j.ijpharm.2008.09.053.

The Effects of Salt on the Physicochemical Properties and Immunogenicity of Protein Based Vaccine Formulated in Cationic Liposome

Weili Yan and **Leaf Huang**[1]

Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Abstract

Recently, we have developed a simple and potent therapeutic cancer vaccine consisting of a cationic lipid and a peptide antigen. In this report, we expanded the utility of this formulation to a protein based vaccine. First, we formulated the human papillomavirus (HPV) 16 E7 protein (E7) in different doses of DOTAP liposome. The results showed that these formulations failed to regress an established tumor. However, when sodium chloride (30 mM) was added to the DOTAP (100 nmol) / E7 (20 μg) formulation, anti-tumor activity was generated in the immunized mice. Correlatively, 30 mM NaCl in the DOTAP/E7 protein formulation increased the particle size from ~350 to 550 nm, decreased the protein loading capacity (from 95 to 90%), and finally increased the zeta potential (from 29 mV to 38 mV). Next, a model protein antigen ovalbumin (OVA) was formulated in different doses of DOTAP liposomes. Similarly, the results showed that 20 μg OVA formulated in 200 nmol DOTAP with 30 mM NaCl had the best OVA- specific antibody response, including both IgG_1 and IgG_{2a}, suggesting both Th1 and Th2 immune responses were generated by this formulation. In conclusion, we have expanded the application of cationic DOTAP liposome formulation to protein based vaccines and also identified that small amounts of salt could change the physicochemical properties of the vaccine formulation and enhance the activity of the DOTAP/protein based vaccine. The enhancement of immune responses by salt is possibly due to its interference of the electrostatic interaction between the cationic lipid and the protein antigen to facilitate the antigen release from the carrier and at the same time activate the antigen presenting cells.

Keywords

Cationic liposome; Protein antigen; Immunogenicity; Salt; Vaccine

1. Introduction

Recent vaccine development efforts have moved away from vaccines containing the whole organism and toward safer subunit vaccines containing only the component necessary to solicit a satisfactory immune response. The new generation vaccine system is based on protein, peptide or DNA, which are considered to be safer than vaccines based on the attenuated or

1Address correspondence and reprint requests to Dr. Leaf Huang, Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina at Chapel Hill, 2316 Kerr Hall, CB# 7360, Chapel Hill, NC 27599-7360, USA. leafh@unc.edu.

killed organism. To generate strong T- or B-cell mediated immune response, the subunit vaccine must efficiently deliver the antigen to the antigen presenting cells (APC) [2] and, at the same time activates the APC [1-3]. We have recently discovered that a cationic liposome, composed of a single cationic lipid DOTAP, can deliver a cytotoxic T lymphocyte (CTL) epitope peptide E7 to the dendritic cells (DC) and immunize the murine host against the growth of the TC-1 tumor cells which contain the human papilloma virus (HPV) DNA [4]. Thus, the therapeutic cancer vaccine contains only two molecules: the antigen and an adjuvant. DOTAP liposome, in addition to its potent delivery activity, must also activate DC.

Interestingly, cationic liposome stimulates the expression of CD80/CD86 on DC, but not the release of TNF-alpha from DC, suggesting the existence of a NF-κB-independent immunostimulation pathway for cationic lipids such as DOTAP. It has been shown that cationic lipid stimulation of expression of CD80/CD86 is dependent on the certain structure of the cationic lipid, suggesting this process is not non-specific [5]. Stimulation of DC by DOTAP liposomes leads to the production of the reactive oxygen species and activation of multiple signaling pathways, including ERK (extracellular-signal-regulated kinase) and p38 [6]. Eventually, DOTAP liposomes induce chemokines/cytokines production and co-stimulatory molecules expression [7].

A protein based vaccine can induce both antibody response and T-cell response in the immunized host. Furthermore, a protein contains multiple epitopes including T-helper (Th) epitopes, which play a key role in tumor immunity, for example, *in vivo* $CD8^+$ T-cell response priming [8] and/or memory generation [9]. The existence of highly diverse haplotypes in MHC (major histocompatibility complex) I and II molecules among the human population also makes the whole protein an attractive molecule to deliver [10].

Plasmid DNA (pDNA)–cationic liposome complexes (i.e. lipoplexes) were well known to lead to systemic gene expression, particularly in the lung [11-14]. Lipoplexes are often prepared in a non-ionic solution due to their well-known tendency to aggregate out of the solution as the salt concentration increases [15]. Aggregation during lipoplex formation in isotonic solution (150 mM sodium chloride) may be due to neutralization of the surface positive charge by the associated counter ion, thus decreasing the repulsion among the lipoplexes. Interestingly, the addition of low concentration of salt (10 mM sodium chloride) during complex formation enhanced gene expression in the lung [16]. Liposomes of various lipid compositions have been widely used to deliver protein as an antigen [17-19]. However, the effect of salt on the physicochemical properties and immunogenicity of cationic liposome/protein complex, especially for T cell response, remains unknown.

We propose that the addition of salt in the cationic liposome/protein complex could also boost the immune response due to the altered physicochemical properties. Here, we report that cationic DOTAP liposome/protein/salt complex serves as an efficient adjuvant/delivery system for a protein antigen and induces both potent antibody and CTL responses.

2. Materials and Methods

2.1 Materials

DOTAP (1, 2-dioleoyl-3-trimethylammoninum propane) and other lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The complete and incomplete Freund's adjuvants

[2]Abbreviation used are: APC: antigen presenting cells; BMDC: bone marrow-derived dendritic cells; CFA: Complete Freund's Adjuvant; CFSE : carboxyfluorescein succinimidyl ester; CTL: cytotoxic T lymphocyte; DC: dendritic cells; DOTAP: 1, 2-Dioleoyl-3-Trimethylammonium-Propane (Chloride Salt); ERK: extracellular-signal-regulated kinase; HPV: human papillomavirus; IFA: Incomplete Freund's Adjuvant; MCP-1/CCL2: monocyte chemoattractant protein-1/CC chemokine 2; MIP-1α/CCL3: macrophage inflammatory protein-1 alpha /CC chemokine-3; OVA: ovalbumin; TLR: Toll-like Receptor; TMB: Tetramethylbenzidine.

(CFA/IFA) were purchased from DIFCO Laboratories (Detroit, MI). Goat anti-mouse IgG, IgG_{2a} and IgG_1 horseradish peroxidase (HRP) conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tetramethylbenzidine (TMB) Substrate Kit was purchased from KPL (Gaithersburg, MD). Ovalbumin (OVA) (Grade II) was purchased from Sigma (San Louis, MS). E7 peptide (RAHYNIVTF) derived from HPV 16 E7 protein (amino acid 49-57) was synthesized and purified in the Peptide Synthesis Facility of the University of Pittsburgh. Murine TC-1 cells were kindly provided by Dr. T.C. Wu at Johns Hopkins University (Baltimore, MD). TC-1 cells are C57BL/6 mouse lung epithelial cells transformed with HPV 16 E6 and E7 oncogenes and activated H-ras. The tumor cell line was maintained in RPMI-1640 medium supplied by Invitrogen (Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

2.2 Purification of recombinant E7 protein

Plasmid pET-E7 was a gift from Dr. Jeong-Im Sin at the Catholic University of Korea (Seoul, Korea). The His-tagged recombinant E7 protein was expressed and purified as previously described with some modifications [19]. Briefly, the pET-E7 plasmid was purified and transformed into BL21 (DE3) cells. A single colony was seeded into LB medium supplemented with kanamycin at a final concentration of 50 μg/ml. The cells were incubated at 37°C shaker until the absorbance at 600 nm was between 0.6 and 0.8. Protein production was induced using 0.5 mM of isopropy-1-thio-β-D-galactopyranoside for 4 h. The cell pellets were collected by centrifugation at 8,000 g for 20 min and then re-suspended in 8 M urea buffer (pH 8.0) (5 ml per 1 mg of wet cell pellet). The cells were broken by sonication and then the cell lysate was cleared by centrifugation at 12,500*g* for 30 min. The supernatant was collected and purified by Ni-agarose column using the protocol provided by the vendor (Bio-rad biotechnology). The protein solution was then desalted and changed to 5 mM Tris-HCl buffer, pH 7.0, by G25 desalting column from Pierce (Rockford, IL). The protein concentration was quantified using the Coomassie Plus protein assay reagent (Pierce). The protein purity (about 90%) was confirmed by electrophoresis on a 15% SDS-PAGE gel (Bio-Rad, CA). Similar to previous reports, the recombinant E7 protein migrated as a 23 kD protein on SDS-PAGE gel [20,21].

2.3 Formulation of protein antigens with DOTAP liposome

Cell culture grade water (Cambrex, Walkersville, MD) was used in all liposome preparations. Briefly, lipid films were made in glass vials by evaporating the chloroform solution under a steady stream of dry nitrogen gas. Traces of organic solvent were removed by keeping the films under vacuum with desiccant overnight. Lipid films were hydrated for 12 h by adding required amount of protein solution in water or specific concentration of NaCl. The suspensions were then sonicated in a bath type sonicator for 10 min and stored at 4°C before use.

2.4 Characterization of DOTAP/protein formulations

The particle size and the zeta (ζ) potential of the liposomal complexes were measured following the manufacturer's instruction using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA) and a ZetaPlus (Brookhaven Instruments, Corp., Holtsville, NY), respectively. The measurement of ζ potential was performed in the 1 mM KCl. Protein incorporation in the liposome was determined by the percentage of liposome-bound protein. In brief, the unbound protein from DOTAP/E7 and was separated from the complex by a Microcon® centrifugal filtrate device (Millipore, Bedford, MA) and the concentration of unbound protein was measured by Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL). The efficiency of protein incorporation was determined as (1- % unbound peptide) and was reported as the mean ± SD (n=3).

2.5 Production of antibodies

All work performed on animals was in accordance with and approved by the institutional IACUC. C57BL/6 mice (n=5) were s.c. immunized on the left flank at day 0 with 20 μg of OVA formulated in DOTAP liposomes, PBS or CFA. At day 14, mice were injected s.c. on the left flank again with the same formulations except that CFA was replaced by IFA. Sera were collected at day 13 and 28 and titrated for OVA specific antibodies by ELISA.

2.6 ELISA for IgG, IgG_1 and IgG_{2a}

Specific serum IgG, IgG_1 and IgG_{2a} levels against OVA protein were determined using ELISA. Briefly, Costar high binding 96-well assay plates were coated with 100 μl (10 mM sodium phosphate buffer, pH 9.6) of OVA protein (1 μg/well) overnight at 4°C. The plates were then washed once with wash buffer (PBS containing 0.01% Tween 20) and blocked for 1 h at 25° C with 100 μl/well ELISA buffer (2% bovine serum albumin (BSA) (Sigma) solution made in PBS). The plates were then washed five times with wash buffer. Mouse serum was serially diluted with ELISA buffer, and 100μl was added into the well and incubated for 2 h at 25°C. After washing five times with wash buffer, goat anti-mouse IgG or IgG_1 or IgG_{2a} HRP (diluted 1:10,000 in 2% BSA) was added (100 μl/well) and incubated for 1 h at 25°C. The plates were washed five times with wash buffer. Finally, the samples were developed with 100 μl TMB substrate for 30 min at room temperature and then stopped with 100 μl of 1M H_2SO_4. The OD of each well was measured using a Microplate Reader System (Bioscan, Washington, D.C) at 405 nm.

2.7 Vaccine treatment for tumor bearing mice

C57BL/6 female mice of 6-7 weeks old were purchased from Charles River Laboratories (Wilmington, MA) and were used in all animal studies. Subcutaneous tumors were established by injecting TC-1 cells (10^5 cells) into the hair-trimmed flank of the mouse on day 0. At day 5, mice (n = 4-6) were subcutaneously injected with 150 μl of selected formulations containing 20 μg E7 protein. The size of the tumor was measured using a caliper two or three times a week. Tumor size was determined by multiplying the two largest dimensions of the tumor.

2.8 *in vivo* CTL assay

In vivo CTL activity of E7-specific cytotoxic T cells was enumerated according to the protocol of Byers et al. [22] with minor modifications. In brief, spleen cells from syngeneic mice were RBC lysed followed by pulsing with 10 μM E7 peptide (RAHYNIVTF) or without peptide in complete medium for 1 h at 37°C. Both spleen cell populations were stained with equal amount of 2 μM PKH26 (red fluorescent dye) (Sigma-Aldrich) according to manufacturer's instruction. Then, the peptide pulsed and unpulsed populations were loaded with either 4 μM or 0.4 μM CFSE (carboxyfluorescein succinimidyl ester) (Molecular Probe) at 37°C for 15 min. CFSE diffuses freely inside the cells and intracellular esterases cleave the acetate groups converting it to a fluorescent, membrane impermeable dye. The two cell populations were mixed 1:1 before tail vein injection to the control or DOTAP/E7 protein immunized mice (10^7 spleen cells per mouse). At 16 h after injection, spleen cells from the recipient mice were isolated and single cell suspension were prepared prior to flow cytometric analysis. The number of $CFSE^{high}$ (loaded with 4 μM CFSE) and $CFSE^{low}$ (loaded with 0.4 μM CFSE) population were determined and the E7 specific lysis percentage was enumerated according to a published equation [22].

2.9 Statistical analysis

Data were analyzed statistically using a two-tailed Student's t-test. Data were considered statistically significant when *p* value was less than 0.05.

3. Results

3.1. DOTAP/NaCl/E7 protein formulation elicits therapeutic anti-tumor activities

Our previous findings showed that DOTAP/E7 peptide induced anti-tumor activity was dependent on the dose of DOTAP. The dose response curve indicated that 100 nmol/mouse generated the optimal effect on the tumor regression. Therefore, we first perform the same experiment to optimize the dose of the DOTAP. The 20 μg E7 protein was formulated with different dose of DOTAP liposomes. However, all these formulations could not induce tumor regression (data not shown). Interestingly, we found one group in which the protein was partially desalted by dialysis showed anti-tumor activity. We also observed that the salt resulted in larger particle size and when this formulation was s.c. injected to the mice, the draining lymph nodes were enlarged, suggesting an enhanced immunological response. Previous report also showed that salt enhanced gene expression of cationic liposome/plasmid DNA complex [16]. Therefore, we systematically studied the effects of salt on the physicochemical characteristics and anti-tumor activities induced by DOTAP/NaCl/E7 protein formulations. In this study, we kept the DOTAP dose as 100 nmol per mouse. The results indicated that the particle size of DOTAP/E7 protein complex increased as the concentration of NaCl increased. When the NaCl concentration was increased to more than 50 mM, the complex precipitated. However, the DOTAP/protein particles are still stable in the presence of 30 mM NaCl although the size increased from 350 nm to 550 nm (Table 1). We also compared the zeta potential of the formulation with 30 mM NaCl to the formulation without NaCl. The results showed that in the presence of 30 mM NaCl, the particles gained more positive charge than the particles without NaCl, although all the protein formulations were less positively charged than empty DOTAP liposomes (Table 1). Table 1 also showed that the loading capacity of DOTAP/protein complex was slightly decreased from 95% to 90% in the presence of NaCl. The reason for the decrease in the loading capacity and increase in the zeta potential by salt is probably due to the interference of electrostatic interaction between the lipid and the protein. Next, the DOTAP/NaCl/E7 protein formulations were utilized to study the therapeutic effect on the tumor bearing mice. TC-1 tumor cells were s.c. injected into mice. Five days later, the mice were treated with DOTAP/NaCl/E7 protein. As shown in Fig. 1, treatment of tumor-bearing mice with DOTAP/E7 protein with 30 mM NaCl caused regression of the tumors and the activity was comparable to the activity of the DOTAP/E7 peptide formulation, which served as a positive control and reported previously [4]. In contrast, E7 protein alone or DOTAP/E7 protein with higher concentration of NaCl did not induce inhibitory effects on the tumor growth. However, DOTAP liposome itself has no effect on the tumor growth demonstrated by our previous work [4].

To make sure the tumor regression was due to antigen specific CTL (cytotoxic T cell), mice with regressed tumors and naïve mice were used to do an *in vivo* CTL assay. A mixture containing equal amounts of E7-pulsed $CFSE^{high}$ and unpulsed $CFSE^{low}$ spleen cells from a syngeneic donor was i.v. injected into tumor regression mice at day 22. The specific lysis of E7-pulsed cells was analyzed by flow cytometry 16 h after adoptive transfer. Mice that received E7 protein formulated in DOTAP liposome with 30 mM NaCl or DOTAP/E7 peptide were capable of killing more than 70% of in vivo targets in 16 h while in the groups of 0 mM NaCl and 150 mM NaCl with DOTAP/E7 protein were not able to kill (Fig.2). This finding suggests that the tumor-killing activity induced by DOTAP/E7 protein with 30 mM NaCl was mainly due to the E7 specific CTL response.

3.2 Model antigen OVA formulated in DOTAP liposome induced antibody response

In order to check if DOTAP can enhance antibody response, we formulated well established model antigen OVA into DOTAP liposomes. The optimal dose of DOTAP to induce the best antibody response could be different from the dose to generate the anti-tumor activity. Therefore, we first varied the dose of DOTAP and kept the antigen amount constant. The

particle size, zeta potential and antigen loading capacity are summarized in Table 2. The zeta potential decreases as the ratio of protein to DOTAP increases, suggesting that the negatively charged protein compensated for the positive charge of DOTAP. However, all these formulations contained net positive charge. Because of electrostatic interaction between the protein and liposome, the loading capacities are all more than 90%. Similar to the salt effect on the properties of DOTAP/E7 protein, 30 mM NaCl increased the particle size, positive charge and also slightly decreased the protein loading capacity of the complex (Table 2).

The data presented in **3.1** indicate that DOTAP/protein/salt complex can prime T cell response and induce a prominent anti-tumor activity. The ability of DOTAP to initiate a B cell response was then tested by immunizing mice against a model antigen OVA and measuring OVA-specific antibodies by ELISA. First, DOTAP alone was used as a delivery and adjuvant system. With increasing dose of DOTAP, antibody responses (both prime and boost) induced by DOTAP/OVA were enhanced compared to OVA alone (Fig 3). The OVA specific total IgG level in the serum of mice immunized with 200 nmol or 400 nmol DOTAP/OVA protein was significantly higher than that of the mice immunized with OVA protein only ($p<0.05$). However, the 200 nmol or 400 nmol DOTAP/OVA generated- antibody response was less than the CFA/IFA system.

In **3.1**, we showed that salt in the vaccine formulation could dramatically enhance the anti-tumor activity. Thirty mM NaCl was also introduced to the DOTAP/OVA formulation to see if the same effect could be observed for antibody production. Although the 30 mM NaCl did not enhance the primary antibody response generated by DOTAO/OVA (data not shown), it significantly boosted the antibody response (Fig 4A). We also checked the IgG subtypes IgG_{2a} and IgG_1 levels in the mouse serum. Fig 4B indicates that 200 nmol DOTAP/OVA could boost both IgG_1 and IgG_{2a} subtypes compared to OVA only ($p<0.05$). Moreover, 30 mM NaCl in the vaccine formulation further enhanced the IgG_1 subtype antibody. Although we observed the enhancement of IgG_{2a} induced by 30 mM NaCl, the statistic analysis showed no significant difference ($p>0.05$).

4. Discussion

In this report, we expanded the cationic DOTAP liposome formulation from a peptide based vaccine to a protein based vaccine. Our data illustrated that DOTAP/protein/salt vaccine formulation has the capacity to generated both CTL and specific antibody responses. Generally speaking, CTL response can not be induced by a protein alone as an antigen because it is taken up and degraded in the endosomes/lysosomes and does not gain access to the MHC I molecules. However, formulation of a protein in a particulate system will facilitate its entry into the cytoplasm, degradation by the immune proteosome and eventually access to the MHC I molecules, leading to a CTL response [23-25]. DOTAP cationic liposome can obviously carry a protein antigen and deliver to the cytoplasm of APC, inducing a strong CTL response. In addition, DOTAP can also facilitate antibody responses (both prime and boost) as shown by our data. Therefore, it has the potential to be applied to vaccines against infectious diseases such as HIV or malaria which requires both arms of the immune response. There are at least three advantages to use cationic liposome/protein complex to deliver a protein antigen. First, cationic liposome/protein complex has a very high loading capacity to formulate negatively charged protein via charge-charge interaction (Table 1 and 2). Second, cationic liposome destabilizes the endosomal membrane by forming ion pair complex with the endosomal anionic lipids, so protein antigen can be efficiently delivered to the cytoplasm [26]. Third, the cationic liposome itself has adjuvant effect on the activation of the antigen presenting cells, such as DC [5,7].

Another finding in this study is that a low concentration of NaCl (30 mM) enhances both the anti-tumor activity and antibody response induced by the DOTAP/protein formulation. The high concentration of the salt compromising the immune response is probably due to the aggregation and precipitation of the liposome/protein complex. The complex became micro meter diameter range in the presence of 75 mM NaCl. The increased particle size may not facilitate the antigen uptake by APC such as DC. Low dose of NaCl (30 mM), however, did not dramatically increase the particle size (< 600 nm), which is still manageable by APC. But the salt could actually decrease the interaction between the cationic liposome and the protein antigen. Consistent with a previous report [27], the loading capacity of DOTAP liposome decreased when the salt was added to the DOTAP/protein complex. The predicted and measured PI values of E7 protein are 4.05 and 5.4, respectively [21] and that of OVA is 4.7 (data provided by Sigma). Therefore, in water (pH 6.7), the interaction between the protein and cationic liposome is through electrostatic and hydrophobic attraction, among which the former interaction is the major driving force. Addition of salt to the liposome/protein complex would decrease the electrostatic interaction, resulting in the observed decrease of the loading capacity (Table 1 and 2). Tsuruta et al [27] studied the interaction between a protein and the DODAB cationic liposome and suggested that in the presence of salt, the protein is basically "lying over" or partially "inserted in the bilayer", although no liposome leakage was observed. However, in water without salt the protein will totally insert into the bilayer and cause the liposome rupture [27]. The authors concluded that efficient exposure of protein antigen to the immunological system resulted from the addition of salt enhanced the immune response. In addition, salt will decrease affinity between the cationic lipid and the protein. When the protein is delivered to the APC, it could more easily be released from the carrier to facilitate the antigen presentation by APC.

Our data also showed the increased zeta potential in the presence of salt, because the salt decreases the electrostatic interaction between the lipid and the protein leading to reduced shielding effect of the protein to the positive charged liposome. These changes could facilitate an enhanced interaction and activation of APC by cationic particles. Our previous reports showed that positive charge of the liposome is essential to initiate the down stream signal transduction in DC, which is required for mounting sufficient immune responses [7].

In summary, DOTAP liposome is a promising adjuvant and delivery system for protein based vaccines. Our data also indicates that addition of a low level of NaCl in the formulation can dramatically change the physicochemical properties of the cationic liposome/protein complex resulting in enhanced immune responses. Whether the observation can be generalized to other protein vaccine formulation remains to be studied. The effect of salt on the immune responses is likely to depend on the properties of both the antigens (PI value, molecular weight, hydrophobicity, etc) and the carriers (charge, size, etc). A protein vaccine formulation should be carefully studied individually to optimize its immune response.

Acknowledgments

This study was partially supported by the Mary Kay Ash Charitable Foundation and the authors' institute.

References

1. Guinn BA, Kasahara N, Farzaneh F, Habib NA, Norris JS, Deisseroth AB. Recent advances and current challenges in tumor immunology and immunotherapy. Mol Ther 2007;15(6):1065–1071. [PubMed: 17375068]
2. Chen WC, Huang L. Non-viral vector as vaccine carrier. Adv Genet 2005;54:315–337. [PubMed: 16096017]

3. Yan W, Huang L. Recent Advances in Liposome-Based Nanoparticles for Antigen Delivery. Polymer Reviews 2007;47(3):16.
4. Chen W, Yan W, Huang L. A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. Cancer Immunol Immunother 2008;57(4):517–530. [PubMed: 17724588]
5. Vangasseri DP, Cui Z, Chen W, Hokey DA, Falo LD, Huang L. Immunostimulation of dendritic cells by cationic liposomes. Mol Membrane Biol 2006;23(5):1–11.
6. Yan W, Chen W, Huang L. Reactive oxygen species play a central role in the adjuvant activity of cationic liposome. J Control Release 2008;130(1):22–28. [PubMed: 18554742]
7. Yan W, Chen W, Huang L. Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines. Mol Immunol 2007;44(15):3672–3681. [PubMed: 17521728]
8. Wang JC, Livingstone AM. Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo. J Immunol 2003;171(12):6339–6343. [PubMed: 14662830]
9. Bourgeois C, Tanchot C. Mini-review CD4 T cells are required for CD8 T cell memory generation. Eur J Immunol 2003;33(12):3225–3231. [PubMed: 14635030]
10. Paschen A, Eichmuller S, Schadendorf D. Identification of tumor antigens and T-cell epitopes, and its clinical application. Cancer Immunol Immunother 2004;53(3):196–203. [PubMed: 14689239]
11. Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. Science (New York, N Y 1993;261(5118):209–211.
12. Wheeler CJ, Felgner PL, Tsai YJ, Marshall J, Sukhu L, Doh SG, Hartikka J, Nietupski J, Manthorpe M, Nichols M, Plewe M, Liang X, Norman J, Smith A, Cheng SH. A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. Proceedings of the National Academy of Sciences of the United States of America 1996;93(21):11454–11459. [PubMed: 8876156]
13. Liu F, Qi H, Huang L, Liu D. Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. Gene therapy 1997;4(6):517–523. [PubMed: 9231067]
14. Li S, Wu SP, Whitmore M, Loeffert EJ, Wang L, Watkins SC, Pitt BR, Huang L. Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. The American journal of physiology 1999;276(5 Pt 1):L796–804. [PubMed: 10330036]
15. Kawakami S, Fumoto S, Nishikawa M, Yamashita F, Hashida M. In vivo gene delivery to the liver using novel galactosylated cationic liposomes. Pharm Res 2000;17(3):306–313. [PubMed: 10801219]
16. Kawakami S, Ito Y, Fumoto S, Yamashita F, Hashida M. Enhanced gene expression in lung by a stabilized lipoplex using sodium chloride for complex formation. The journal of gene medicine 2005;7(12):1526–1533. [PubMed: 16170832]
17. Davidsen J, Rosenkrands I, Christensen D, Vangala A, Kirby D, Perrie Y, Agger EM, Andersen P. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6′-dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses. Biochim Biophys Acta 2005;1718(12):22–31. [PubMed: 16321607]
18. Vangala A, Kirby D, Rosenkrands I, Agger EM, Andersen P, Perrie Y. A comparative study of cationic liposome and niosome-based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice. The Journal of pharmacy and pharmacology 2006;58(6):787–799. [PubMed: 16734980]
19. Cui Z, Huang L. Liposome-polycation-DNA (LPD) particle as a carrier and adjuvant for protein-based vaccines: therapeutic effect against cervical cancer. Cancer Immunol Immunother 2005;54 (12):1180–1190. [PubMed: 15846491]
20. Zhang LF, Zhou J, Chen S, Cai LL, Bao QY, Zheng FY, Lu JQ, Padmanabha J, Hengst K, Malcolm K, Frazer IH. HPV6b virus like particles are potent immunogens without adjuvant in man. Vaccine 2000;18(1112):1051–1058. [PubMed: 10590325]
21. Pahel G, Aulabaugh A, Short SA, Barnes JA, Painter GR, Ray P, Phelps WC. Structural and functional characterization of the HPV16 E7 protein expressed in bacteria. The Journal of biological chemistry 1993;268(34):26018–26025. [PubMed: 8245034]
22. Byers AM, Kemball CC, Moser JM, Lukacher AE. Cutting edge: rapid in vivo CTL activity by polyoma virus-specific effector and memory CD8+ T cells. J Immunol 2003;171(1):17–21. [PubMed: 12816977]

23. Falo LD Jr, Kovacsovics-Bankowski M, Thompson K, Rock KL. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nat Med 1995;1(7):649–653. [PubMed: 7585145]

24. de Jong S, Chikh G, Sekirov L, Raney S, Semple S, Klimuk S, Yuan N, Hope M, Cullis P, Tam Y. Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN. Cancer Immunol Immunother 2007;56(8):1251–1264. [PubMed: 17242927]

25. Liang MT, Davies NM, Blanchfield JT, Toth I. Particulate systems as adjuvants and carriers for peptide and protein antigens. Current drug delivery 2006;3(4):379–388. [PubMed: 17076640]

26. Xu Y, Szoka FC Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry 1996;35(18):5616–5623. [PubMed: 8639519]

27. Tsuruta LR, Quintilio W, Costa MH, Carmona-Ribeiro AM. Interactions between cationic liposomes and an antigenic protein: the physical chemistry of the immunoadjuvant action. J Lipid Res 1997;38(10):2003–2011. [PubMed: 9374123]

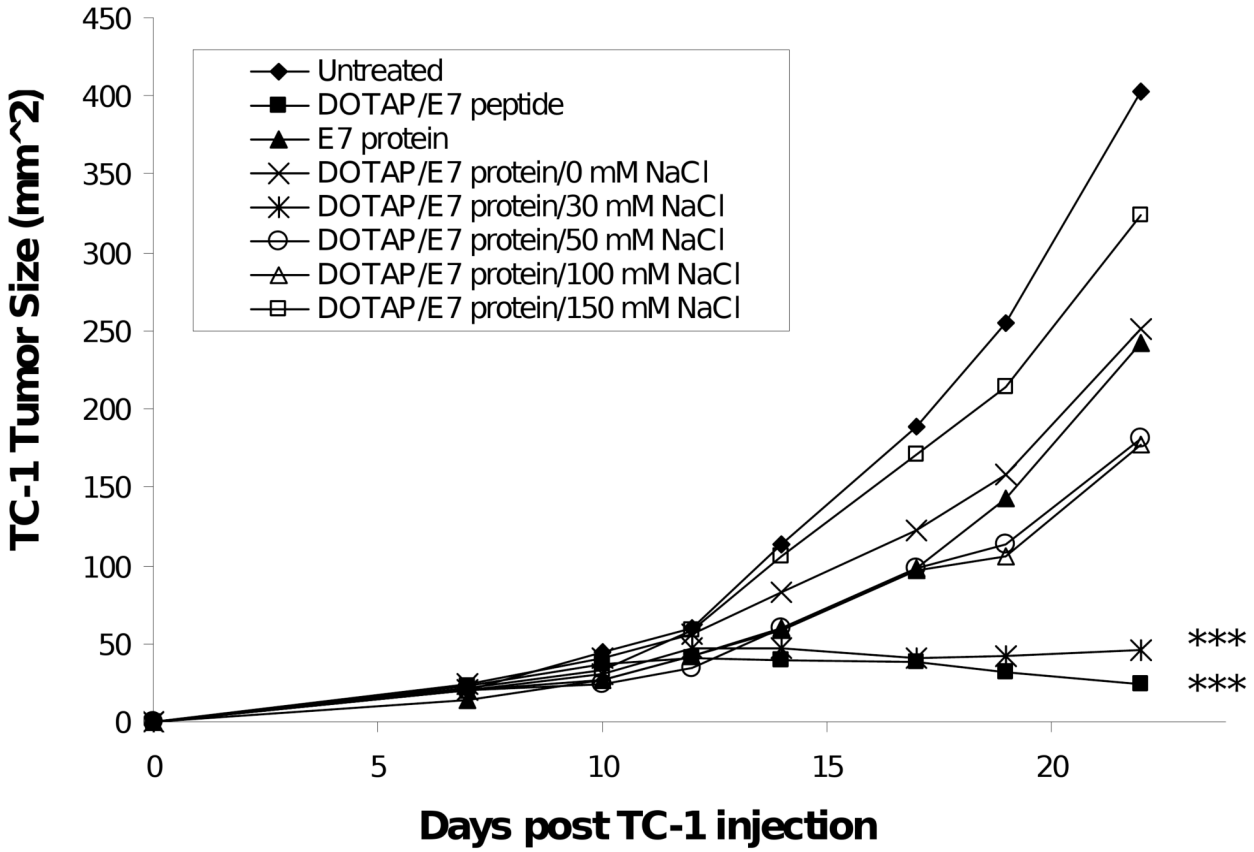


Figure 1.

Kinetics of TC-1 tumor growth in mice treated with DOTAP/E7 protein formulations in the presence of different concentration of NaCl. C57BL/6 female mice of seven weeks old (n= 4 to 6) were s.c. injected with TC-1 tumor (10^5 cells per mouse) at day 0. At day 5, mice received 20 μg E7 protein formulated in liposome made by 100 nmol DOTAP lipid in the presence of 0, 30, 50, 100, 150 mM NaCl. Mice left untreated were used as the negative control. Tumor sizes were measured by a caliper every two or three days and determined by multiplying the two largest dimensions of the tumor. TC-1 tumor size of each group at day 23 was compared to the untreated control group and was analyzed statistically (***$p < 0.001$).

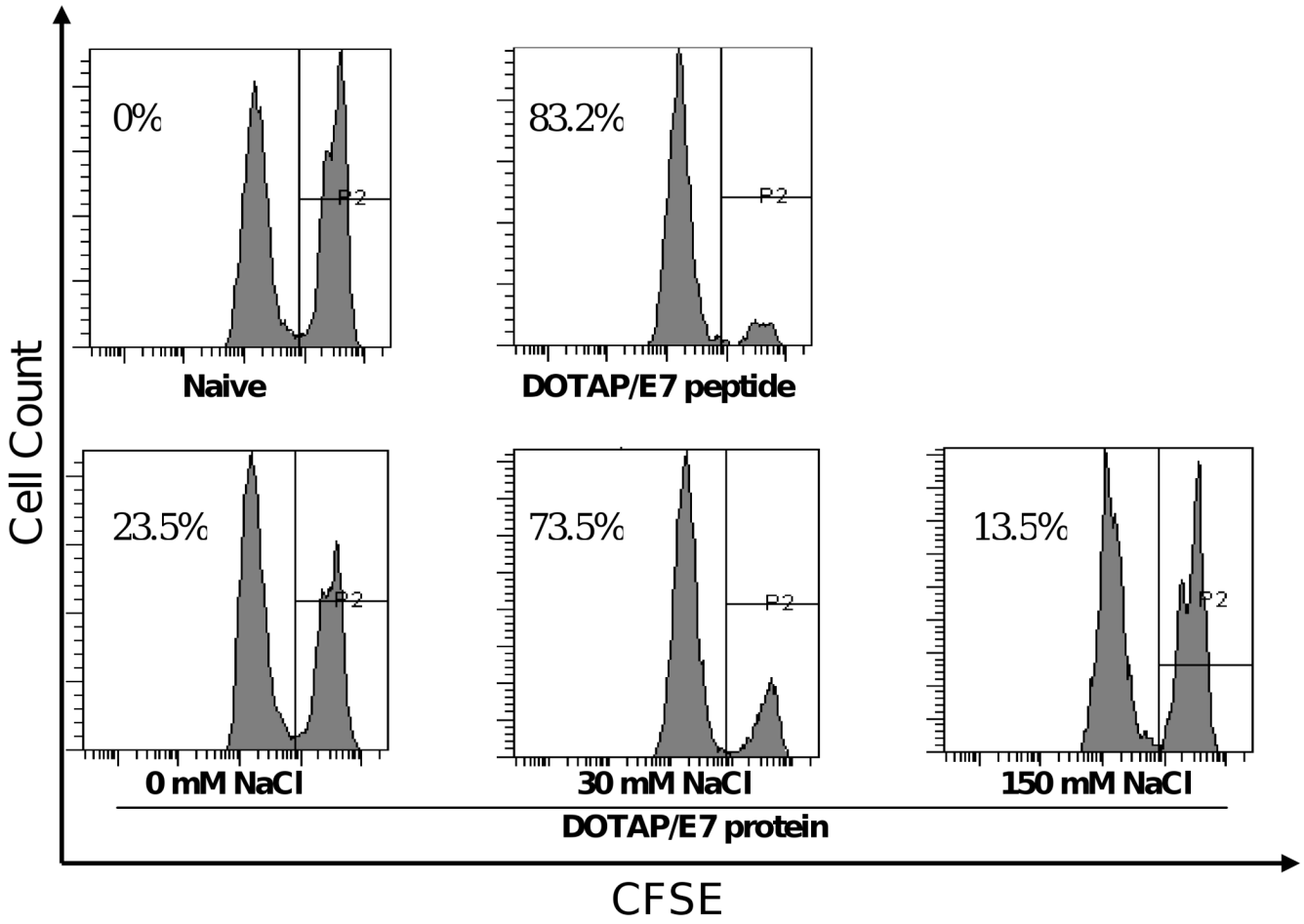
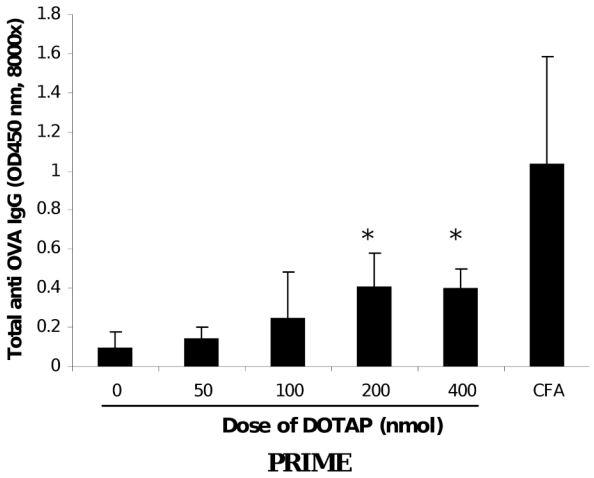


Figure 2.

Immunization with E7 protein formulated in DOTAP with 30 mM NaCl elicits functional $CD8^+$ T cells. At day 23, C57BL/6 mice from tumor growth study, were i.v. injected with equivalent amount of E7 peptide-pulsed specific (labeled with 4 μM CFSE) and nonspecific (labeled with 0.4 μM CFSE) PKH-26 labeled spleen cells from a syngeneic donor. 16 h later, spleen cells were harvested from the adoptively transferred mice and the proportions of the $CFSE^{high}$ and $CFSE^{low}$ cells were analyzed by flow cytometry. Percentage of specific anti-E7 killing was shown in the upper left corner of each panel. Data was show in the represent of three similar results.

A



B

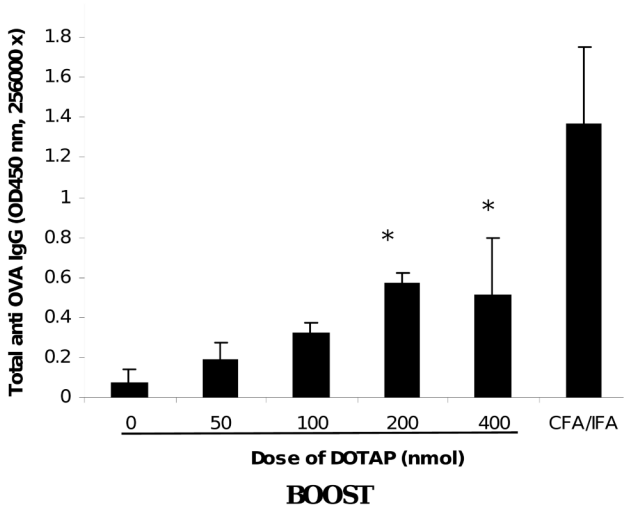


Figure 3.

Anti-OVA antibody responses can be generated by DOTAP/OVA protein formulations. C57BL/6 female mice of seven weeks old (n= 5) were s.c. immunized on Days 0 and 14 with 150 μl of each formulation. OVA-specific total serum IgG was evaluated on Day 13 (**A**) and 28 (**B**) by ELISA (prime or boost as indicated). Data represent the mean ± S.D. *$p < 0.05$ compared to OVA alone group (0 nmol DOTAP).

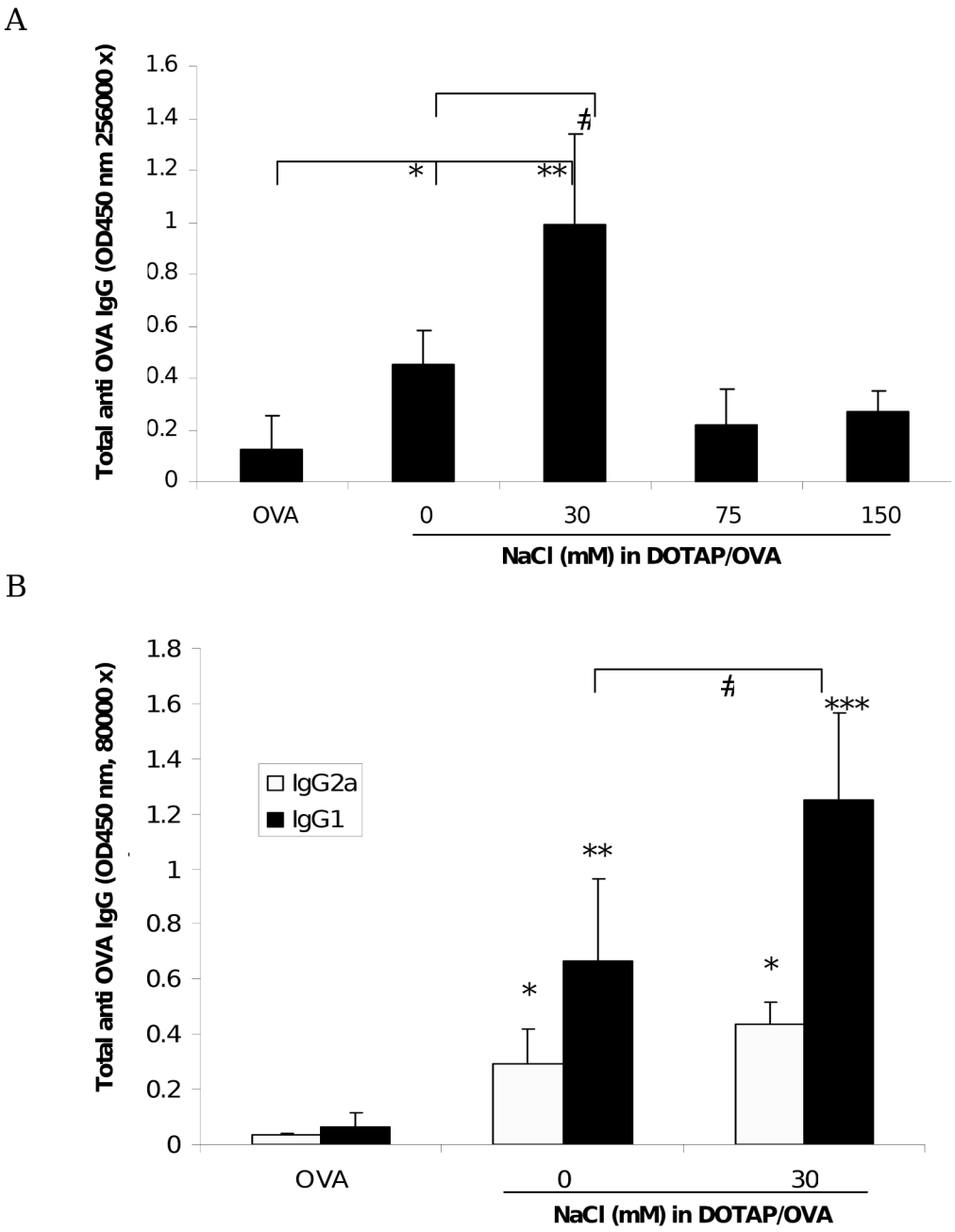


Figure 4.

Low concentration of NaCl can further boost the anti-OVA IgG antibody response induced by DOTAP/OVA protein formulation. C57BL/6 female mice of seven weeks old (n= 5) were s.c. immunized on Days 0 and 14 with 150 μl of each formulation. **(A)** The total anti-OVA IgG antibody response (day 28) was boosted by 30 mM NaCl in the 200 nmol/OVA protein formulations in 150 μl. (**B**) OVA- specific IgG_1 and IgG_{2a} subtypes (day 28) were generated by DOTAP/OVA protein in the presence of 30 mM NaCl. Data represent the mean ± S.D. *$p < 0.05$, **$p<0.01$, ***p<0.001 compared to OVA alone group; #$p<0.05$, compared to 0 mM NaCl.

Table 1

Physical properties of DOTAP/E7 proteins in the presence of 30 mM NaCl

Samples	Mean diameter (nm)[c]	Mean charge (mV)[c]	Mean protein incorporation (%)[c]
DOTAP[a]	152.4 ± 13.4	52.3 ± 3.2	N/A
DOTAP/E7[b]	354.2 ± 23.2	28.9 ± 1.0	95.2 ± 2.3
DOTAP/E7/NaCl	559.4 ± 32.3	38.2 ± 3.1	89.7 ± 1.0

[a] DOTAP concentration, 1 mM.

[b] E7 protein concentration, 0.2 mg/ml.

[c] Values represent means ± SD of $n = 3$ experiments.

Table 2

Physical properties of DOTAP/OVA[a] formulations

Samples	Mean diameter (nm)[b]	Mean charge (mV)[b]	Mean protein incorporation (%)[b]
0.5 mM DOTAP	334.5 ± 34.5	18.6 ± 1.7	94.8 ± 2.8
1 mM DOTAP	324.4 ± 25.7	26.3 ± 2.3	95.8 ± 1.3
2 mM DOTAP	339.6 ± 28.9	32.8 ± 3.7	97.5 ± 2.3
2 mM DOTAP / 30 mM NaCl	589.8 ± 46.3	43.7 ± 4.2	90.2 ± 3.4
4 mM DOTAP	319.3 ± 24.7	38.4 ± 1.2	98.4 ± 1.2

[a] OVA protein concentration, 0.2 mg/ml.

[b] Values represent means ± SD of $n = 3$ experiments.