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Induction of systemic and mucosal immune responses by intranasal administration of alginate microspheres encapsulated with tetanus toxoid and CpG-ODN

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Abstract

In the induction of systemic and mucosal immunity, particulate antigens are more effective than soluble antigens; possibly because they are more efficiently endocytosed by mucosal-associated lymphoid tissue (MALT) M cells. In this study, we determined the systemic and mucosal immune responses in rabbits following intranasal immunization with encapsulated tetanus toxoid (TT) and CpG-ODN in alginate microspheres. The microspheres were less than 4 μm in diameter. Encapsulation efficiency of TT and CpG-ODN was determined as 47.7 ± 6.6 and 34.2 ± 7.4, respectively. Release of TT and CpG-ODN in a simulated model with nasal cavity was 14.2 ± 3.06 and 36.7 ± 2.4% after 4 h. Encapsulated TT preserved its intact structure, but its immunoreactivity was decreased to about 91 ± 5%. The highest serum IgG and antitoxin, and nasal lavage IgA titers were observed in groups immunized with microsphere formulations. CpG-ODN as an adjuvant could increase the serum IgG and antitoxin titers when co-administered with TT solution, but its co-encapsulation with TT in alginate microspheres failed to potentiate the systemic immune response while induced high IgA titers in nasal lavages. No hemolysis was occurred on incubation of alginate microspheres and human RBCs. Also after nasal administration of plain microspheres to human volunteers, no local irritation was observed. Intranasal administration of microspheres encapsulated with vaccines showed to be an effective way for inducing a variety of immune responses and that a strong systemic IgG and mucosal IgA responses can be induced in rabbits with intranasal administration of alginate microspheres encapsulated with TT.

Keywords: Nasal immunization; Alginate; CpG-ODN; Microsphere; Tetanus toxoid

1. Introduction

It is well known that protection against pathogenic organisms correlates better with the presence of antibody in local secretions than with serum antibody (Husband, 1993). It has also been shown that antibodies resulting from parenteral immunization do not necessarily reach mucosal surfaces where most infectious agents enter the host (Bowersock et al., 1996). Even after highly immunogenic antigens (e.g. polysaccharide–protein conjugates) are administered by intramuscular injection, systemic IgM or IgG could not effectively prevent the primary colonization and invasion of pathogens into bronchoalveolar tissue (Cho et al., 1998).

The most effective way to induce mucosal immunity in the upper respiratory tract is intranasal immunization (Rebelatto et al., 2001). Immunization at the mucosal surfaces which produces the protective antibody, secretory immunoglobulin A (IgA), is very important (Gombotz and Wee, 1998; McGhee et al., 1992). Development of effective delivery systems for the presentation of antigens to mucosal surfaces is critical to the success of these vaccines (Gombotz and Wee, 1998). 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 (Rebelatto et al., 2001). It has been shown that after nasal administration, polymeric particles can be taken

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up by the nasal-associated lymphoid tissues, followed by inducing the immune responses to the encapsulated antigens (Carr et al., 1996; Lemoine et al., 1998).

Alginate microspheres act as adjuvants, and vaccine-containing alginate microspheres are effective for nasal vaccination in animal species (Rebelatto et al., 2001).

Oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG-ODNs) appears to be a promising class of adjuvants for a wide variety of vaccine candidates (McCluskie and Davis, 1998). CpG-ODNs affect various components of the immune system and can induce strong humoral or cellular immune responses according to their sequences.

In the present study, tetanus toxoid (TT)-containing alginate microspheres were prepared and their efficiency, 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 tested when it was co-encapsulated with TT in microspheres or simply mixed with toxoid in solution.

2. Materials and methods

2.1. Materials

Sodium alginate, bicinchoninic acid (BCA), bovine serum albumin (BSA) and Span-80 were purchased from Fluka (Buchs, Switzerland). Calcium chloride, *n*-octanol, sodium citrate and isopropyl alcohol were from Merck (Darmschtadt, Germany). Tetanus toxoid solution (2500 Lf/ml) and alum-adsorbed tetanus toxoid (50 Lf/ml) were from Razi Inc. (Hesarak, Iran). CpG-ODN was purchased from Microsynth (Balgach, Switzerland). Anti-rabbit IgG and IgA were purchased from Sigma (MO, USA) and Bethyl Laboratories Inc. (TX, USA), respectively. White albino rabbits weighing 2–2.5 kg were bred and provided by animal house of Mashhad University of Medical Sciences.

2.2. Preparation of alginate microspheres encapsulated with TT and/or CpG-ODN

Briefly, 1 ml of aqueous solution of sodium alginate (3%, w/v) containing 360 Lf TT (and 100 μg CpG-ODN) was dispersed in 10 ml *n*-octanol solution containing 2% (w/v) of Span-80. The emulsion was prepared by probe sonication (Soniprep-150, MSE, Sussex, UK) in amplitude of 18 for 90 s. The emulsion was rapidly added to a solution (60 ml) of calcium chloride in octanol (0.33%, w/v). After 10 min, 2 ml of isopropyl alcohol was added dropwise. The microspheres were collected by filtration.

2.3. Morphology and size analysis of alginate microspheres

Optical microscope (Carl Zeiss, Oberkochen, FRG) and scanning electron microscope (Leo, Oberkochen, Germany) were used for both studying the morphological features of microspheres and analyzing the size distribution. The volume mean diameter of microspheres was also determined by a particle size analyzer (Zetasizer 2000, Malvern, UK).

2.4. Determination of the encapsulation efficiency of tetanus toxoid and CpG-ODN in alginate microspheres

Five milligrams of TT- and/or CpG-containing microspheres were dissolved in 750 μl sodium citrate (0.1 M, pH 7.4). Bicinchoninic acid (BCA) protein assay was used to determine the TT concentration in the microsphere solution.

The amount of oligodeoxynucleotide was estimated spectrophotometrically based on absorbance at 260 nm (Barman et al., 2000).

2.5. In vitro release study

The release profile of tetanus toxoid from alginate microspheres was studied with a diffusion chamber, which mimics the hydration conditions of the nasal mucosa (Cornaz et al., 1996). The donor compartment contained air saturated with water and the receiver contained 25 ml of PBS (pH 7.4), working at 37 °C. The microspheres (25 mg) were laid on a filter paper in contact with the liquid phase of the receiver compartment. During 4 h, every 30 min, 400 μl samples were drawn from the receiver compartment and TT or CpG-ODN released from microspheres was quantified. Each experiment was done in triplicates.

2.6. Structural stability and immunoreactivity of encapsulated TT

The molecular stability of encapsulated TT was evaluated by SDS-PAGE method.

The immunoreactivity of TT extracted from microspheres was determined by an ELISA method (Diwan et al., 2001). Briefly, wells were coated with 50–1000 ng/well (100 μl of each concentration in quadruplicate) 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 with 1% BSA, 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 mice immunized three times by s.c. injection of 2 Lf alum-adsorbed TT. The proper working dilution of hyperimmune serum was determined by a proprietary ELISA assay. After 1 h of incubation and washing, 100 μl of the working dilution of HRP-conjugated goat anti-mouse IgG was added. TMB:peroxidase was used for color development.

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 alginate microspheres;
(2) 10 Lf TT solution;
(3) 10 Lf TT in alginate microspheres;
(4) 40 Lf TT solution;
(5) 40 Lf TT + 10 μg CpG-ODN both in solution;
(6) 40 Lf TT in alginate microspheres;

(7) 40 Lf TT + 10 μg CpG-ODN both in microspheres;
(8) 10 Lf alum-adsorbed TT (IM injection).

Ten milligrams of microspheres (5 mg in each nostril, drown into polyethylene tubes) were nasally administered. The solutions (200 μl, 100 μl in each nostril) were administered using a pipette.

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

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 ELISA assay (Diwan et al., 2002).

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 method described by Dokmetjian et al. (2000). The L+/100 and L+/1000 dose 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, kills 100% of mice in 4 days. Tetanus toxin was diluted to L+/100 or L+/1000 doses/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 three 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 (1990). Human RBCs were suspended in McIlvaine's buffer (citric acid, NaCl and Na_2HPO_4), pH 7. Two hundred microlitres of RBC suspension (12% hematocrit) was incubated with 200 μl of alginate microspheres suspension (containing 0.25, 0.5 or 1 mg microspheres) for 30 min at 37 °C. The absorbance of the supernatant was recorded at 540 nm.

2.11. Local irritation studies in human volunteers

Ten milligrams of blank alginate microspheres was dispersed into the right nostril of four healthy volunteers. 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 was carried out by unpaired Student's *t*-test. *P*-values less than 0.05 were regarded as significant.

3. Results

3.1. Morphology and size of alginate microspheres

Spherical, discrete and smooth microspheres were obtained. Mean diameter of alginate microspheres encapsulated with TT and TT + CpG-ODN was 2.2 ± 0.1 and 3.2 ± 0.2 μm ($n = 4$), as observed under optical microscope. Percent of microspheres larger than 10 μm in diameter was determined as 1.5 and 1.7%, respectively. Using particle size analyzer, volume mean diameter of microspheres was determined to be 1.3 ± 0.4 and 2.0 ± 0.5 μm, respectively.

3.2. Encapsulation efficiency of tetanus toxoid and CpG-ODN in alginate microspheres

The encapsulation efficiency of tetanus toxoid and CpG-ODN in alginate microspheres was found to be $47.7 \pm 6.6\%$ ($n = 5$) and $34.2 \pm 7.4\%$ ($n = 3$). The loading of TT and CpG-ODN in microspheres was around 4 Lf/mg and 1 μg/mg, respectively. The yield of alginate microspheres was determined as $90 \pm 33\%$.

3.3. Release profile of TT and CpG-ODN from alginate microspheres

The tetanus toxoid was released from microspheres with a low burst release of $6.6 \pm 3.5\%$ in 30 min. This was followed by a slow and sustained release profile. After 4 h, $14.2 \pm 3.06\%$ of encapsulated TT was released (Fig. 1a). The release profile of CpG-ODN was started with a high burst release of 28.3 ± 5.2 in the first 30 min and reached to 36.7 ± 2.4 after 4 h (Fig. 1b). The diffusion cell method has several benefits over usual in vitro release studies. In this model, microspheres are in contact with a wetted and warm surface (filter paper) in a humid atmosphere, similar to nasal cavity. In this model, the release rate of encapsulate presumably is lower than usual models. Normally, microspheres are immersed in a stirring medium, thus it seems that our results could be better extrapolated to in vivo situations.

3.4. Structural stability and immunoreactivity of encapsulated TT

The identical bands were seen for encapsulated and original TT (Fig. 2). This is indicative of preservation of protein structure of tetanus toxoid in the microsphere preparation process. The immunoreactivity of extracted TT was also compared with original TT by an ELISA method. The results are showing that the immunoreactivity of encapsulated TT decreased to about $91 \pm 5\%$ when compared to that of original TT. This decrease in immunoreactivity could be attributed to some physical and chemical harsh conditions such as organic solvents, surfactants and sonication treatment.

3.5. Serum anti-TT IgG titers

Sera TT IgG titers were determined by ELISA (Fig. 3). The highest IgG titers among the nasally immunized animals

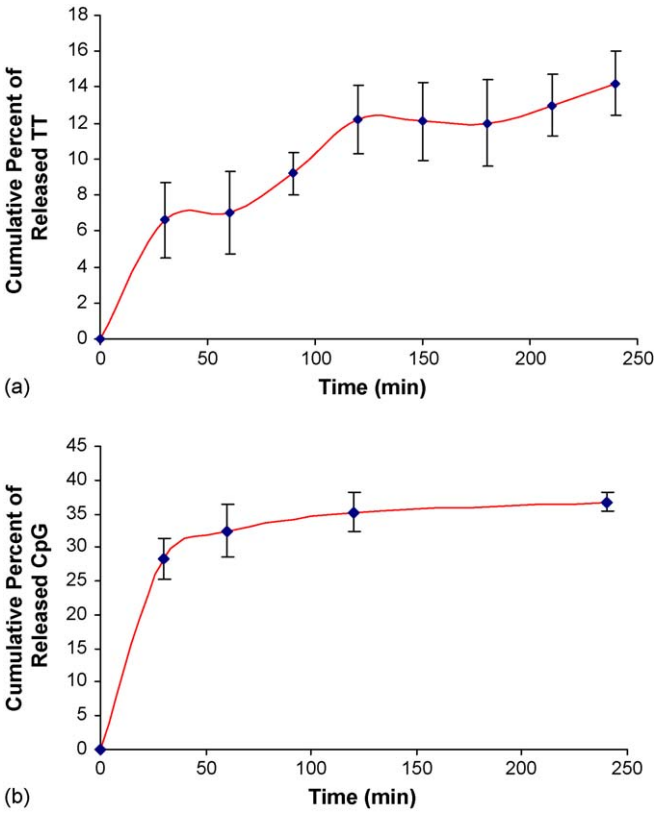

Fig. 1. In vitro release of encapsulated tetanus toxoid (a) and CpG-ODN (b) from alginate microspheres. Microspheres (25 mg) were laid on a paper filter mounted in a diffusion cell. Each 30 min until 4 h, 400 μl samples were drawn from the receiver compartment and immediately replaced with fresh buffer. Each experiment was done in triplicate. Error bars represent the standard error of the mean ($n = 3$).

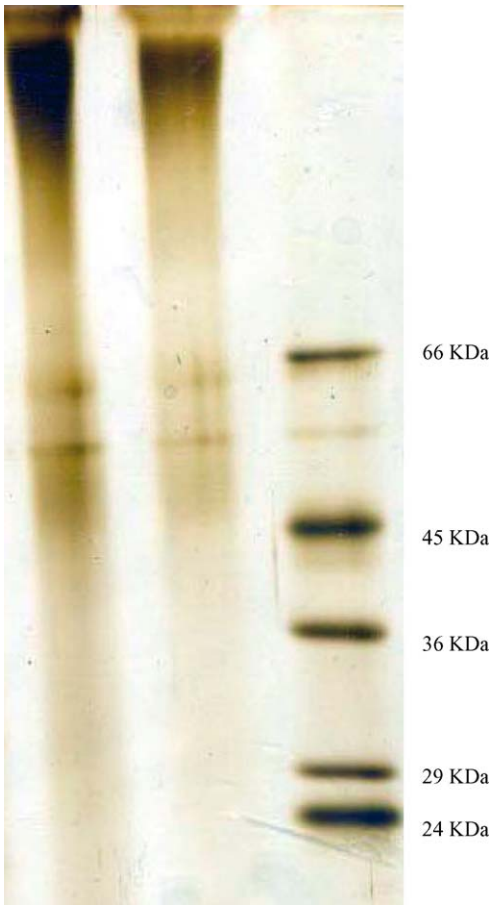

Fig. 2. The SDS-PAGE gel. Dissolved TT-containing microspheres in sodium citrate, original TT and a molecular weight reference marker were loaded onto a 10% acrylamide gel. Protein bands were visualized by silver nitrate staining.

were seen in groups immunized with microsphere formulations ($P < 0.001$, 6th week; $P < 0.01$, 10th week). CpG-ODN as an immunopotentiating adjuvant could increase the serum IgG titers when co-administered with TT solution ($P < 0.05$), but its co-encapsulation with TT in alginate microspheres failed to potentiate the immune response. Positive controls were intramuscularly injected with 10 Lf alum adsorbed TT and showed the highest IgG titers. The sera IgG titers in animals nasally immunized with blank microspheres or 10 Lf TT (solution or microsphere) were not determined.

3.6. Nasal lavage anti-TT IgA titers

The highest mucosal IgA titers were seen in animals immunized with microsphere formulations (Fig. 4). Co-encapsulation of CpG-ODN with TT in alginate microspheres was greatly increased the IgA titers, compared with microspheres encapsulated with TT alone. However, when CpG-ODN was simply mixed with TT solution could not increase the mucosal IgA titers. Intramuscular injection of alum-TT, resulted the lowest sIgA titers, compared with nasally immunized animals. The lavage IgA titers in animals nasally immunized with blank microspheres or 10 Lf TT (solution or microsphere) were not determined.

3.7. Serum anti-TT antitoxin titers

Nasal immunizations with 10 Lf TT, both encapsulated and in solution, resulted in zero or trace antitoxin titers. However, encapsulation of 40 Lf TT in microspheres as well as co-administration of TT with CpG solution could significantly increase the antitoxin titers ($P < 0.01$). Co-encapsulation of TT and CpG in microspheres could also increase the antitoxin titers, compared with TT solution ($P < 0.05$) (Fig. 5). The sera

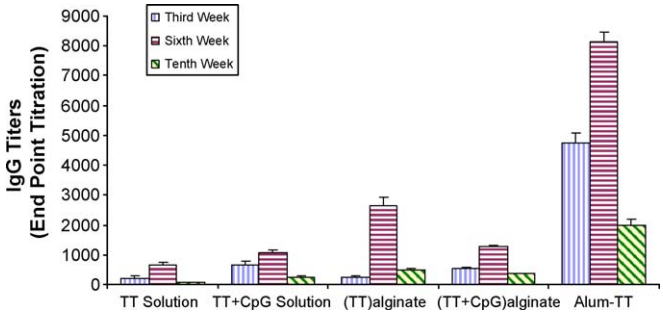

Fig. 3. Serum anti-TT IgG titers (mean ± S.E.). Rabbits ($n = 4$) were nasally (intramuscularly for alum-TT) immunized with 10 or 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT IgG titers (end-point titration) were determined by an ELISA method.

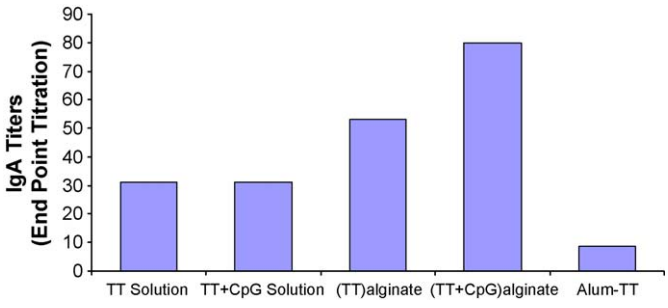

Fig. 4. Nasal lavage anti-TT IgA titers. Rabbits ($n=4$) were nasally (intramuscularly for alum-TT) immunized with 10 or 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and nasal lavages were collected at week 10. Lavages were pooled and anti-TT IgA titers (end-point titration) were determined by an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

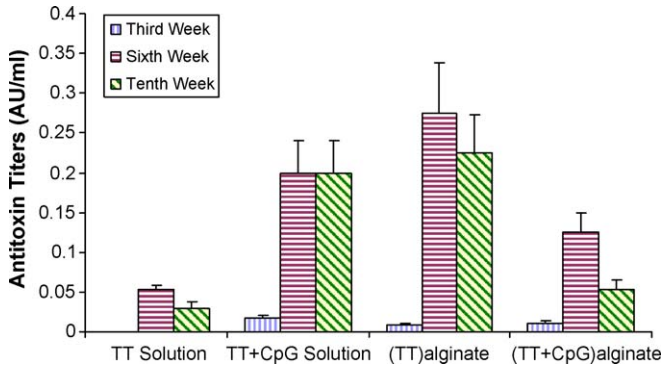

Fig. 5. Serum anti-TT antitoxin titers (mean ± S.E.). Rabbits ($n=4$) were nasally immunized with 10 or 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT antitoxin titers (AU/ml) were determined by toxin neutralization (TN) bioassay.

antitoxin titers in animals nasally immunized with blank microspheres or 10 Lf TT (solution or microsphere) were zero. Animals injected with 10 Lf alum-TT, as positive controls, showed higher antitoxin titers (4750, 8125 and 2000 IU/ml, in 3rd, 6th and 10th weeks) in comparison with nasally immunized animals ($P<0.0001$).

3.8. Hemolysis and nasal irritation

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

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

4. Discussion

The results obtained in this study indicate that intranasal administration of alginate microspheres encapsulated with tetanus toxoid can induce strong immune responses. Animals immunized with TT-containing alginate microspheres showed higher systemic and mucosal immune responses, compared to liquid formulations ($P<0.01$). Microspheres encapsulated with TT + CpG-ODN produced lower serum IgG ($P<0.01$), comparable antitoxin titers ($P=0.06$) and higher nasal lavage IgA titers, compared with TT-microspheres. In a previous study (Diwan et al., 2002), we immunized mice intramuscularly with PLGA nanospheres encapsulated with TT or TT + CpG-ODN. Co-encapsulation of TT with CpG-ODN in PLGA nanospheres was able to potentiate the systemic humoral and cellular immune responses (significantly higher IgG, IgG1, IgG2a, IgG2b and IFN-γ titers), when compared to TT-nanospheres and TT or TT + CpG solutions.

Particles larger than 3 μm in diameter have been shown in humans to be retained in the nasal cavity when inhaled (Stuart, 1984) and it has been observed in calves that tonsils could absorb resin particles of 1–5 μm in diameter (Payne et al., 1960). M-cells, thought to be the principal uptake site of particulate antigen, are found in the distal regions of the nose, the nasopharyngeal and palatine tonsils, and bronchial-associated lymphoid tissues (BALT) in the lung (Eyles et al., 1999).

The combined systemic and mucosal responses observed in intranasally immunized animals may be attributed to the wide variation in the size of the microspheres. The smaller particles are translocated to regional lymph nodes and inducing a systemic immune response, and larger particles being retained in the NALT and inducing a mucosal immune response (Rebelatto et al., 2001). In tour study, TT + CpG microspheres were larger than the TT microspheres. Thus, one explanation is that TT microspheres, having smaller diameters, have been taken up more efficiently by NALT microfold cells, leading to a higher systemic immune response. Larger diameter of TT + CpG microspheres have supposedly resulted in a less uptake by M cells, hence the coencapsulated CpG-ODN failed to exert its adjuvant potential. Compared to TT microspheres, more TT + CpG microspheres have remained in the IgA inductive sites of NALT and produced higher mucosal IgA titers. These microspheres induced higher mucosal IgA which could be attributed to both size of microspheres and adjuvant effect of CpG-ODN.

One of the limitations of alginate microspheres, rooted from their internal porous structure, lies in their limited ability to prolong the release of entrapped drug. It has been reported that 90% of encapsulate have been released from alginate microspheres in 15 min (Chan et al., 1997). Regards to the higher immune stimulating potential of particulate antigens in nasal immunization (Eyles et al., 1999), lower release rate and more particulate form of antigen is preferred. The prepared microspheres showed a total release of 14% in 4 h. So the obtained immune responses could be mainly attributed to alginate encapsulated TT and possibly the released TT have a minor contribution. The release of CpG-ODN was started with a higher burst release of 28.3% and reached to 36.7 ± 2.4 after 4 h. This could be attributed to the low molecular weight (6058.7 Da) of CpG-ODN compared to TT (150,000 Da). The electrostatic interaction of cationic CpG-ODN and negatively charged polymer matrix did not prohibit the release. The similar results were seen in our previous study, in that the release of CpG-ODN from PLGA nanospheres was higher in comparison with TT (Diwan et al., 2002).

The SDS-PAGE electrophoresis method could establish that TT could withstand the organic solvents, homogenization and sonication steps and preserve its native structure, as it is evident from identical bands for native and encapsulated TT (Fig. 2). It is still quite possible that antigenic epitopes on TT molecule be affected by organic solvents, sonication, etc. In this study, the immunoreactivity of the antigen encapsulated in alginate microspheres was, for the first time, studied by an ELISA method. Results revealed that the preparation process has minimal effect on the antigen and decrease its immunoreactivity only by $9 \pm 5\%$.

In the present study, lack of membrane toxicity and local irritation of alginate microspheres in human nose was studied for the first time. Different concentrations of blank alginate microspheres were incubated with human RBCs and no hemolysis was observed. This could be interpreted as a safety issue for alginate microspheres. Tolerability of microspheres by the users is of high practical importance. Any local irritation caused by microspheres could result in sneezing and rhinorrhea which, both of them could expel out the particles and decrease in drug delivery efficiency. Nasal application of blank alginate microspheres to four human volunteer did not cause any local irritation. This finding could also demonstrate one safety aspect of alginate microspheres for practical use.

In the present study, administration of 40 Lf of TT solution as nasal drop, could induce systemic and mucosal immune responses (Figs. 3–5). Regards to protective levels of tetanus antitoxin serum titers (0.01 AU/ml), administration of 40 Lf TT solution could result in protective levels of antitoxin. Co-administration of TT and CpG-ODN solution increased both serum IgG and antitoxin titers ($P < 0.01$), but IgA titers in nasal lavages remained unchanged. In our previous study (Diwan et al., 2002), s.c. injection of TT + CpG-ODN solution to mice induced higher humoral and cellular immune responses, compared to TT solution.

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 could not induce mucosal responses and the lowest IgA titers was seen in nasal lavages of this group (Fig. 4).

Our previous gamma-scintigraphic study in human nose showed that the alginate microspheres have a high mucoadhesion potential. In that study, we compared the clearance rate of alginate, PLGA and Sephadex microspheres from human nose and showed that the clearance half-life of alginate microspheres in human nose is more than 4 h (Tafaghodi et al., 2004).

The induction of systemic and mucosal immune responses was also examined by Rebelatto et al. (2001) by intranasal administration of pig serum albumin in alginate microspheres. High levels of anti-PSA IgG1 antibodies were found in the serum, nasal secretions, and to a lesser extent in the saliva of calves vaccinated intranasally, but not orally, with PSA-microspheres. There was no significant increase of PSA-specific IgA.

5. Conclusion

The combined results of these studies suggest that intranasal administration of microspheres encapsulated with vaccines is an effective way for inducing a variety of immune responses. We also showed that a strong systemic IgG and mucosal IgA responses can be induced in rabbits with intranasal administration of alginate microspheres encapsulated with TT. CpG-ODN as an immunomodulating adjuvant could also exert its adjuvant effect both in solution and encapsulated in microspheres. We also showed that the microsphere size has a determinative role in the induction of the systemic and mucosal immune responses. Lack of membrane toxicity, as studied by a standard hemolysis test, and local irritation of alginate microspheres in human nose was also indicated for the first time. The immunoreactivity of TT extracted from microspheres have been decreased to about $91 \pm 5\%$ that of original TT. This is the first report on the quantitative effect of preparation conditions of alginate microspheres on immunoreactivity of encapsulated antigen.

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