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ORIGINAL ARTICLE

Evaluation of Biodegradable Microparticles for Mucosal Vaccination Against Diphtheria Toxoid: Nasal Efficacy Studies in Guinea Pigs

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ABSTRACT

Objectives: In this study, poly-(ε-caprolactone) (PCL) and poly-(lactic-co-glycolic acid) (PLGA) microparticles encapsulating diphtheria toxoid (DT) were investigated for their potential as a mucosal vaccine delivery system.

Materials and Methods: Antigen-containing microparticles were prepared using the double emulsion (*w/o/w*) solvent evaporation method.

Results: The average geometric diameter of the particles was found to be between 7 and 24 µm, which is suitable for uptake by the antigen-presenting cells in the nasal mucosa. Although the differences were insignificant, the PLGA polymer-containing formulations exhibited the highest encapsulation efficiency. Microparticle formulations, prepared with both PLGA and PCL polymers, were successfully produced at high production yields. The *in vitro* release profile was presented as a biexponential process with an initial burst effect due to the release of the protein adsorbed on the microsphere surface, and the subsequent sustained release profile is the result of protein diffusion through the channels or pores formed in the polymer matrix. DT-loaded microparticles, DT solution in phosphate-buffered saline (PBS), and empty microparticles (control) were administered *via* nasal route and subcutaneously to guinea pigs. The antibody content of each serum sample was determined using an enzyme-linked immunosorbent assay (ELISA).

Conclusion: Absorbance values of the ELISA test showed that PLGA- and PCL-bearing microparticles could stimulate an adequate systemic immune response with intranasal vaccination. In addition, PLGA and PCL microparticles resulted in significantly increased IgG titers with intranasal administration as a booster dose following subcutaneous administration. PCL polymer elicited a high immune response compared with PLGA polymer ($p < 0.05$).

Key words: Diphtheria toxoid, immunity, intranasal, microparticulate formulations

INTRODUCTION

The effects of emerging technologies have shown not only the success of the correlation between vaccination and immunogenic components of vaccines but also the effectiveness of delivery systems. Therefore, studies on an effective vaccine formulation or delivery system are of great importance for developing modern vaccines. Most important viral and bacterial infections occur through mucosal membranes such as the respiratory, intestinal, tear, or urogenital tracts.[1] Adjuvants have been developed to facilitate and improve the immune response obtained after mucosal immunization.[2] Particulate adjuvants or antigen delivery systems are considered an alternative to other immune stimulating adjuvants.[3] The concept of a polymeric delivery system has emerged for targeting specific regions of proteins or antigens. This approach for developing mucosal vaccine delivery systems has become mandatory, especially for protecting proteins against fragmentation of antigens in the mucosal environment and for increasing their uptake by the immune system. Polymeric particles are adsorbed at higher efficiency rates compared with soluble molecules in mucosal epithelial tissues and thus, high antigen concentrations are provided in the area requiring an immune response.[4,5] Biodegradable and biologically compatible polyesters and poly-lactic-co-glycolics (PLG) are some of the most preferred

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polymeric particulate systems because they have been used in humans for many years on surgical suture materials, as well as controlled-release drug systems.[6]

Diphtheria is a bacterial disease caused by a toxin produced by *Corynebacterium diphtheria* and causes clinical symptoms.[7] Because the ability to create long-term immunological memory of the vaccine applied is parenterally weak, booster doses should be administered intermittently. Booster vaccination is recommended as a strategy to reduce the disease's health and economic burden in these populations.[8] The reason for not providing a formation of permanent immunological memory is thought to arise from the inability to stimulate local humoral immunity in the respiratory tract.[9,10] Because mucosal immunization stimulates both systemic and mucosal immunity, it is considered an alternative route to parenteral administration. In addition, side effects are minimized with the application of mucosal vaccines, which is an easier route than the parenteral route. Therefore, mucosal immunization holds notable importance when the vaccination needs to be repeated at certain intervals, as in diphtheria.[11] The nasal mucosa is an important part of the mucosal immune system. It is the first point of contact for antigens inhaled into the organism. Thus, pathogen neutralization occurs at the first point at which pathogens enter the organism with nasal vaccination. As a result, both systemic and mucosal immune responses could be accomplished.[7,12] Recent studies have shown that the nasal route is more sufficient for the systemic transport of low-molecular-weight polar drugs, peptides, and proteins than the mucosal delivery routes. Traditional drugs applied nasally are used for treating local diseases such as nasal allergy, nasal congestion, and nasal infection.[13,14]

The purpose of this study was to develop microparticulate vaccine systems against diphtheria with long-term immunological memory. One of our main objectives was to eliminate repetitive doses to improve patient comfort. In addition, alternative intranasal administration was studied instead of parenteral root, which is more difficult and painful.

MATERIALS AND METHODS

Materials

Poly(lactide-co-glycolide) (PLGA, 50:50) was obtained from Boehringer Ingelheim. Poli-(ε-caprolactone) (PCL, M.A. 14 Kda) was purchased from Sigma. Polyvinyl alcohol (PVA; 87-89% hydrolyzed, molecular weight 13,000-23,000) was purchased from Aldrich Chemical Company (USA). Diphtheria toxoid (800 Lf/ampoule, Japanese Lot) was provided by the Refik Saydam National Public Health Agency (Türkiye) as a standard. Dichloromethane (DCM) was purchased from Sigma-Aldrich (Germany). The other agents were all special reagent grade.

Preparation of the DT-loaded microparticles

Microparticles were prepared by double emulsion-solvent evaporation technique as follows:[15] 250 µL of diphtheria toxoid in ultrapure water was emulsified with 2.5 mL of 5% (*w/v*) PLGA or 5% (*w/v*) PCL in DCM using an Ultraturrax model T 25 (IKA Laboratory Technology, Staufen, Germany) at 8,000 rpm for 5 min or Sonicator model Sonopuls HD 2070 (Bandelin Electronics GmbH&Co, Germany) at 60 W for 30 s. The resulting water-in-oil emulsion (2.5 mL) was then emulsified at 8,000 rpm for 5 min in a Eurostar mechanical stirrer (IKA Laboratory Technology, Staufen, Germany) with 50 mL of 5% (*w/v*) polyvinyl alcohol to produce a water-in-oil-in-water emulsion. This emulsion was stirred magnetically overnight under pressure at room temperature to allow evaporation of the organic solvent and the formation of microparticles. Microparticles were isolated by centrifugation (10 min at 4,000 x g), washed three times in 10 mL of ultrapure water, and freeze-dried. The prepared formulations are given in Table 1.

Characterization and quantification of DT-loaded microparticles

Particle appearance and particle size analysis

Photomicrographs of microparticles were taken using a Leica DM 4000B microscope. Lyophilized microparticles were dispersed in purified water and analyzed using (Sympatec Helos H0728) particle size analyzer.

The surface morphologies of the microspheres were also observed using an environmental scanning electron microscope (ESEM) (FEI Quanta Model 200 FEG, Tokyo, Japan). A small aliquot of the microspheres was mounted onto metal stubs using double-sided adhesive tape for sample preparation. After being vacuum coated with a thin layer (100-150 A°) of gold, the microspheres were examined by ESEM operated at 5 kV accelerating voltage. The photomicrographs were then taken at a magnification of 10,000.

Table 1. The description of the formulations

Formulation code	Diphtheria toxoid (mg)	Route of administration	Polymer used	1st step of homogenization
D1	75.8	Intranasal	PLGA	Ultraturrax
D2	75.8	Intranasal	PLGA	Sonicator
D3	75.8	Intranasal	PCL (70-90 kDA)	Ultraturrax
D4	75.8	Intranasal	PCL (70-90 kDA)	Sonicator
D5	75.8	Intranasal	PCL (14 kDA)	Ultraturrax
D6	75.8	Intranasal	PCL (14 kDA)	Sonicator

PLGA: Poly-(lactic-co-glycolic acid), PCL: Poly-(ε-caprolactone)

In vitro release of protein from PLGA and PCL microparticles

In vitro drug release was determined by the statical method.[16] Microparticles were suspended in pH 7.4 PBS contain 0.01% NaN_3. The samples were retained in a water bath at 37 ˚C and shook at 40 rpm. At appropriate time intervals, the release medium was completely withdrawn after centrifugation at 7,500 rpm and replaced with fresh buffer. The diphtheria toxoid concentration in the supernatant was determined by the mBCA technique. In addition, all release data (n: 3) were calculated (with SPSS) according to the percentage released amount of diphtheria toxoid to determine the release kinetics.

Diphtheria toxoid loading in microparticles

The protein contents of the microparticles were assayed by the digestion technique. For this technique, a NaOH/SDS solution (1 N NaOH, 5% *w/v* SDS) was added to the microparticles and dissolved.[3] The mixture was neutralized with 1 N HCl and centrifuged. The diphtheria toxoid content was determined using the microbicinchoninic acid (mBCA) (Molecular Devices, $SPECTRA_{max}$ 190 pc) total protein assay at 560 nm.

Stability studies

D1 and D5 formulations were stored in a refrigerator at 2-8 ˚C and in a climate cabinet at 25 ± 2 ˚C, 60 ± 5% relative humidity for 3 months. The diphtheria toxoid content was determined by mBCA assay. At the end of 3 months, non-reduced-PAGE analysis was performed to understand whether the protein integrity of the diphtheria toxoid was preserved.

Polyacrylamide Gel Electrophoresis

Diphtheria toxoid calibration samples and formulations that remained at 2-8 ˚C for three months were subjected to PAGE gel under non-reducing conditions using an electrophoresis system. Samples and reference materials for diphtheria toxoid were run on discontinuous gels prepared by stacking and separating in a sample buffer.

Immunization studies

The immunization program we followed included intranasal vaccination and triple repeat blood sample collection from animals; thus, guinea pig was selected as the most suitable experimental animal, and ethical approval was obtained from the Refik Saydam Hıfzıssıhha Center Presidency Scientific Committee and the Ethics Committee (06.01.2011/14886). For *in vivo* studies, weights ranging from 300 to 350 g of 54 male albino guinea pigs (7-8 years old) were used. The morphology of the D1, D2, D3, D4, D5, and D6 coded formulations, the sizes and distribution of particles, the encapsulation and production efficiency, and *in vitro* release results were considered. For the application of D1 and D5 coded formulations, *in vivo* studies were conducted. In the control group, diphtheria toxoid with PBS (pH 7.4) was preferred.

Many formulations containing diphtheria toxoids are 25-30 Lf in the first vaccination and booster dose application for the pediatric population, whereas it contains 2 Lf and above diphtheria toxoids for adults.[17] A formulation of 25 Lf/50 µL (25 µL/nostril) was applied to the nostrils of male guinea pigs using a micropipette without using anesthetics for intranasal vaccination.[18] With the application to both nostrils, the membrane area and, thus the absorption of formulations were aimed to increase. Guinea pigs were kept still for approximately 2 min to avoid any loss after administration of the vaccine into the nose. For subcutaneous vaccination, 25 Lf/500 µL of formulation was injected into male guinea pigs without anesthetics.

For *in vivo* studies, formulations containing diphtheria toxoid were applied in certain ways and days to nine groups (n: 6) of male guinea pigs (Table 2). To obtain information about whether the experimental animals had ever experienced diphtheria toxoid or not, blood samples were taken from each animal a week before the first vaccination. In this way, exposure to stress conditions of experimental animals was prevented and the best response after immunization was aimed to be obtained. To determine the changes in immune responses of guinea pigs, 1 mL of blood samples from each one was taken from their hearts, when immunoglobulin A (IgA) and IgG antibody formation took place on the 20th and 42nd days.[19] During blood collection, guinea pigs were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg). The collected

Table 2. Groups of guinea pigs immunized with adjuvant-free, microencapsulated diphtheria toxoid and control (n: 6)

Group code	Formulation	Polymer used	Administration route	Dose (Lf)		
				1st Immunisation 0 day	2nd Immunisation 7th day	3rd Immunisation 21th day
Control 1 (F1)	Dtxd-solution	-	*i.n.*	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)
Control 2 (F2)	Placebo MP	PLGA	*i.n.*	50 µL (*i.n.*)	50 µL (*i.n.*)	50 µL (*i.n.*)
Control 3 (F3)	Placebo MP	PCL	*i.n.*	50 µL (*i.n.*)	50 µL (*i.n.*)	50 µL (*i.n.*)
MP-D1 (F4)	Dtxd-MP	PLGA	*i.n.*	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)
MP-D5 (F5)	Dtxd-MP	PCL	*i.n.*	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)
MP-D1 (F6)	Dtxd-MP	PLGA	*s.c.* + *i.n.*	25 Lf/500 µL (*s.c.*)	-	25 Lf/50 µL (*i.n.*)
MP-D5 (F7)	Dtxd-MP	PCL	*s.c.* + *i.n.*	25 Lf/500 µL (*s.c.*)	-	25 Lf/50 µL (*i.n.*)
Control 4 (F8)	Dtxd-solution	-	*s.c.* + *i.n.*	25 Lf/500 µL (*s.c.*)	-	25 Lf/50 µL (*i.n.*)

Dtxd: Diphtheria toxoid, MP: Microparticle, *s.c.*: Subcutan, *i.n.* : Intranasal

blood samples were centrifuged at 10,000 rpm for 10 min after waiting 1 h at room temperature and then another 1 h in the refrigerator (2-8 ˚C). The serum samples were collected from the supernatant and kept at -20 ˚C before analysis. Immune responses in collected serum samples were evaluated using an ELISA kit (total IgG guinea pig).

Anti-DT antibody assays

DT-specific IgG antibodies were measured by ELISA. The immunization chart is given in Table 2. In this assay, the IgG in the sample reacts with the anti-IgG antibodies that have been adsorbed on the surface of polystyrene microtiter wells. After removing unbound proteins by washing, anti-IgG antibodies conjugated with horseradish peroxidase were added. These enzyme-labeled antibodies form complexes with previously bound IgG. Following another washing step, the enzyme bound to the immunosorbent is assayed by adding a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of IgG in the tested sample; thus, the absorbance at 450 nm is a measure of the concentration of IgG in the test sample. The quantity of IgG in the test sample can be interpolated from the standard curve constructed from the standards and corrected for sample dilution.

RESULTS

Preparation and characterization of DT-loaded PCL and PLGA microparticles

The microparticles prepared using the *w/o/w* solvent evaporation method had a regular morphology and a smooth surface. The particle size of the formulations was between 7 and 34 µm (Figure 1). D5 formulation was also observed under scanning electron microscopy (SEM) with a smooth spherical shape (Figure 2).

DT loading

DT-loaded microparticles were prepared using different polymers (PCL and PLGA) by variation in the weight of the polymer dissolved in DCM to investigate the eventual modifications of the particle size, protein loading, and efficiency of entrapment (Table 3). It was indicated that the type of polymer used also affected the encapsulation efficiency. It was determined that formulations prepared with PLGA polymer showed higher encapsulation efficiency than those prepared with PCL polymer.[20,21]

Stability studies

D1 and D5 formulations were stored for 3 months at 2-8 ˚C and 25 ± 2 ˚C (60 ± 5% RH). Bands of digestion and calibration samples were obtained as shown in Figure 3. According to the bands of integrity, we can conclude that diphtheria toxoids maintained their stability during the formulation process. The number of changes during stability studies under different conditions for the active substance and encapsulated DT are given in Table 4.

To examine the stability of diphtheria toxoid, it was kept in refrigerated conditions for multiple months. It has been stated that DT remains stable for years when stored at 2-8 ˚C.[22]

PAGE electrophoresis

In our previous study, the structural integrity of the diphtheria toxin was analyzed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Our standard diphtheria toxin has a broad band around 66 kDa, with two fragments (A fragment around 45 kDa, and B fragment around 29 kDa). Preservation of the integrity and stability of the diphtheria toxin in 3 months under both conditions did not show any significant differences in the non-denaturing environment (p >0.05). Samples extracted from microparticles under both stability conditions were applied to the polyacrylamide gel under non-denaturing conditions (Figure 3). There was only one band around 66 kDa. This shows that the antigen encapsulated in the formulations remains intact.

In vitro release studies

Rapid drug release from polymeric particles, called "burst release" were examined in all formulations (Figure 4). This rapid initial release is attributed to the fraction of the drug adsorbed

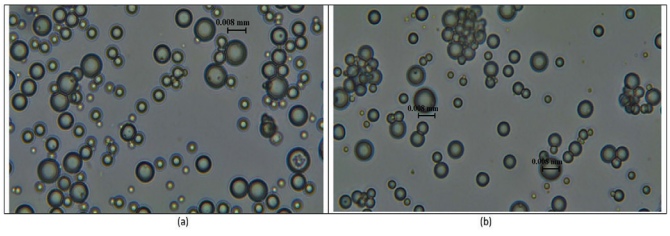

Figure 1. Optical micrograph of (a) PLGA and (b) PCL (90 kDa) microparticles entrapping BSA produced by the standard double emulsion solvent evaporation method

PLGA: Poly-(lactic-co-glycolic acid), PCL: Poly-(ε-caprolactone), BSA: Bovine serum albumin

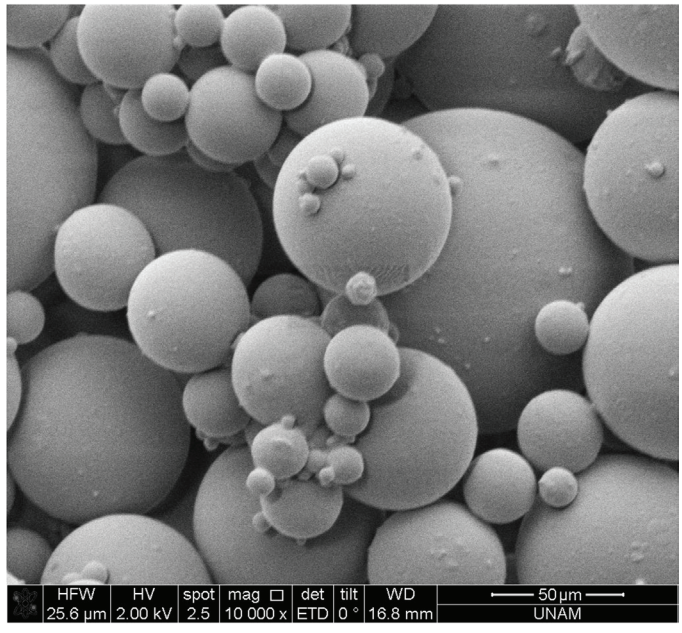

Figure 2. Environmental scanning electron microscopy images of D5 microparticles

Table 3. Characterization and physicochemical properties of the DT-loaded microparticles

Code	Type of polymer	1st step of homogenization	Antigen (mg)	Encapsulation efficiency (%) ± SD*	Yield (%)	Geometric diameter (μm) ± SD*
D1	PLGA	Ultraturrax	75.8 (144 g protein)	60.6 ± 0.39	88.38	6.93 ± 0.45
D2	PLGA	Sonicator	75.8 (144 g protein)	53.7 ± 1.53	87.23	7.98 ± 0.83
D3	PCL (70-90 kDa)	Ultraturrax	75.8 (144 g protein)	46.8 ± 1.97	73.57	22.47 ± 0.31
D4	PCL (70-90 kDa)	Sonicator	75.8 (144 g protein)	52.6 ± 1.08	74.00	34.4 ± 1.22
D5	PCL (14 kDa)	Ultraturrax	75.8 (144 g protein)	55.7 ± 0.86	88.30	8.88 ± 0.02
D6	PCL (14 kDa)	Sonicator	75.8 (144 g protein)	45.9 ± 1.68	88.96	12.6 ± 0.19

SD: Standard deviation, *n: 6, DT: Diphtheria toxoid, PLGA: Poly-(lactic-co-glycolic acid), PCL: Poly-(ε-caprolactone)

Table 4. Diphtheria toxoid integrity during stability studies (n: 3)

Formulation code	Condition	Remaining amount of diphtheria toxoid (%) ± SD		
		Initial	1st month	3rd month
DT alone	5 ± 3 °C	99.85 ± 0.27	99.78 ± 0.18	99.44 ± 0.33
D1	5 ± 3 °C	60.6 ± 0.39	58.7 ± 1.30	57.5 ± 1.54
D5	5 ± 3 °C	55.7 ± 0.86	54.63 ± 1.53	52.48 ± 1.69
D1	25 ± 2 °C	60.6 ± 0.39	57.3 ± 0.99	52.39 ± 1.72
D5	25 ± 2 °C	55.7 ± 0.86	52.8 ± 1.15	46.63 ± 0.57

SD: Standard deviation, DT: Diphtheria toxoid

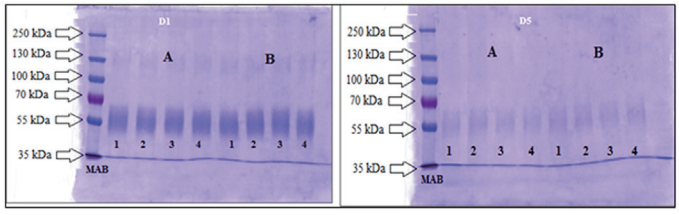

Figure 3. Bands obtained by polyacrylamide gel electrophoresis for D1 and D5 coded formulations (A) 2-8 °C, (B) 25 ± 2 °C, 1) initial, 2) 1st month, 3) 2nd month, 4) 3rd month

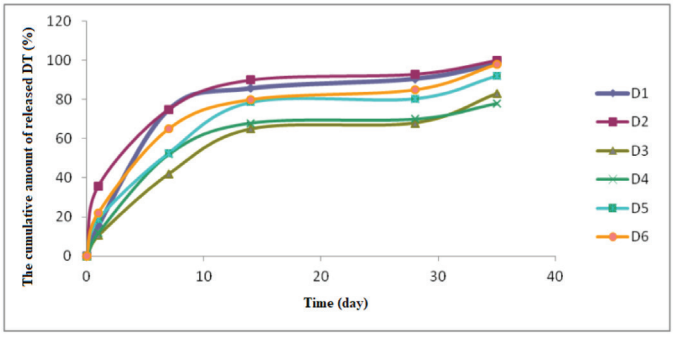

Figure 4. *In vitro* release profiles of diphtheria toxoid from the formulations

or weakly bound to the surface of the microspheres. Similar to our previous investigation, we obtained triphasic release profiles generally found in formulations prepared with PLGA and PCL polymers.[23,24] It was observed that D1 and D2 coded formulations prepared with the PLGA polymer had a faster release than those prepared with PCL polymers. At the end of five weeks, the PLGA polymer used formulation released all active ingredients. On the other hand, PCL formulations (coded as D3, D4, D5, and D6) could not release all the active ingredients in 5 weeks. In addition, previous studies have revealed that the release rates of particulate systems prepared with PLGA are higher than those of particulate systems prepared with PCL.[25] Furthermore, all of the prepared formulations were compatible with the Higuchi kinetics, which is an expected outcome in matrix systems. In addition, silver staining electrophoresis was applied to *in vitro* release samples, and no extra band formation was observed (data not shown). The obtained single band indicates that the structural integrity of the diphtheria toxoid is preserved during microparticle formation.

Immunization studies

The first vaccination experiment was performed to investigate, whether DT-loaded microparticles can induce a systemic immune response following nasal administration to guinea pigs. The results of guinea pig serum antibody (IU/milliliter) are summarized in Figure 5.

By comparing the groups of F2 and F4, the formulation coded D1 increased the IgG titer on the 42nd day, when administered intranasally. When the IgG titers of group F6 were examined, increased concentrations were observed after intranasal application. Although this group first applied subcutaneously, continuous intranasal administration showed increased titers. According to these results, we concluded that formulation D1 can be a good candidate for a booster (rappel) formulation.

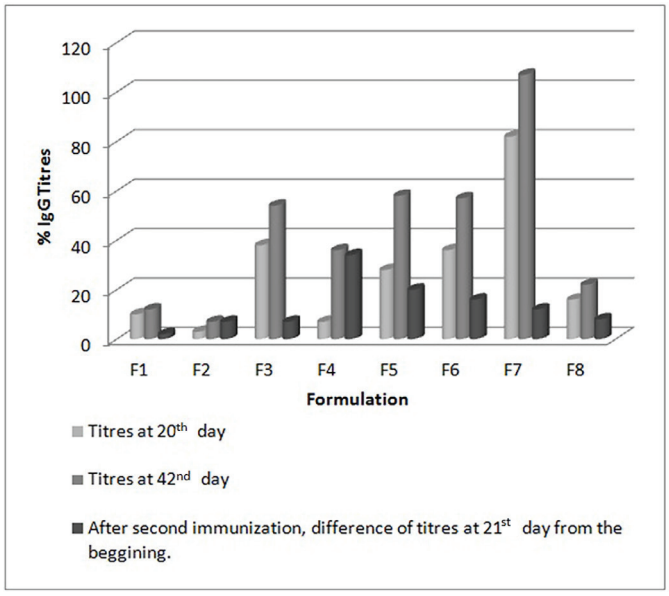

Figure 5. IgG titers at specified intervals
IgG: Immunglobulin G

When the groups of F3 and F5 were compared, D5 formulation administered intranasally was found to have an increment effect on the IgG titer on the 42nd day. When the blood concentrations of group F7 were examined, similar increasing titers were observed as formulation D1.

When D1- and D5-coded formulations were compared with each other, D5-coded formulation was observed to stimulate antibody formation stronger than the D1-coded formulation, and the difference was statistically evaluated by the *t*-test and was determined to be significant ($p < 0.05$). The reason is a result of the PCL polymer. In the formulation in which PCL polymer alone is applied intranasally (group F3), there is a significant increase in immune response obtained from the F1 group. This increase in IgG is thought to play an active role in the stimulation of systems forming the immune response of PCL polymer. Murillo et al. observed the same adjuvant effect, when they applied the PCL polymer to animals *in vivo*.[26] However, we concluded that the PLGA polymer did not show an adjuvant effect with nasal application.

The immune response of the F4 and F5 groups in which only intranasal application was performed was higher than those of the F6 and F7 groups in which subcutaneous injection followed intranasal application. In the first and second immunizations, the reason why the immune response obtained after intranasal application is higher than the others is thought to be from a disease that maintains the upper respiratory tract. This finding supports the request of World Health Organization (WHO) to encourage mucosal immunization with diseases that affect the upper respiratory tract like diphtheria.[22]

DISCUSSION

The purpose of this study was to develop microparticulate systems containing diphtheria toxoid as the model antigen and to evaluate their intranasal administration. New approaches to diphtheria vaccination that generate immune responses equal to or surpass those generated by traditional vaccines must have an increased safety profile. In this study, we selected mucosal vaccination because of capacity to stimulate both systemic and mucosal immune responses. It has been shown that using the nasal route, most soluble antigens can induce immune responses.[11] Conversely, it should not be ignored that these antigens can induce poor immunogenicity with immunological tolerance.[1-3] In our previous study, we found that nasal administration of diphtheria toxoid alone induces very weak immune responses.[27] In contrast, the use of polymeric-DT microparticulate formulations considerably enhance their immunogenicity for intranasal immunization of guinea pigs. These microparticulated formulations containing diphtheria toxoid were prepared using the double emulsion (*w/o/w*) solvent evaporation method. PLGA and PCL polymers were used in microparticulate formulations because of their biodegradable and biocompatible properties. Furthermore, recent studies have shown that PLGA copolymers significantly affect the stability and biological activities of active substances, especially depending on the hydrophobicity of the polymer and the presence of acidic degradation products.[28]

Sayin et al.[19] established that chitosan can enhance immune responses *via* the nasal route. They studied medium molecular weight chitosan, which could successfully enhance the absorption of diphtheria toxoid nasally in rat models. In the nasal cavity, biodegradable chitosan increases the residence of the antigen and can open tight junctions between the cells, thereby promoting absorption into the nasal cells.[19] On the other hand, organic solvent residues in the formulation, while preparing chitosan solutions can cause the lower stability of these molecules against proteolysis and lower absorption when administered intranasally.[9] We also used similar organic solvents while preparing microsphere formulations. However, in order to remove the organic solvent residue, during the preparation step, the solvent was allowed to evaporate overnight under pressure, and the washing procedure was applied under high-speed centrifugation three times. The encapsulation yields and immune response of the formulations show that we eliminated this negative condition.

Isaka et al.[7] previously demonstrated that immunization of mice with alum-adsorbed DT was considerably enhanced by booster immunization *via* the same route. Immunization of the mice in this way generated similar IgG and neutralizing antibody titers in parenterally immunized mice, and generated high levels of local IgA.[10] The development of an alternative microparticulate formulation for mucosally delivered diphtheria toxoid booster vaccines may eliminate some of the side effects associated with the conventional vaccine with aluminium salts and may also be more acceptable for frequent boosting against diphtheria. Similar results were obtained using our previous *in situ* gel formulations.[27] When the release kinetics were compared, it was shown that the microsphere formulation was more effective than the *in situ* gel formulations. In addition, it is one step ahead of other formulation designs because of the adjuvant effect of

the PCL polymer, which is used for microsphere formulations.

The morphological properties, particle size, encapsulation efficiency, and production yield of microparticles were investigated during pre-formulation studies. According to the obtained results, formulations were optimized and certain amounts of diphtheria toxoid were added to the formulations (D1, D2, D3, D4, D5, and D6). For these formulations; morphological properties, particle size, and distribution, encapsulation efficiencies, production yields, drug release profiles, and stability of microparticles were investigated. Formulations coded D1 and D5 were considered to be superior to other formulations given in the *in vitro* tests (*e.g.*, encapsulation efficiencies, particle size and distribution, particle size and distribution). Therefore, these formulations were tested in guinea pigs to determine immune responses that would be produced following intranasal and subcutaneous administration. Absorbance values of the ELISA test showed that formulations coded D1 and D5 could stimulate adequate systemic immune response with intranasal vaccination.

Additionally, D1 and D5 formulations exhibited a significant increment in IgG titers with intranasal administration as a booster dose following subcutaneous administration. PCL polymer elicited a high immune response compared with PLGA polymer (p <0.05). However, the PCL polymer, when used alone, was also found to have an adjuvant effect.

In this study, we developed intranasal vaccines as an alternative to parenteral formulations. Therefore, it was investigated for any inflammation, edema, or other side effects that occurred in the injection and nasal area for all guinea pigs within 42 days. The absence of adverse effects was also positive for our formulations for mucosal administration.

In our previous study, *in situ* gel formulations improved the residence time of the diphtheria toxoid in the mucosa for its therapeutic efficacy. [27] Although these gel formulations can be applied easily by droplet into intranasal way, both Kruskal-Wallis and Wilcoxon tests indicated that the microparticulated formulation (D1 and D5) resulted in an increased systemic immune response according to the *in situ* gel formulation (F3 and F7). This may have been caused by the late migration of DT in gel formulations.

CONCLUSION

This study demonstrates that microparticles of PCL and PLGA encapsulated with diphtheria toxoid can be produced using a modified double emulsion (*w/o/w*) technique. The morphology, size, and distribution of particles, encapsulation, production efficiency, and *in vitro* release of the formulations were investigated, and two candidate formulations were applied to the nostrils of male guinea pigs.

The immune response of formulations applied through the intranasal route was higher in groups, in which the subcutaneous injection followed the intranasal application. This finding supports the recommendation of WHO to encourage mucosal immunization with diseases that affect the upper respiratory tract, such as diphtheria.

The biodegradable property of the PLC polymer, its hydrophobicity, and its resistance to acidicu pH indicated make this delivery system a potential carrier for mucosal intranasal vaccines. Compared with PLGA microparticles loaded with the same amount of toxoid, this hydrophobic polymer (PCL) has a high immune response. In addition, PCL formulations without diphtheria toxoid have an immune response because of their adjuvant effect.

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Ethics

Ethics Committee Approval: The Refik Saydam Hıfzıssıhha Center Presidency Scientific Committee and the Ethics Committee (06.01.2011/14886).

Informed Consent: Not applicable.

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Authorship Contributions

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