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Induction of high antitoxin titers against tetanus toxoid in rabbits by intranasal immunization with dextran microspheres

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ABSTRACT

Poor absorption of protein antigens through the mucosal membranes necessitates the use of mucoadhesive delivery systems. Regarding the advantages of mucosal immunization and also the penetration enhancement potential of dextran microspheres, in this study the adjuvant potential of these microspheres was compared with CpG-ODN.

Cross-linked dextran microspheres (CDMs) were loaded with tetanus toxoid (TT). In vitro release studies were performed in a model, simulating the nasal cavity. The immunoreactivity of encapsulated TT was assayed by ELISA. Membrane toxicity and local irritating potential of CDM was examined by erythrocyte hemolysis and nasal administration to human nose, respectively. The various formulations were nasally administered to rabbits ($n = 4$). Alum-adsorbed TT (AATT) was injected as the positive control. The serum IgG and nasal lavage sIgA titers were determined by ELISA method. Serum antitoxin titers were determined by toxin neutralization (TN) bioassay method. Mean diameter of CDM was 128.1 ± 25.8 μm. Mean encapsulation efficiency was $20.3 \pm 3.2\%$ ($n = 3$). Antigenicity of encapsulated TT was $90.5 \pm 1.8\%$ ($n = 3$) that of original TT. Hemolysis studies showed no membrane disruption by CDM and none of the human subjects reported nasal irritation. Among the nasally immunized animals, the highest antitoxin titers was seen in the group immunized with CDM + TT ($P < 0.0001$). The serum IgG titers of the CDM + TT group was higher than the TT solution group ($P < 0.05$). The adjuvant potentials of CDM and CpG-ODN in inducing IgG titers was not significantly different ($P > 0.05$). The lowest sIgA titers in the bronchial lavage were seen in the group of animals received AATT parenterally.

Considering the proper release characteristics, desirable preservation of the antigen activity of TT, good mucoadhesion properties and also safety of CDM + TT, these microspheres could be regarded as an efficient mucosal adjuvant and antigen delivery system. These microspheres could induce very high antitoxin titers following nasal administration, while the CpG-ODN could not induce such titers. The antitoxin titers induced by CDM + TT was 175 times higher than the protective levels.

1. Introduction

Mucosal administration of vaccines offers several advantages. Neither sterile needles nor trained personnel is needed, reduces costs and increases patient compliance and, safety and minimization of adverse effects may be increased (Jepson et al., 2004; van der Lubben et al., 2001; Walker, 1994). Mucosal delivery of vaccines could make possible home administration of vaccines. These advantages would not only be of benefit, in less developed countries, but could also increase acceptability of and access to vaccinations in developed nations (Walker, 1994).

The introduction of proteins, including vaccines, without adjuvants into mucosal inductive sites may induce systemic unresponsiveness (mucosally induced tolerance). This modality could result in loss of the host's ability to respond to infection. Thus, mucosal adjuvants are required not only to boost mucosal and systemic immunity, but also to prevent the induction of mucosally induced tolerance (Fujihashi and Koga, 2002).

Illum et al. (1987) used Sephadex® (cross-linked dextran microspheres, CDMs) as well as Spherex (cross-linked starch microspheres) as carriers for drug delivery. Other derivatives of dextran and starch including diethyl aminoethyl dextran (DEAE-Dextran) (Illum et al., 1987) and polyacryl starch (Degling and Stjarnkvist, 1995) have also been used for mucosal drug delivery. The cross-linked dextran and starch microspheres have been used in mucosal delivery of several peptide and protein drugs including insulin

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(Chandler et al., 1991; Pereswetoff-Morath and Edman, 1995; Ryden and Edman, 1982), HCG (Illum et al., 1990), octreotide (Oechslein et al., 1996) and G-CSF (Gill et al., 1998).

Diwan et al. (2001, 1997) attached tetanus and diphtheria toxoids covalently to dextran microspheres and used parenterally in rat which induced considerable immune responses. These microspheres have also been investigated for encapsulation of liposomes (Stenekes et al., 2001). Several mechanisms have been reported for absorption enhancement effects of cross-linked starch and dextran microspheres: (1) Deposition of the microspheres in the less or no ciliated anterior part of the nasal cavity and slower nasal clearance (Illum et al., 2001). (2) Due to bioadhesive nature of microspheres, the formulation is retained in the nasal cavity for an extended time period (Illum et al., 2001). (3) The gelled system provides a local high drug concentration in close contact with the epithelial absorptive surface (Illum et al., 2001). (4) The absorption of water by the microspheres from the mucus layer may induce reversible shrinking of the epithelial cells and widening of the tight junctions for about 15 min. In this time, the transport of hydrophilic compounds could be increased (Bjork and Edman, 1990; Pereswetoff-Morath, 1998). (5) As in the presence of calcium-chelating agents, the tight junctions will be loosened and passage of hydrophilic molecules from the paracellular way will be increased (Oechslein et al., 1996).

There are only few reports in the literature on using pre-formed cross-linked starch and dextran microspheres for mucosal delivery of antigens (Pereswetoff-Morath and Edman, 1996). There are controversial results reported in the literature after mucosal immunization by these microspheres (O'Hagan, 1996; O'Hagan et al., 1993; Pereswetoff-Morath and Edman, 1996). However, in recent oral immunization studies, the laboratory-made cross-linked dextran and starch microspheres with less than 5-μm diameter had better results (Rydell and Sjoholm, 2004, 2005; Stertman et al., 2004, 2006).

In the present study, the potential of nasally administered CDM in inducing immune responses against encapsulated antigen (TT) was studied. The adjuvant potential of these microspheres was compared with CpG-ODN as a known immunomodulator. The routine vaccine (i.e. injectable AATT) was also used as the positive control.

2. Materials and methods

2.1. Materials

Sephadex® G-150 fine (CDM) was from Pharmacia (Sweden). Tetanus toxoid (TT) 2500 Lf/ml and alum-adsorbed TT 50 Lf/ml were obtained from Razi Ins. (Hesarak, Iran). Anti-rabbit IgG and IgA antibodies were from Sigma (Missouri, USA) and Bethyl Laboratories Inc. (Texas, USA). CpG-ODN was purchased from Microsynth (Balgach, Switzerland).

2.2. Loading of TT in CDM

To 5 ml of TT solution (1200 Lf/ml) in PBS (pH 7.4), 300 mg of CDM was added and stirred for 1 h at room temperature. Microspheres were then washed two times with PBS. For this purpose, microspheres were incubated in 5 ml PBS and stirred for 2 min. The separated microspheres were freeze-dried on a Heto DW3 freeze drier (Allerd, Denmark).

2.3. Morphology and size analysis of CDM

Optical microscope (Carl Zeiss, Oberkochen, FRG) and scanning electron microscope (Leo, Oxford, UK) were used for studying both morphological features and size distribution of CDM. For the latter purpose, the diameter of 300 microspheres was determined under the optical microscope equipped with an eyepiece reticule.

2.4. Encapsulation efficiency of TT

Supernatants of the washed microspheres were collected and the amount of non-included TT in supernatant was determined using Bicinchoninic acid (BCA) protein assay. Bovine serum albumin (BSA) (Fluka, Switzerland) was used as standard protein.

2.5. In vitro release studies

The release profile of tetanus toxoid from CDM was studied using a diffusion chamber, which mimics the conditions of nasal mucosa (Cornaz et al., 1996). The donor compartment contained humid air and the receiver contained 25 ml of PBS (pH 7.4, 37 °C). Microspheres (25 mg) were laid on a filter paper (Whatman no. 40) in contact with the liquid phase of the receiver compartment. During 4 h, every 30 min, 400 μl samples were taken from the receiver compartment and released TT or CpG-ODN was quantified. Each experiment was carried out 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 CDM 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 as 1:80,000 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. In vivo nasal immunization studies

White albino rabbits weighing 2.0–2.5 kg (four animals per group) were nasally immunized with the following formulations in days 0, 14 and 28 of experiment:

- Group 1: 10 Lf TT solution (nasal)–TT sol (10 Lf).
- Group 2: 40 Lf TT solution (nasal)–TT sol.
- Group 3: 40 Lf TT solution + 10 μg CpG-ODN (nasal)–TT + CpG sol.
- Group 4: 40 Lf TT in dextran microspheres (nasal)–CDM-TT.
- Group 5: dextran microspheres with no TT (nasal)–CDM.
- Group 6: 10 Lf TT alum-adsorbed (i.m. injection)–alum-TT.

Ten milligrams of dry microspheres filled in polyethylene tubes (5 mg in each nostril) were nasally administered. Solutions (200 μl, 100 μl in each nostril) were administered using an automatic pipetter.

Each animal was bled in weeks 3, 6 and 10. After the third bleeding, the trachea of the animals was cut and nasal cavity was washed with 10 ml of 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 an ELISA method (end-point titration).

End-point titers were determined as the highest dilution with absorbances two times higher than the normal sera (Diwan et al., 2002).

2.9. Toxin neutralization (TN) test

The toxin neutralization (TN) test was used for determination of serum anti-TT antitoxin titers. The 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 doses of tetanus toxin are the minimal amounts of tetanus toxin, which upon mixing with 0.01 and 0.001 unit [AU] of the 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 the 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 s.c. injection of 0.5 ml to 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 Mc Ilvaine's buffer (citric acid, NaCl, and Na_2HPO_4) pH 7. Two hundred microliters of RBC suspension (adjusted to 12% hematocrit) was incubated with 200 μl of dextran microsphere suspension (containing 0.25, 0.5 or 1 mg microspheres) for 30 min at 37 °C. The absorbance of the supernatant was read at 540 nm.

2.11. Local irritation studies in human volunteers

Ten milligrams of blank CDM microspheres was dispersed 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 was carried out by unpaired Student's *t*-test and one-way ANOVA.

2.13. Ethics in animal investigations

The protocols of human and animal studies were approved by regional ethics committee.

3. Results

3.1. Morphology and size characteristics of CDM

The original microspheres were spherical and smooth, but as shown in Fig. 1, freeze-drying process has changed both the features. It could be attributed to volume expansion of water in freezing process, which has changed the internal matrix of microspheres.

An optical microscope equipped with an eyepiece reticule was used to determine the size of microspheres. The mean diameter of CDM was determined as 128.1 ± 25.8 μm.

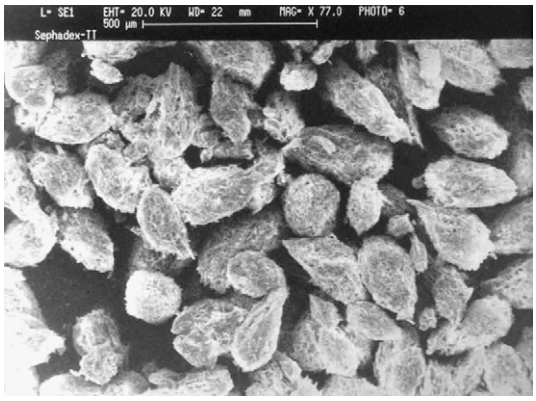

Fig. 1. The SEM image of CDM microspheres after freeze-drying.

3.2. Loading percent of tetanus toxoid (TT) in CDM

The loading percent of TT in CDM-TT microspheres was determined as 20.3 ± 3.2%, using Bradford protein assay method.

3.3. Release studies of TT from CDM

The release profile of TT from CDM-TT was studied using a diffusion chamber. As it is shown in Fig. 2, nearly all the encapsulated TT has been released in the first hour and reach a plateau in the following 3 h.

3.4. Structural stability and immunoreactivity of encapsulated TT

In SDS-PAGE gel, identical bands were observed for the original TT and TT extracted from CDM-TT. So, it could be concluded that in loading procedure of TT in CDM, the structure of encapsulated TT is preserved (Fig. 3).

The antigenic activity of the encapsulated TT was determined by an ELISA method, based on the standard curve (absorbance at 495 nm vs. amount (mg) of tetanus toxoid, $y = 0.001x + 0.0281$, $R^2 = 0.9969$). The antigenicity of encapsulated TT was 90.5 ± 1.8% that of original TT. The depletion in antigenicity could be related to freezing and sorption stresses.

3.5. Serum anti-TT IgG 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 bled at weeks 3, 6 and

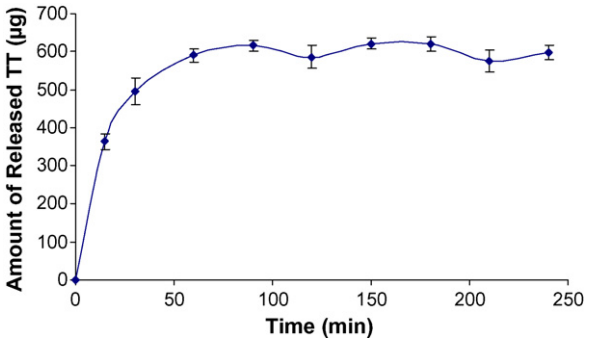

Fig. 2. In vitro release of encapsulated tetanus toxoid from CDM-TT microspheres. Microspheres (25 mg) were laid on a paper filter mounted in a diffusion cell. Each 30 min until 4 h, 400 μl samples were drawn from the receiver compartment and immediately replaced with fresh buffer. Each experiment was done in triplicate. Error bars represent the standard error of the mean ($n = 3$).

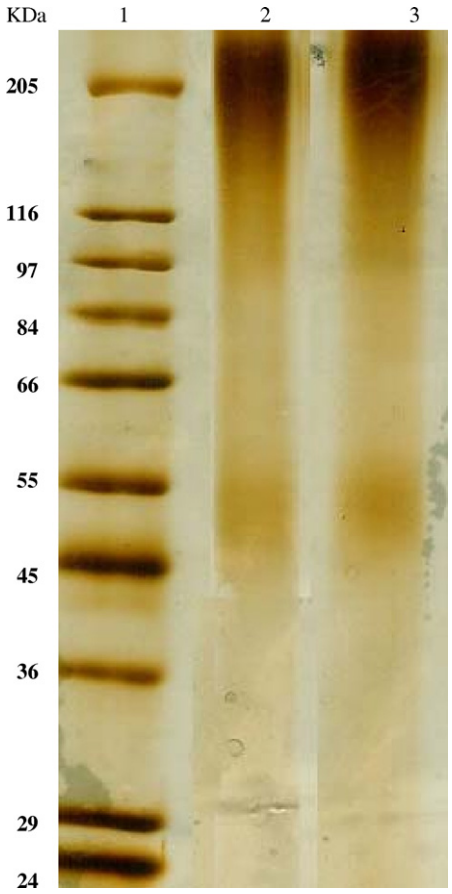

Fig. 3. The SDS-PAGE gel. A molecular weight reference marker (1), the released TT from freeze-dried microspheres (2) and original TT (3) were loaded onto a 10% acrylamide gel. Protein bands were visualized by silver nitrate staining.

10 post-immunization. Sera anti-TT IgG titers were determined by an ELISA method (Fig. 4). Regards to the trace antitoxin titers seen in the sera of animals immunized with 10 Lf TT, the antibody titers were not determined.

In the 10th week, the highest IgG titers amongst all the nasally immunized animals were seen in groups immunized with CDM + TT ($P < 0.05$). In this group, the IgG titers in the 3rd and 6th weeks were not significantly different with TT + CpG sol ($P > 0.05$). The group of positive control, i.e. animals given 10 Lf alum-TT intramuscularly, showed the highest IgG titers ($P < 0.0001$). Nasal administration of 10 Lf TT did not induce any detectable IgG titers.

In all the groups, the highest serum IgG titers were achieved in the 6th week and after three immunizations. The IgG titers were

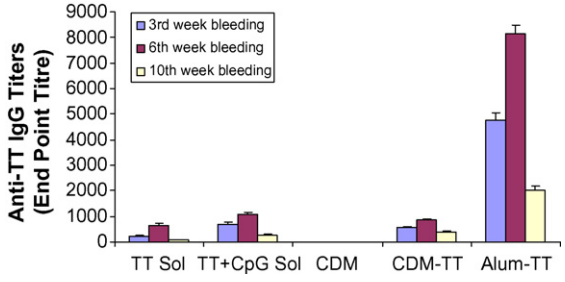

Fig. 4. Serum anti-TT IgG titers (mean ± S.E.). Rabbits ($n = 4$) were nasally (intramuscularly for alum-TT) immunized with 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT IgG titers (end-point titration) were determined by an ELISA method. TT sol: 40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

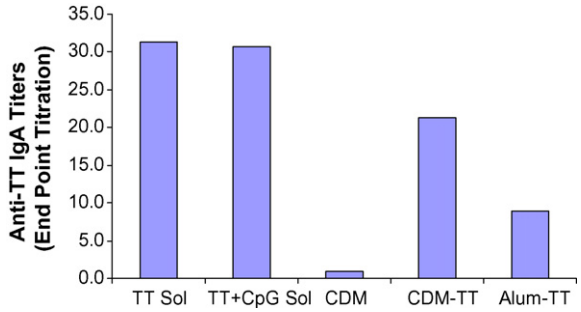

Fig. 5. Nasal lavage anti-TT IgA titers. Rabbits ($n = 4$) were nasally (intramuscularly for alum-TT) immunized with 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and nasal lavages were collected at week 10. Lavages were pooled and anti-TT IgA titers (end-point titration) were determined by an ELISA method. TT sol: 40 Lf TT solution; TT + CpG sol: 40 Lf TT + 10 μg CpG-ODN; CDM: blank CDM microspheres; CDM-TT: CDM microspheres loaded with 40 Lf TT; alum-TT: alum-adsorbed TT.

gradually decreased between weeks 6 and 10, when no boosting immunization was performed. Nasal administration of blank CDM microspheres, as negative control, resulted in undetectable IgG titers.

3.6. Nasal lavage anti-TT IgA titers

Following the above-mentioned immunizations, at the week 10, nasal lavages were collected, pooled and anti-rabbit IgA titers were determined by an ELISA method (Fig. 5). Among the groups immunized with various formulations, the highest mucosal sIgA titers were seen in animals immunized with TT solution (with or without CpG). Co-administration of CpG-ODN with TT did not induce any more sIgA titers. Administration of CDM + TT was adversely affected the mucosal sIgA titers. As expected, intramuscular injection of alum-TT, resulted in the lowest sIgA titers, compared with nasally immunized animals. Nasal administration of plain CDM, as the negative control, resulted in undetectable sIgA titers.

3.7. Serum anti-TT antitoxin titers

After the above-mentioned immunization protocol, serum anti-TT antitoxin titers (AU/ml) were determined by TN method (Fig. 6). Nasal immunization with 10 Lf TT solution resulted in trace antitoxin titers. However, when the TT was increased to 40 Lf, all formulations produced detectable anti-TT antitoxin titers. Among the nasally immunized animals, the highest antitoxin titers in weeks 3, 6 and 10 were achieved with CDM + TT ($P < 0.001$). Nasal administration of plain CDM, as the negative control, resulted in 0

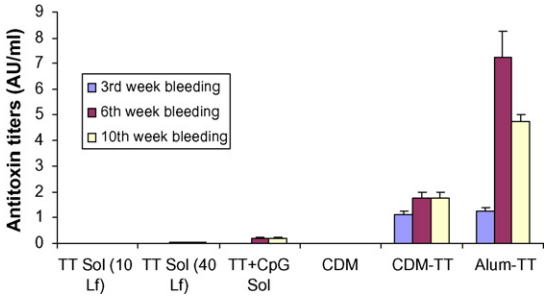

Fig. 6. Serum anti-TT antitoxin titers (mean ± S.E.). Rabbits ($n = 4$) were nasally immunized with 40 Lf TT and 10 μg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT antitoxin titers (AU/ml) were determined by toxin neutralization (TN) bioassay. TT sol: 40 Lf TT solution;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

antitoxin titer. Animals injected with 10 Lf alum-TT, as the positive controls, showed higher antitoxin titers in weeks 6 and 10, compared with nasally immunized animals with CDM + TT ($P < 0.01$) and TT solutions ($P < 0.0001$). In the 3rd week, nasally immunized animals with CDM + TT showed similar antitoxin titers with those immunized intramuscularly ($P > 0.05$).

3.8. Hemolysis and nasal irritation

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

CDM microsphere powder 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

Pre-formed cross-linked dextran (Sephadex®) microspheres have been studied for nasal delivery of peptides and proteins (Chandler et al., 1991; Gill et al., 1998; Illum et al., 1990; Oechslein et al., 1996; Ryden and Edman, 1982), but as carrier for nasal vaccination, they have not shown promising results and in both studies little or no immune responses were induced (Pereswetoff-Morath and Edman, 1996; Rydell and Sjoholm, 2005). So they have concluded that the dextran microspheres were not taken up through the nasal mucosa to any significant extent and do not act as adjuvants (Rydell and Sjoholm, 2005). Neither formaldehyde treatment nor conjugation of a mucosal adjuvant (cross-reacting material—CRM197) to microparticles had much effect (Rydell and Sjoholm, 2005).

In this study tetanus toxoid as a model antigen was encapsulated in CDM and its adjuvant potential was evaluated. Among the nasally immunized animals, the lowest serum IgG titers in all bleedings was seen in animals immunized with TT solution ($P < 0.05$). The adjuvants, CpG-ODN and CDM, similarly increased the IgG titers in the 3rd and 6th weeks ($P > 0.05$), while in the 10th week, CDM showed the highest titers ($P < 0.05$). This equivalent or higher adjuvant potential of CDM compared with CpG-ODN, shows the potential of these microspheres for further studies. The highest titers seen in the 10th week, 4 weeks after the last immunization could be interpreted as potential of microspheres for induction of longer lasting IgG titers.

The most confident and reliable criteria for evaluation of TT vaccines is toxin neutralization test, and it is still the only criteria which is accepted by WHO for quality control of TT vaccines (Dokmetjian et al., 2000). The antitoxin titers induced with microsphere formulation is far higher than the other nasally applied formulations ($P < 0.0001$). The protective antitoxin titer for protection against tetanus is 0.01 AU/ml, so a marginal protection has been induced even with TT solution. Addition of CpG-ODN adjuvant has induced higher antitoxin titer (4–7 times, $P < 0.01$), in a manner that in the 6th week, the antitoxin titer is 20 times higher than the protective level. CDM however showed very high antitoxin levels (175 times higher than the protective levels). We have also evaluated the adjuvant potential of alginate microspheres (Tafaghodi et al., 2006b), PLGA nanospheres (Tafaghodi et al., 2007) and liposomes (Tafaghodi et al., 2006a, 2008). This is the highest antitoxin level we have seen with nasal immunization with TT formulations. While the antitoxin titers induced by i.m. injection of alum-TT, is far higher than the soluble antigen (tens to hundreds times higher, $P < 0.0001$), the titers are equivalent (3rd week, $P > 0.05$) or 2–4 times higher (6th and 10th weeks, $P < 0.01$ and <0.001) than CDM + TT.

The discrepancies between ELISA and TN bioassay results have been studied, and reported that serum samples are overestimated by ELISA as compared to TN assay. This is attributed to the ratio of symmetric to asymmetric IgG molecules. While higher proportions of asymmetric molecules present in the serum samples which have lower toxin neutralizing activity in the ELISA method, both kinds of IgG molecules react similarly. Therefore, this technique fails to discriminate between the two types of antibodies and only an *in vivo* serum neutralization procedure (TN) reflects the true neutralizing activity of the serum (Dokmetjian et al., 2000).

As it has been shown, for the uptake of particulate antigens by mucosa-associated lymphoid tissue (MALT) M cells, the particles should be below 10 μm in diameter (Eldridge et al., 1991; Florence, 1997; O'Hagan, 1996). Hence it is obviously known that CDM used in this study (128 μm diameter) could not have any efficient interaction with M cells and other mechanisms should be involved in their adjuvant potential and the high protective immunity induced by them. Dextran microspheres are classified according to their internal pore size as G-25, G-50, G-100, G-150 and G-200. Numbers correspond to the maximum weight of the molecules which can be entrapped within the microspheres. For example, Sephadex® G-150 can encapsulate molecules as high as 300 kDa (Voet and Voet, 1990). TT molecular weight is about 150 kDa (Wassilak et al., 1994), so it could be penetrated into the gel matrix of microspheres. In this study, after incubation of microspheres with TT solution, they were washed to remove the surface adsorbed and absorbed antigen. Therefore, the 20.3% encapsulated TT is mainly entrapped in the gel matrix inside the microspheres. It has been suggested that CDM could withdraw water from their adjacent mucosal cells, followed by shrinkage of cells and temporary widening of intercellular tight junctions (Pereswetoff-Morath, 1998; Pereswetoff-Morath and Edman, 1995). It has been shown that the widening of tight junctions is lasting for about 15 min, so the fast release of encapsulated antigen is highly critical for efficiency of this mechanism. The release studies were performed in a diffusion cell simulating nasal cavity (Cornaz et al., 1996; Tafaghodi et al., 2006b). Microspheres were not immersed in the release medium, but like the nasal mucosa they were just in contact with a wet and warm membrane, in a humid and warm atmosphere (provided by covering the donor compartment). As it is shown in Fig. 2, the encapsulated TT has shown a fast release, in a manner that nearly all the encapsulated TT has been released in the first hour.

In contrast to nasal solutions, dextran and starch microspheres will be settled down in the anterior part of the nasal cavity, where non or few cilia are found (Illum et al., 2001), so it is expected that the residence time of these particulate delivery systems be more than that of solution dosage forms. We have shown in an *in vivo* gamma-scintigraphic study on human nose that after 4 h, about 27% of nasally administered microspheres were still remained in the nasal cavity (Tafaghodi et al., 2004). This is indicative of the mucoadhesion potential of these microspheres.

The lowest lavage sIgA titers were seen in animals immunized with i.m. injection of alum-TT. It has become apparent that locally administered mucosal vaccines are often able to induce a systemic immune response in addition to the mucosal response. This is not true for parenterally administered vaccines, which are not often able to induce an effective mucosal immune response. Moreover, parenteral vaccination is associated with various degrees of discomfort for the recipient, which adds to the attraction of mucosal vaccination methods (Rydell and Sjoholm, 2005).

Among the nasally applied formulations, the highest sIgA titers were induced by TT solution. The sIgA titers induced with microsphere formulation was lower than that of the solutions. As efficient interaction of antigen with MALT has a determinant role in induc-

tion of sIgA titers, the lower sIgA titer induced with microsphere could be attributed to the size of microspheres and their failure in interaction with MALT.

Although during the encapsulation of antigens in particulate drug delivery systems, such as microspheres, the antigen encounters various stresses which could affect its structural features or immunoreactivity, encapsulation of TT in CDM did not cause any change in the structure of TT (Fig. 3). However, its immunoreactivity decreased to about 90.5% that of original (non-encapsulated TT). This could be resulted from adsorption and absorption of TT on microspheres and also from freezing stress.

One of the advantages of CDM as a mucosal delivery system is their lack of toxic effects on mucus membranes. Moreover, for a nasal delivery system one important prerequisite is the lack of any effect on ciliary beat frequency (CBF) and CDM has not shown any effect on the CBF of isolated rat trachea (Pereswetoff-Morath et al., 1996). In this study administration of CDM into the right nostril of four healthy volunteers did not show any stimulant effect. *In vivo* experiments on human subjects have also not shown any toxic effect such as changing in mucociliary clearance rate, congestion or decongestion on nasal mucosa (Pereswetoff-Morath, 1998). Moreover, nasal administration of starch microspheres twice daily for 8 weeks in rabbits (Bjork et al., 1991) or CDM once daily for 4 weeks in rat (Pereswetoff-Morath et al., 1996) resulted in slight hyperplasia in nasal epithelium. The membrane toxicity of CDM has also been studied on erythrocytes and as stated before (Bjork et al., 1991), no hemolysis was occurred.

5. Conclusion

With regard to the proper release characteristics, desirable preservation of the antigenic activity of TT, good mucoadhesion properties and also safety of TT-containing CDM, these microspheres could be used as an efficient mucosal adjuvant and antigen delivery system. The adjuvant effect of CDM could be mainly attributed to temporary widening of tight junctions by the CDM and also to their mucoadhesion potential. These microspheres could induce very high antitoxin titers following nasal administration, while the CpG-ODN could not induce such titers. The antitoxin titers induced with TT-microsphere was 175 times higher than the protective levels.

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