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Research paper

Nasal immunization studies using liposomes loaded with tetanus toxoid and CpG-ODN

Mohsen Tafaghodi [a,b,*], Mahmood-Reza Jaafari [a,c], Sayyed Abolghasem Sajadi Tabassi [a,d]

[a] School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Pharmaceutical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Pharmacological Research Center for Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran

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Abstract

To increase the systemic and mucosal immune responses against the nasally administered tetanus toxoid, liposomes as a drug delivery system and CpG-ODN as an adjuvant were evaluated. Rabbits were nasally immunized with entrapped tetanus toxoid (TT) and CpG-ODN in neutral liposomes and systemic and mucosal immune responses were determined. Liposomes containing TT and CpG-ODN were prepared by dehydration–rehydration method. The volume mean diameter of liposomes was 2.3 ± 0.6 μm. Encapsulation efficiency of TT and CpG-ODN was determined as 54.0 ± 8.8 and 60.1 ± 7.4, respectively. The leakage of the encapsulated TT from liposomes reached 7.38% after 3 months. Encapsulated TT kept its intact structure, and its immunoreactivity was also completely preserved, as shown by SDS–PAGE and ELISA methods. The highest serum IgG and antitoxin titers were observed in groups immunized with solution formulations ($P < 0.001$). However the highest mucosal sIgA titers were achieved by liposomes encapsulated with TT. CpG-ODN as an adjuvant was able to increase the serum IgG and antitoxin titers when co-administered with TT solution ($P < 0.05$) or co-encapsulated with TT in liposomes ($P < 0.01$), but failed to increase the sIgA titers in nasal lavages. No hemolysis occurred on incubation of liposomes and human RBCs. Also after nasal administration of plain liposomes to human volunteers, no local irritation was seen. Intranasal administration of liposomes encapsulated with vaccines showed to be an effective way for inducing the mucosal immune responses.

Keywords: Nasal immunization; Neutral liposome; CpG-ODN; Tetanus toxoid; Adjuvant; Antitoxin; sIgA; IgG

1. Introduction

The World Health Organization has indicated that the development of improved immunization strategies for tetanus, as well as diphtheria, is a priority. The development of effective mucosally delivered vaccines is one strategy for potentially ameliorating some of the problems associated with the present vaccine [1], such as need to frequent injections, need to trained personnel for injections and failure of completing the boosting immunizations in far regions and in underdeveloped countries. It is well known that protection against pathogenic organisms correlates better with the presence of antibody in local secretions than with serum antibody [2]. It has also been shown that antibodies resulting from parenteral immunization do not necessarily reach mucosal surfaces from where most infectious agents enter the host [3]. One of the most effective methods to induce mucosal immunity in the upper respiratory tract is intranasal immunization [4]. Immunization at the mucosal surfaces which produces the protective antibody, i.e. secretory immunoglobulin A (sIgA), is very important,

* Corresponding author. School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran. Tel.: +98 511 8823255; fax: +98 511 8823251.
E-mail address: m-tafaghodi@mums.ac.ir (M. Tafaghodi).

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especially for pathogens that reside and colonize on mucosal surfaces [5,6].

Development of effective delivery systems for the presentation of antigens to mucosal surfaces is critical to the success of these vaccines [5]. In the induction of systemic and mucosal immunity, particulate antigens are more effective than soluble antigens; possibly because of more efficient endocytosis of particulate antigens by mucosal-associated lymphoid tissue (MALT) M cells [4]. Liposomes have been identified as effective immunological adjuvants and have potential for the intranasal and oral delivery of protein antigens [1]. Liposomes can effectively entrap various drugs and biologicals, which are slowly released over an extended period of time in vivo, and are also efficiently taken up by antigen-presenting cells [7,8], suggesting that liposomes can serve as an efficient delivery system for CpG-based vaccines [9]. The physicochemical properties of liposomes can influence their utility as a delivery system and vaccine adjuvant. Of them, it could be referred to the liquid-crystalline phase-transition temperature (T_c) of the lipids, the charge of the lipids, inclusion of cholesterol and liposomes size [1,10]. Neutral liposomes can be prepared by uncharged phospholipids, such as, phosphatidylcholine (PC), and in most cases cholesterol is included to increase the stability of phospholipid bilayers and decrease the leakage of liposomes [1].

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) containing immunostimulatory sequences (known as CpG motifs) are potent stimulators of both innate immunity and specific adaptive immune responses [11]. Bacterial DNA and CpG-ODNs directly activate monocytes/macrophages, dendritic cells, NK cells and B cells, induce the production of pro-inflammatory cytokines (e.g. IL-6, IL-12, IFNs, TNFα) and upregulate the expression of MHC I, MHC II and co-stimulatory molecules [9]. In animal studies, CpG-ODNs exhibit strong Th1 [12,13] and mucosal [14] adjuvant effects to a wide range of antigens. The immunostimulatory activity of CpG-ODNs requires cellular uptake by endocytosis following their binding to a cell receptor belonging to the Toll-like receptor family, TLR9. Endosomal acidification and digestion of the ODN followed by interaction with specific protein kinases results in rapid generation of reactive oxygen intermediates, leading to activation of MAPK and NF-κB pathways and subsequent cytokine production [9].

In the present study, liposomes containing the tetanus toxoid (TT) were prepared by dehydration–rehydration method. The stability and immunoreactivity of encapsulated antigen were checked with SDS–PAGE and ELISA. The stability of liposomes was evaluated with an in vitro release study. The mucosal irritation potential of liposomes was evaluated in human volunteers. Erythrocyte hemolysis test was also carried out for the evaluation of membrane toxicity of liposomes. The efficiency of these liposomes, as nasal delivery system and adjuvant, was evaluated by nasal immunization in rabbits, followed by determination of systemic and mucosal immune responses. The adjuvant effect of CpG-ODN in mucosal immunization was also studied when it was co-encapsulated along with TT in liposomes or simply mixed with TT in solution.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC) was purchased from Fluka (Buchs, Switzerland). Cholesterol (Chol) was purchased from Merck (Darmstadt, Germany). Phosphorothioated CpG oligodeoxynucleotide (#1826) was purchased from Operon Technologies Inc. (Alameda, CA). Tetanus toxoid (TT) solution (2500 Lf/ml) and alum-adsorbed tetanus toxoid (50 Lf/ml) were from Razi Inc. (Hesarak, Iran). Anti-rabbit IgG and IgA were purchased from Sigma (Missouri, USA) and Bethyl Laboratories Inc. (Texas, USA), respectively.

White albino rabbits weighing 2–2.5 kg were provided by Pasteur Institute (Tehran, Iran).

2.2. Preparation of liposomes encapsulated with TT

Liposomes were prepared as dehydration–rehydration vesicles (DRV) [15] with lipid composition of PC/Chol (16.5 μmol/ml from each one). Briefly, the lipid phase was dissolved in chloroform: methanol; 2:1 v/v, in a round-bottomed flask. The solvent was removed by rotary evaporation. The lipid film was then freeze-dried (Heto Drywinner, DW3, Heto-Halter, Allerod, Denmark) for 2 h to ensure total removal of the solvent. The lipid film was hydrated with distilled water at 45 °C and vortexed for 30 min. The resulting multilamellar vesicles (MLVs) were converted to small unilamellar vesicles (SUVs) using probe-type sonicator (Soniprep-150, MSE, Sussex, UK). The resulting SUVs were mixed with encapsulate (TT and CpG-ODN), flush freezed in dry ice-acetone and freeze-dried overnight. The dried broken liposome powder was rehydrated at 45 °C for 30 min with distilled water, using a volume equivalent to one-tenth of the total SUV used. Rehydration was aided by gentle vortexing. The liposomes were then diluted with PBS (phosphate-buffered saline) to the final volume.

2.3. Morphology and size analysis of liposomes

Optical microscope (Carl Zeiss, Oberkochen, FRG) was used for studying the morphological features of liposomes. The volume mean diameters of liposomes were determined by a laser diffraction size analyzer (Zetasizer 2000, Malvern, UK).

2.4. Determination of the encapsulation efficiency of tetanus toxoid (TT) and CpG-ODN in liposomes

Encapsulation efficiency was determined with an indirect method by determination of non-entrapped TT and

CpG in supernatant of liposome suspensions. Liposome suspensions were centrifuged (13,000*g*, 15 min) and supernatants were separated. Liposomes were washed three times and all supernatants combined.

The amount of TT in supernatants was determined by micro-BCA protein assay method [12]; and CpG-ODN was quantified by spectrophotometric assay at 260 nm [13].

2.5. Evaluation of leakage of tetanus toxoid from liposomes

Leaking of encapsulated tetanus toxoid from liposomes was assayed in 1-month intervals for 3 months. One milliliter of liposome suspension (in triplicate) was kept in fridge (2–8 °C). At sampling times liposomes were centrifuged and 200 μl of supernatant was drawn for quantification of released TT. A mild vortexing was used for re-suspending of liposomes.

2.6. Structural stability and immunoreactivity of encapsulated TT

2.6.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The molecular stability of encapsulated TT was evaluated by SDS–PAGE method. Supernatant of original liposome suspension, original TT and a molecular weight reference marker were loaded onto a 10% acrylamide gel. Protein bands were visualized by silver nitrate staining.

2.6.2. Enzyme-linked immunosorbent assay (ELISA)

The amount of TT in supernatant of original liposome suspension was determined by micro-BCA protein assay and immunoreactivity of TT was determined by an ELISA method [16]. Briefly, a 96-well ELISA plate (Nunc-immunosorb, Maxisorb, Denmark) was coated with 50–1000 ng/well (100 μl of each concentration in quadruplicate) of the above-mentioned TT solution and standard TT solution in phosphate buffer (0.05 M, pH 7.4) and incubated at 37 °C, for 60 min. After blocking the unreacted sites with 1% BSA (300 μl/well) and washing with phosphate-buffered saline (PBS, 0.05 M, pH 7.4)–Tween 20 (0.05%), 100 μl of the working dilution of mice hyperimmune sera was added to each well.

The hyperimmune serum (as a source of anti-TT IgG) was from animals immunized three times by s.c. injection of 10 Lf alum-adsorbed TT. The proper working dilution of hyperimmune serum was determined by a proprietary ELISA.

After 1 h of incubation at 37 °C followed by washing, 100 μl of the working dilution of goat anti-mouse IgG conjugated to horseradish peroxidase was added to each well and the plate was again incubated for 60 min at 37 °C followed by four times washing. To develop the color, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB, KPL): peroxidase (1:1) solution was added, and the reaction was stopped by addition of 50 μl/well of 1 M phosphoric acid. The absorbances were read at 450 nm.

2.7. Nasal immunization studies

White albino rabbits weighing 2–2.5 kg (four animals per group) were nasally immunized with the following formulations in days 0, 14 and 28 of experiment:

(1) Blank neutral liposomes
(2) 40 Lf TT solution
(3) 40 Lf TT + 10 μg CpG-ODN both in solution
(4) Neutral liposomes encapsulated with 40 Lf TT
(5) Neutral liposomes co-encapsulated with 40 Lf TT and 10 μg CpG-ODN
(6) 10 Lf Alum-adsorbed TT (IM injection)

Animals were first injected with 40 mg/kg ketamine HCl to prevent the sneezing after administrations. Liposomal suspensions and TT and CpG solutions (200, 100 μl in each nostril) were administered using an automatic pipette.

Each animal was bled in days 21, 42 and 63. After the third bleeding, animals were sacrificed, trachea was cut and nasal cavity was washed with 10 ml sterile normal saline.

Sera and nasal lavages of each group were pooled and frozen until immunological assays.

2.8. Determination of serum anti-TT IgG titers and nasal lavages anti-TT IgA titers

Anti-TT antibodies in the rabbit serum and nasal lavage were detected and quantified by end-point titration using an enzyme-linked immunosorbent assay (ELISA) [12]. Briefly, 96-well ELISA plates were coated with 100 μl/well of 0.001% (w/v) TT solution in 0.05 M PB buffer (pH 7.4) and incubated for 60 min at 37 °C. After washing, the unreacted sites were blocked with 300 μl/well of 1% (w/v) BSA. Plates were washed two times followed by the addition of test samples in duplicate. Serum obtained from unimmunized animals served as the negative control for serum IgG titration; normal saline was used as negative control for lavage IgA titration. Serial dilutions of the sera and lavage were added to each plate in duplicate and incubated for 60 min at 37 °C. After washing three times, 100 μl/well of goat anti-rabbit IgG or goat anti-rabbit IgA horseradish peroxidase conjugates was added at 1:17,000 and 1:10,000 dilutions, respectively. Following a 60 min incubation at 37 °C and four washes, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the wells. As color developed, the reaction was stopped by addition of 5 N sulfuric acid (50 μl/well) and the absorbance was read at 450 nm.

All washes were done with 0.05 M PBS-T (pH 7.4). Endpoint titers for IgG and IgA were defined as the highest serum or lavage dilution that resulted in an absorbance value (OD 450) two times greater than that of negative control.

2.9. Toxin neutralization (TN) test

For determination of serum anti-TT antitoxin titers, the TN test was performed at L+/100 and L+/1000 levels by the methods described elsewhere [17]. The L+/100 and L+/1000 doses of tetanus toxin are the minimal amounts of tetanus toxin, when mixed, respectively, with 0.01 and 0.001 antitoxin unit [AU] of standard tetanus antitoxin kill 100% of mice in 4 days. Tetanus toxin was diluted to L+/100 or L+/1000 doses per ml. Various dilutions of standard tetanus antitoxin and serum samples were mixed with L+/100 or L+/1000 doses of toxin. The volume was made up to 1 ml with normal saline. The toxin-antitoxin or toxin-serum mixtures were incubated at room temperature for 1 h. Each mixture was assayed by injecting 0.5 ml subcutaneously into 3 mice. Mice were observed for 5 days for tetanic symptoms and deaths. The titers of samples were calculated against the standards in terms of AU/ml.

2.10. Erythrocyte hemolysis test

The experiment was essentially performed as mentioned by Bjork and Edman [18]. Ten milliliters of fresh heparinized human blood was centrifuged at 2000*g* for 10 min. The cells were then washed three times by suspending them in Mc Ilvaine's buffer (citric acid, NaCl, Na_2HPO_4), pH 7.0. Two hundred microliters of RBC suspension (12% hematocrit) was incubated with 200 μl of liposome suspensions (containing 50, 100 or 200 μl of liposome suspension diluted to 200 μl with Mc Ilvaine buffer) for 30 min at 37 °C. The mixtures were then centrifuged at 1100*g* for 35 s. The absorbance of the supernatant was recorded at 540 nm. 100% hemolysis was achieved by adding 0.2 ml deionized water to 0.2 ml RBC suspension and the percentage of total hemolysis was then calculated.

2.11. Local irritation studies in human volunteers

One hundred microliters of blank liposomes was sprayed into the right nostril of four healthy volunteers and any symptoms of local irritations including sneezing, coughing, tearing, nasal stinging and burning was recorded in a 1-week follow up period.

2.12. Statistical analysis

Statistical analysis of the results was carried out using unpaired Student's *t*-test.

2.13. Ethics in animal and clinical investigations

The protocol of animal and clinical studies was approved by Regional Ethics Committee.

3. Results

3.1. Morphology and size of liposomes

The liposomes were morphologically multilamellar vesicles, as observed under optical microscope. Using particle size analyzer, volume mean diameter of liposomes was determined to be 2.3 ± 0.6 ($n = 3$).

3.2. Encapsulation efficiency of tetanus toxoid (TT) and CpG-ODN in liposomes and leaking of TT from liposomes

The encapsulation efficiency of tetanus toxoid (TT) and CpG-ODN in liposomes was determined to be 54.0 ± 8.8 ($n = 5$) and 60.1 ± 7.4 ($n = 3$), respectively.

The cumulative percent of leaked TT in 3 months was, respectively, as 4.86%, 5.93% and 7.38% of encapsulate.

3.3. Structural stability and immunoreactivity of encapsulated TT

The structural stability of liposomal form of TT was evaluated using a SDS–PAGE method. The identical bands were seen for encapsulated and original TT. This is indicative of preservation of protein structure of tetanus toxoid in the liposome preparation process and that the physical and chemical stresses encountered have not affected this structure.

The immunoreactivity of liposomal TT was also compared with original TT by an ELISA method. The results are showing that the immunoreactivity of encapsulated TT has been completely conserved. The immunoreactivity of encapsulated TT was $100.5 \pm 1.3\%$ ($n = 3$) that of original TT.

3.4. Serum anti-TT IgG titers

Rabbits ($n = 4$) were nasally immunized with 40 Lf TT and 10 μg CpG-ODN at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT IgG titers were determined by an ELISA method (Fig. 1). The highest IgG titers among the nasally immunized animals were seen in groups immunized with TT and CpG solution ($P < 0.05$). CpG-ODN as an immunopotentiating adjuvant could increase the serum IgG titers when co-administered with TT solution ($P < 0.05$), or co-encapsulated with TT in neutral liposomes ($P < 0.01$). Positive controls were intramuscularly injected with 10 Lf alum adsorbed TT and showed the highest IgG titers ($P < 0.0001$). In all of groups, the highest serum IgG titers were achieved in the sixth week, following three immunizations. The IgG titers were gradually decreased between weeks 6 and 10, when no boosting immunization was done (Table 1).

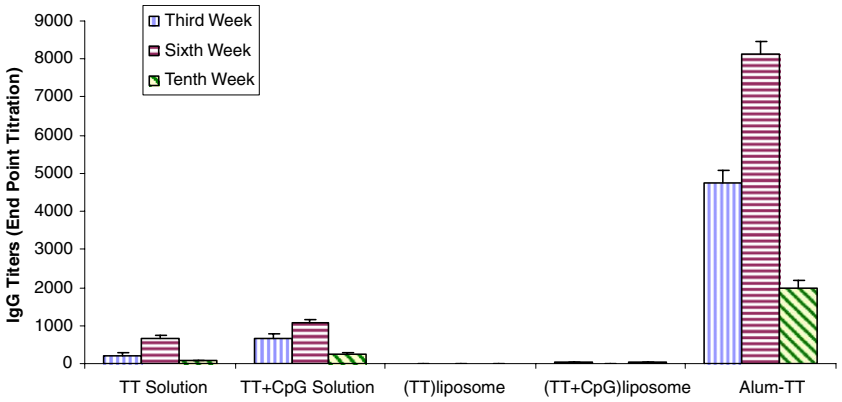

Fig. 1. Serum anti-TT IgG titers. Rabbits ($n = 4$) were nasally (intramuscularly for Alum-TT) immunized with 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT IgG titers (end point titration) were determined by an ELISA method. Error bars represent SE.

Table 1
Serum IgG and antitoxin, and nasal lavage IgA titers. Rabbits ($n = 4$) were nasally immunized with 10 or 40 Lf TT and 10 μg CpG-ODN at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10

Nasally administered formulations	Serum IgG titers (EPT[a])			Serum antitoxin titers (AU/ml)			Nasal lavage IgA titers (EPT[a])
	Third week	Sixth week	Tenth week	Third week	Sixth week	Tenth week	Tenth week
Blank liposomes	nd[b]	nd[b]	nd[b]	0	0	0	nd[b]
TT solution (10 Lf)	nd[b]	nd[b]	nd[b]	0	0	0	nd[b]
TT solution (40 Lf)	215	640	85	0	0.05	0.03	31
TT + CpG solution (40 Lf)	675	1063	263	0.02	0.20	0.20	31
(TT) liposomes (40 Lf)	4	2	4	0	0	0	64
(TT + CpG) liposomes (40 Lf)	12	31	29	0.009	0.013	0.013	13
Alum-TT (10 Lf)[c]	4750	8125	2000	1.40	7.20	4.70	9

Nasal lavages were collected only at tenth week.
[a] End point titration (EPT).
[b] Not determined (nd).
[c] Alum adsorbed TT was injected intramuscularly.

3.5. Nasal lavage anti-TT IgA titers

Following the above-mentioned immunizations, at the tenth week, nasal lavages were collected, pooled and anti rabbit IgA titers were determined by ELISA (Fig. 2).

Among the groups immunized with various formulations, the highest mucosal IgA titers were seen in animals immunized with liposomes encapsulated with TT. Co-encapsulation of CpG-ODN with TT in liposomes greatly suppressed the IgA titers, compared with liposomes encapsulated with TT alone. However, when CpG-ODN was simply mixed with TT solution did not change the mucosal IgA titers. Intramuscular injection of alum-TT resulted the lowest sIgA titers, compared with nasally immunized animals (Table 1).

3.6. Serum anti-TT antitoxin titers

Following above-mentioned immunization protocol, serum anti-TT antitoxin titers (AU/ml) were determined by a bioassay method, toxin neutralization (TN) (Fig. 3). The highest antitoxin titers were induced with nasal administration of TT and CpG-ODN solution ($P < 0.05$). The antitoxin titers induced by liposome formulations were lower than solution formulations ($P < 0.05$). CpG-ODN as an immunomodulator adjuvant could increase the antitoxin titers, both co-administered with TT solution or co-encapsulated with TT in liposomes ($P < 0.05$). Nasal administration of blank liposomes, as negative control, resulted in zero antitoxin titer. Animals injected with 10 Lf alum-TT, as positive controls, showed far higher antitoxin titers in comparison with nasally immunized animals ($P < 0.0001$) (Table 1).

3.7. Hemolysis and nasal irritation

Different concentrations of liposomes were incubated with erythrocyte suspension, but no hemolysis was observed.

Liposomes suspension was nasally administered to four human volunteers, but no irritation was reported. Both immediately after administration and in 1-week following up, there was no report of sneezing, coughing, stinging or burning sensation in the nose.

4. Discussion

Most infectious diseases begin at a mucosal surface, therefore as a preventive measure, one must consider ways to enhance local immunity to prevent the attachment and

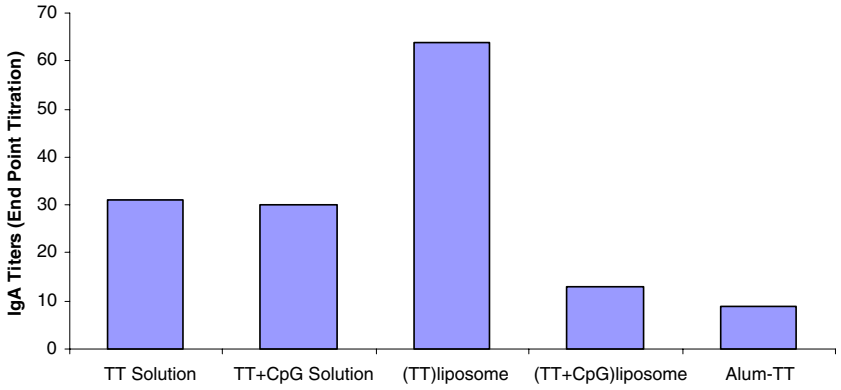

Fig. 2. Nasal lavage anti-TT IgA titers. Rabbits ($n = 4$) were nasally (intramuscularly for Alum-TT) immunized with 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and nasal lavages were collected at week 10. Lavages were pooled and Anti-TT IgA titers (end point titration) were determined by an ELISA method.

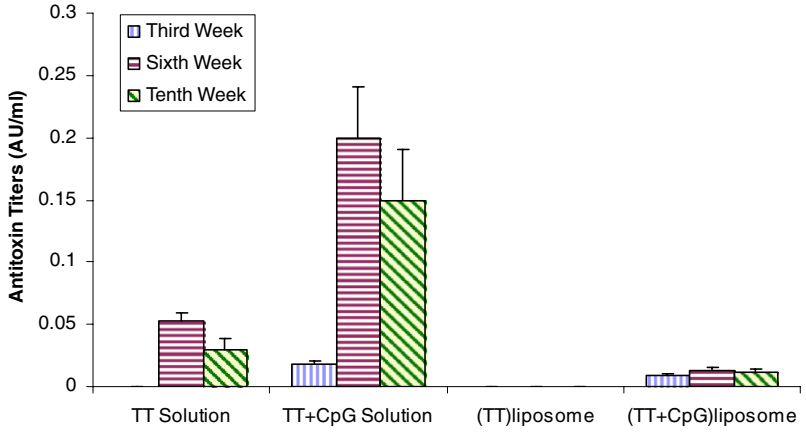

Fig. 3. Serum anti-TT antitoxin titers. Rabbits ($n = 4$) were nasally immunized with 10 or 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT antitoxin titers (AU/ml) were determined by toxin neutralization (TN) bioassay. Error bars represent SE.

invasion of microbes [19]. Despite this understanding, vaccination usually involves parenteral administration of antigens. This route of immunization stimulates the immune system to produce IgG antibody in the serum but fails to generate a mucosal antibody response. In contrast, vaccines that are delivered either orally or intranasally stimulate IgA antibody response along the mucosal surfaces of the gastrointestinal or respiratory tracts. IgG facilitates the phagocytosis of bacteria and activates the complement whereas IgA principally acts by preventing attachment and colonization of bacteria on mucosal surfaces [20].

In the present study, neutral liposomes as antigen delivery system and particulate adjuvant were used for efficient targeting of antigens to the mucosal associated lymphoid tissues (MALT). The adjuvant potential of liposomes in enhancing the systemic and mucosal immune responses was compared to immunopotentiating adjuvant, CpG-ODN, which could directly activate the cells of the immune system.

The results presented herein indicate that intranasal administration of liposomes encapsulated with tetanus toxoid (TT) suppressed the systemic immune responses, while highly potentiating the mucosal sIgA titers. Animals immunized with TT-containing liposomes showed higher mucosal immune responses, compared to liquid formulations (Fig. 2). Liposomes encapsulated with TT + CpG-ODN produced higher serum IgG ($P < 0.01$), antitoxin titers and lower nasal lavage IgA titers, compared with TT-liposomes.

It has been reported that liposomes, administered i.n., provide a promising adjuvant system for stimulation of antibody responses in general, and mucosal sIgA responses in particular [21]. Thus, the liposomal formulations with various compositions and sizes have been widely used for mucosal immunizations and different results have been obtained.

In an effort for the induction of antibody responses against opacity (Opa) proteins of Neisseria meningitidis, mice were intranasally immunized with liposomes containing Opa proteins. These antigens were combined with or without adjuvants. After intranasal immunization with any of these formulations, anti-Opa IgA antibodies were found in nasal lavages. However, non-adjuvanted liposomal Opa formulations were poorly immunogenic [22].

Nasal immunization of normal mice with HIVgp160-encapsulated hemagglutinating virus of Japan (HVJ)-liposome induced high titers of gp160-specific neutralizing IgG in

serum and IgA in nasal wash, saliva, fecal extract, and vaginal wash, along with both Th1- and Th2-type responses [23].

Liposomal formulations of an influenza subunit preparation were intranasally administered to mice and resulted in a stimulated serum IgG response relative to the response to i.n. immunization with the antigen alone. In addition, the liposomal vaccine induced a secretory IgA (sIgA) response in the mucosa of the lungs and nasal cavity. Immune stimulation was observed with negatively charged liposomes but not with zwitterionic liposomes, consisting of PC and cholesterol alone [21].

Nasally administered liposomes encapsulated with inactivated whole measles virus vaccine, but not the free virus, induced a secretory IgA (s-IgA) response in the lungs and nasal cavity. The liposomes induced a moderate increase in the serum IgG response, but no t-IgA response, following intramuscular immunization [24].

In our study, while the highest mucosal sIgA titers were obtained with liposomal formulation, the systemic responses and protection were even lower than that of TT solution. It has been suggested that particle size may determine the type of the immune response elicited by vaccine-containing microspheres administered by the mucosal route. Particles larger than 3 μm in diameter have been shown in humans to be retained in the nasal cavity when inhaled [25] and it has been observed in calves that tonsils could absorb resin particles of 1–5 μm in diameter [26]. An explanation for our observation is that the liposomes (even with <5 μm in diameter) mainly remain in the IgA inductive sites and are not able to translocate to regional lymph nodes, which is needed for induction of systemic immune responses [4]. Our previous gamma-scintigraphic study in human nose has also shown that the neutral liposomes have a high mucoadhesion potential and after a 4-h follow up, only $18 \pm 2.9\%$ of administered liposomes were cleared from the human nose [27]. So, liposomes have sufficient time to interact with IgA inductive sites at nasal mucosa and induce high sIgA titers, but as they are not able to penetrate into the microfold cells of nasal associated lymphoid tissue (NALT), they fully failed to induce the systemic responses. On the other hand, because of the stability of liposomes (7.38% of encapsulate leaked in a 3-month in vitro follow up), encapsulated TT has not been released, and has not been able to induce the systemic immunity as the TT solution.

In the preparation of liposomes with DRV method, the freezing of antigen could potentially affect the structural stability and immunoreactivity of encapsulated antigen. The identical bands for liposomal and original TT, as well as the preserved immunoreactivity of liposomal TT, indicate that TT has well tolerated the formulation process.

In the present study, administration of 40 Lf of TT in solution as a nasal drop led to systemic and mucosal immune responses (Table 1). As regards protective levels of tetanus antitoxin serum titers (0.01 AU/ml)[17], administration of 40 Lf TT solution could result in protective levels of antitoxin. Co-administration of TT and CpG-ODN in solution increased both serum IgG and antitoxin titers ($P < 0.01$), but IgA titers in nasal lavages remained unchanged. Co-encapsulation of CpG-ODN and TT in liposomes could also induce the protective levels of antitoxin titers, while no antitoxin titers were achieved by liposomes encapsulated with TT alone (Fig. 3).

Intramuscular injection of alum-adsorbed TT which is the usual route of vaccination against tetanus was also used as positive control. Rabbits immunized with alum-TT showed highest serum IgG and antitoxin titers ($P < 0.001$), but as expected, parenteral immunization did not induce mucosal responses and the lowest IgA titers were seen in nasal lavages of this group (Fig. 2).

In the present study, lack of membrane toxicity and local irritation of neutral liposomes in human nose was studied. Different concentrations of blank liposomes were incubated with human RBCs and no hemolysis was observed. It could be interpreted as a safety issue for these liposomes. Tolerability of liposomes by the users is of high practical importance. Any local irritation aroused by liposomes could result in sneezing and rhinorrhea; both of them could expel out the suspension and decrease in drug delivery efficiency. Nasal application of blank liposomes to four human volunteers did not cause any local irritation. This finding also demonstrates one safety aspect of these liposomes in practical use.

5. Conclusion

The combined results of these studies suggest that intranasal administration of liposomes encapsulated with vaccines is an effective way for inducing mucosal immune responses. We also showed that little or no systemic IgG and high mucosal IgA responses can be induced in rabbits with intranasal administration of liposomes encapsulated with TT. CpG-ODN as an immunomodulating adjuvant could also exert its adjuvant effect both in solution and encapsulated in liposomes. Lack of membrane toxicity, as studied by a standard hemolysis test, and local irritation of neutral liposomes in human nose were also indicated for the first time. The immunoreactivity of TT extracted from liposomes was completely preserved ($100.5 \pm 1.3\%$ that of original TT).

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