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Research paper

In vitro study of the immune stimulating activity of an athrophic rhinitis vaccine associated to chitosan microspheres

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Abstract

Chitosan microspheres (CMs) were prepared by an ionic gelation process with tripolyphosphate and characterized. *Bordetella Bronchiseptica* Dermonecrotoxin (BBD), a major virulence factor of a causative agent of atrophic rhinitis (AR), was loaded on to the CMs for nasal vaccination. BBD-loaded CMs were observed as aggregated shapes although unloaded CMs were observed as relatively spherical ones. The average particle size of the BBD-loaded CMs was 4.39 μm. The lower the molecular weight of chitosan and the higher the medium pH, the greater was the release of BBD from the BBD-loaded CMs in vitro due to weaker intermolecular interaction between chitosan and BBD. Tumor necrosis factor α and nitric oxide from RAW264.7 cells exposed to BBD-loaded CMs were gradually secreted with time, suggesting that released BBD from CMs had immune stimulating activity of AR vaccine in vitro.

Keywords: Chitosan microspheres; *Bordetella bronchiseptica dermonecrotoxin*; Vaccine delivery; Nasal vaccination; Macrophage; Tumor necrosis factor α; Interferon γ; Nitric oxide

1. Introduction

Microspheres have received much attention for delivering therapeutic peptides, proteins, antigens, oligonucleotides and genes by intravenous, oral, and mucosal administration [1]. Several types of microspheres have been demonstrated to significantly enhance the systemic and/or mucosal immune responses after mucosal vaccination [2]. Mucosally delivered antigens are frequently not immunogenic. Adjuvants are therefore required to be co-delivered with the antigens to enhance the immune responses. Microcapsules or microspheres are one of the most common forms used for the controlled-vaccine delivery formulations [3]. The ideal controlled-release of vaccine should deliver antigen in such a way that a long-lasting boosting effect is achieved with a single administration and provides effective antibody responses against an infectious organism [3].

Chitosan is a valuable excipient for mucosal drug delivery systems due to its biocompatibility, biodegradability, low cost and the ability to open intercellular tight junctions [4]. Molecular weight and degree of deacetylation of chitosan determine the properties of chitosan. A high molecular weight of chitosan has been reported to enhance the absorption of various compounds across the mucosal barrier [4,5]. Chitosan has been found to induce a wide range of antigens from bacteria, viruses and tumors. With respect to mucosal drug delivery, chitosan also has strong mucoadhesive properties [6–13]. Also, an advantage of chitosan microspheres (CMs) is that the loading is performed by incorporation in an aqueous solution without exposure to organic solvents [14]. For this reason chitosan has been used as an immunological adjuvant or a vaccine carrier for mucosal vaccination.

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Swine respiratory diseases have induced severe economic losses in the swine industry. Atrophic rhinitis (AR) is a serious and widely prevalent infectious disease of swine. The signs of AR usually appear by 8–12 weeks of age, and the disease progresses throughout the growing period. The most characteristic lesion is severe atrophy of the nasal turbinate bones accompanied by lateral deviation or shortening of the nose [15]. Two infectious agents, *Bordetella bronchiseptica* and *Pasteurella multocida* type D, are associated with the etiology of AR. Toxigenic strains of *B. bronchiseptica* vaccination are widely used to reduce the incidence of AR [16].

In this study, CMs were prepared and characterized for the controlled release of the antigen of the AR vaccine. Release of *B. bronchiseptica Dermonecrotoxin* (BBD) from BBD-loaded CMs was performed in vitro. Immune stimulating activity of BBD-loaded CMs as AR vaccine was checked in a murine macrophage cell-line.

2. Materials and methods

2.1. Materials

Three kinds of chitosan as base form with average molecular weights of 10, 100 and 300 K measured by gel permeation chromatography were kindly provided by Jakwang (Ansung, Korea). Deacetylation of the used chitosan was 90.8%. Sodium tripolyphosphate (TPP) was purchased from Sigma (Missouri, USA). Lipopolysaccharide (LPS) was purchased from Sigma (St Louis, MO, USA). All other chemicals were reagent grade chemicals.

2.2. AR vaccine

2.2.1. Bacterial strain

Bordetella bronchiseptica was used as a causative agent of AR. This strain was isolated from porcine nasal cavity with a selective medium, G20G, and was identified by colony morphology, biochemical and automatic bacteria identification system, Vitek (Hazelwood, MD, USA).

2.2.2. Detection of dermonecrosistoxin (DNT) gene from isolated bacterial strain and preparation of DNT

DNT gene was detected by PCR amplification as described by Shin et al. [16]. DNT was extracted as described by Shin et al [16]. Briefly, bacterial cells were cultured in tryptic soy broth (TSB, Difco Co.) at 37 °C overnight with shaking. The bacteria were harvested and washed twice with phosphate-buffered saline (PBS, pH 7.4). It was centrifuged at 20,000 rpm for 1 h at 4 °C after sonication of bacterial cells with an ultrasonic homogenizer (Bahedelin Co., Germany) for 1 min. The supernatants were taken by centrifugation and analyzed by SDS-PAGE and Western blot.

2.3. Preparation of CMs

CMs were prepared according to the procedure previously developed by Park et al. [17] based on the ionotropic gelation of chitosan with TPP anions. Briefly, chitosan was dissolved in 2% aqueous acetic acid to give a polymer concentration of 0.25 w/v %. Five ml of 15 w/v% TPP was dropped into 25 ml of 0.25 wt% chitosan solution under magnetic stirring and sonication (5 W, constant duty cycle). The CMs were obtained by centrifugation for 15 min at 3000 rpm.

2.4. Loading of AR vaccine

BBD (12 mg/ml) dispersed in 0.5 ml of phosphate buffered saline (PBS, pH 7.4) containing 20 mg of CMs were kept at 37 °C for 24 h under shaking. After incubation, the suspension was centrifuged at 2500 rpm for 15 min to remove unloaded BBD. Loading content of BBD in CMs was determined by quantifying unloaded BBD in the supernatant with the Micro BCA protein assay method [18] and with performing chitosan control experiments and calibration curves at each incubation time point. The loading content was calculated according to following equation [19]:

$$\text{Loading content (\%)} = \frac{\text{total amount BBD} - \text{free BBD}}{\text{total BBD}} \times 100$$

2.5. Observation of CMs and BBD-loaded CMs by scanning electron microscopy (SEM)

Drop of CMs and BBD-loaded CM solution were placed on a stud. After air-drying at room temperature, the samples were gold coated using a JEOL JFC-110E Ion Sputtering device (JEOL, Tokyo, Japan). The coated samples were observed using JSM 5410LV field emission SEM (JEOL, Tokyo, Japan) [17].

2.6. Measurement of dynamic light scattering (DLS)

Particle sizes and distribution were determined using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics. Ltd, Osaka, Japan) with a He–Ne laser beam a 90° scattering angle at 25 °C [17].

2.7. Release of BBD from BBD-loaded CMs in vitro

The in vitro BBD release from BBD-loaded CMs was carried out by filling 20 mg of BBD-loaded CMs into 1.5 ml microtubes and was performed at 37 °C and pH 7.4 of PBS using a shaking water bath. Aliquots (1 ml) were withdrawn from the release medium and replaced by

an equal volume at each sampling time [17]. The amount of BBD released was determined by the Micro BCA protein assay method [18].

2.8. Measurement of secreted tumor necrosis factor α(TNF-α) and nitric oxide (NO)

A murine macrophage cell line, RAW264.7, was cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3% fetal bovine serum (FBS) at 37 °C under 5% CO_2. After a 18 h incubation of the cells in the 6 well plates containing 4.4×10^5 cells per well, they were stimulated with 1 μg/ml of stimulants as indicated at each experiment. *E. coli* LPS (1 μg/ml, Sigma) and unloaded CMs were used as positive and negative controls, respectively. After stimulation, secreted concentrations of TNF-α and IFN-γ were measured by enzyme-linked immunosorbent assay (ELISA) method (Endogen Co.).

Production of NO in culture medium was measured by nitrite accumulation with the Griess reaction [20]. Briefly, 100 μl aliquots of the culture supernatants were incubated with same volume of the solution containing 1% sulfanilamide (Sigma) and 0.1% N-(1-naphthyl)ethylene-diamine dihydrochloride (Sigma) in 2.5% phosphoric acid at room temperature. After 10 min, absorbance was measured at 540 nm using a spectrometer, and nitrite concentrations were calculated based on the standard curve generated with nitrite.

2.9. Statistical analysis

All sample collections and experiments were run in triplicate under the same condition. Genometric means of the concentration of the samples were calculated, and the significance of differences among the groups were determined by an unpaired *t*-test. Probability values ($P < 0.05$) were considered as significant.

3. Results and discussion

3.1. Characterization of CMs

Fig. 1 shows SEM micrographs of CMs (a) and BBD-loaded CMs (b). The results indicated that morphologies of CMs were observed as spherical shapes although BBD-loaded CMs became bigger and more aggregated ones than CMs themselves.

Particle size distribution of CMs (a) and BBD-loaded CMs (b) measured by ELS is shown in Fig. 2. The results indicated that the average particle sizes of CMs themselves and BBD-loaded CMs were 1.94 ± 0.72, and 4.39 ± 0.68 μm with uniformity, respectively, indicating that the particle sizes of CMs became bigger after loading of BBD into CMs. It has already been reported that

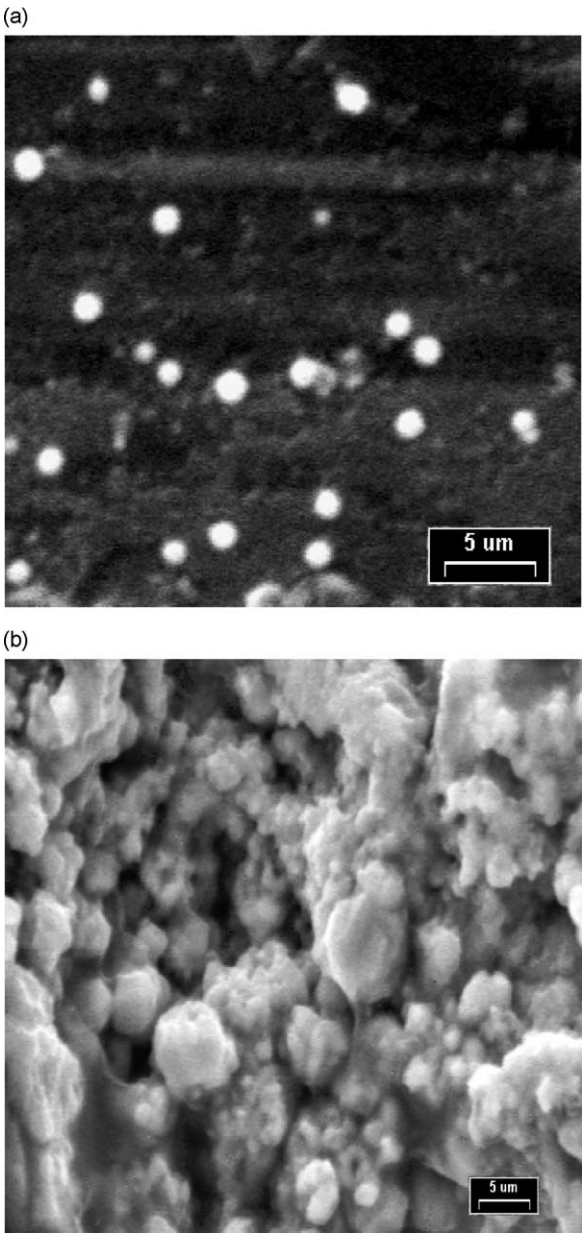

Fig. 1. SEM photographs of CMs (a) and BBD-loaded CMs (b) (2000 ×).

an appropriate size of microspheres is very important to target vaccines for uptake into the mucosal associated lymphoid tissues (MALTs) of the gut or the respiratory tract when applied intranasally for the delivery of vaccine [21,22]. Also, the majority of the microspheres below 10 μm in diameter were transported through the efferent lymphotics within macrophages. Therefore, it will be expected that BBD-loaded CMs will be effective for delivery of vaccine to the MALTs of the respiratory tract, although microspheres are not commonly used in veterinary vaccines [23].

3.2. Release of BBD from BBD-loaded CMs in vitro

Release of BBD from BBD-loaded CMs in PBS (pH 7.4) at 37 °C according to molecular weight of chitosan is shown

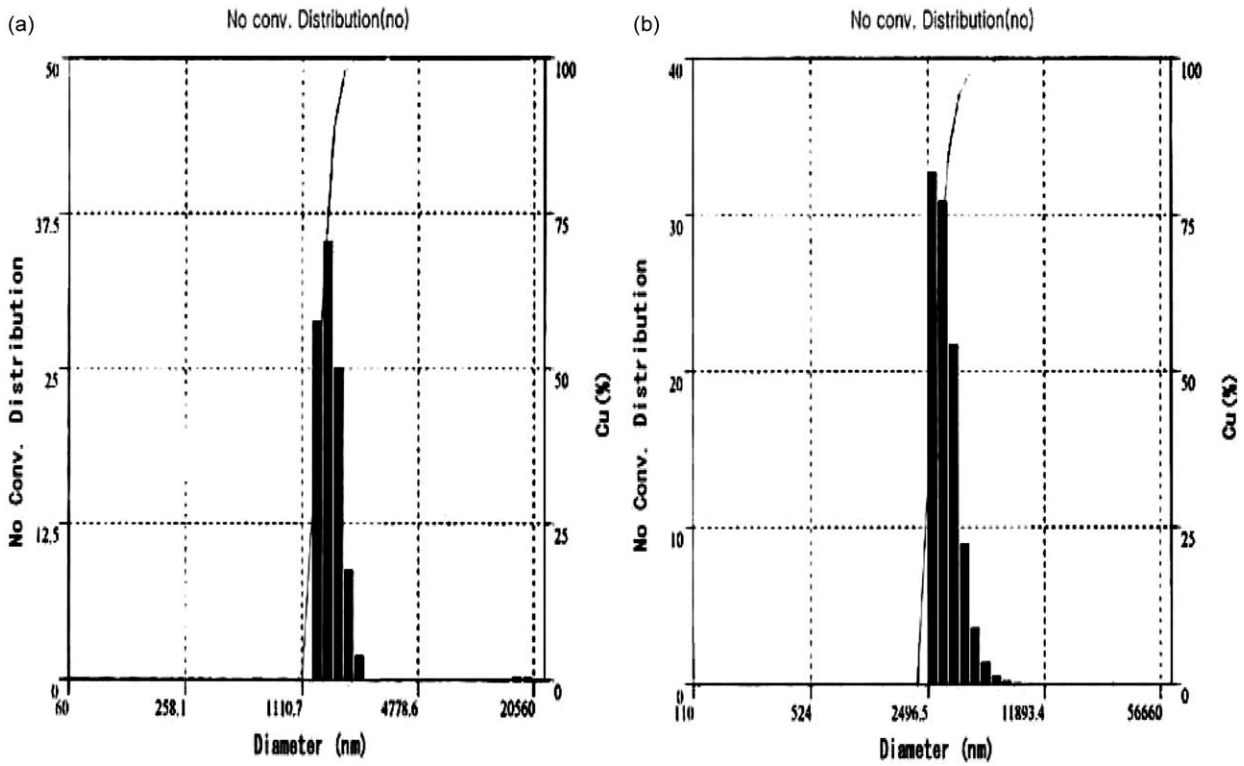

Fig. 2. Particle size distribution of CMs (a) and BBD-loaded CMs (b). Electrophoretic light scattering spectrophotometer (ELS 8000) was used with a 90° scattering angle at 25 °C.

in Fig. 3. The results indicated that more BBD was released with a decrease of molecular weight of chitosan without much difference of loading contents of BBD (65–75 wt%). It suggests that higher molecular weight of chitosan could form much stronger intermolecular interaction with the BBD than low molecular weight of chitosan, because amine groups in the chitosan increased with an increase of molecular weight of chitosan, resulting in a very slow release rate of BBD. These phenomena have also been observed by Xu et al. [19] and Lorengo-Lamosa et al. [24].

Fig. 4 shows release of BBD from BBD-loaded CMs at 37 °C according to medium pH. The results indicated that more BBD was released with an increase of medium pH. This is due to the fact that positive charges of chitosan are increased with a decrease of medium pH because p*K*a value of chitosan is about 6.5, and can strongly interact with

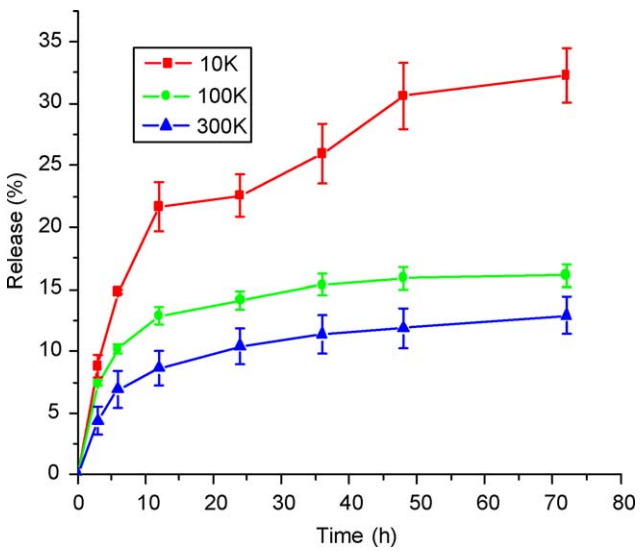

Fig. 3. Release of BBD from BBD-loaded CMs in vitro according to molecular weight of chitosan at pH 7.4 and 37 °C (data shown are the mean ± SD, $n = 3$).

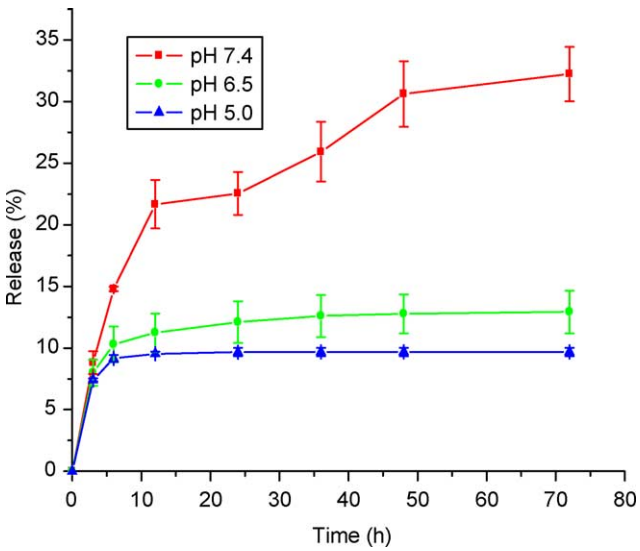

Fig. 4. Release of BBD from BBD-loaded CMs in vitro against medium pH (molecular weight of chitosan: 10 K) at 37 °C (data shown are the mean ± SD, $n = 3$).

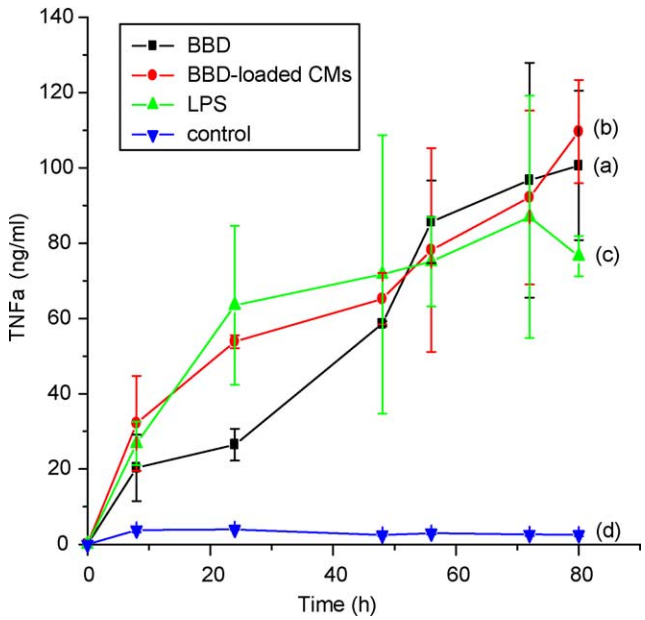

Fig. 5. Secretion of TNF-α from murine macrophage stimulated with BBD (a), BBD-loaded CMs (b), LPS (c) and control (d) ($n = 3$).

the BBD as the medium pH is decreased, resulting in a slow release of BBD at low pH. Mi et al. also reported that increased electrostatic attraction between the substrate of antigen and CMs resulted in a decreased release rate of the Newcastle disease (ND) vaccine's antigen from ND vaccine-loaded CMs in vitro [3].

3.3. Secretion of TNF-α, IFN-γ and NO from RAW264.7 cells

The main goal of the present work was to investigate the immune stimulating activity of CMs as vaccine delivery carriers for nasal administration. Using BBD as a model vaccine, we checked secretion of TNF-α, IFN-γ and NO from RAW264.7 cells as indicators in the induction of immune reaction because the macrophages produce these materials more efficiently than other materials. Concentrations of TNF-α, IFN-γ and NO were measured in the culture supernatant. The profiles of proinflammatory mediators including TNF-α and NO are shown in Figs. 5 and 6, respectively. Production of TNF-α and NO from RAW264.7 cells was increased in a time-dependent manner after exposure to BBD-loaded CMs, BBD, or LPS although that of TNF-α and NO from the cells after exposed to CMs as a negative control were not increased. The production patterns of TNF-α and NO were similar between BBD-loaded CMs and BBD itself with request to time-course ($P < 0.05$). It indicates that BBD has the ability to stimulate the immune system to a similar level to with BBD itself even when loaded into CMs. Also, BBD-loaded CMs continuously stimulated TNF-α secretion from RAW264.7 cells whereas concentration of TNF-α secreted by LPS began to decrease at 80 h post-stimulation ($P < 0.05$). It indicates that BBD-loaded CMs can steadily maintain

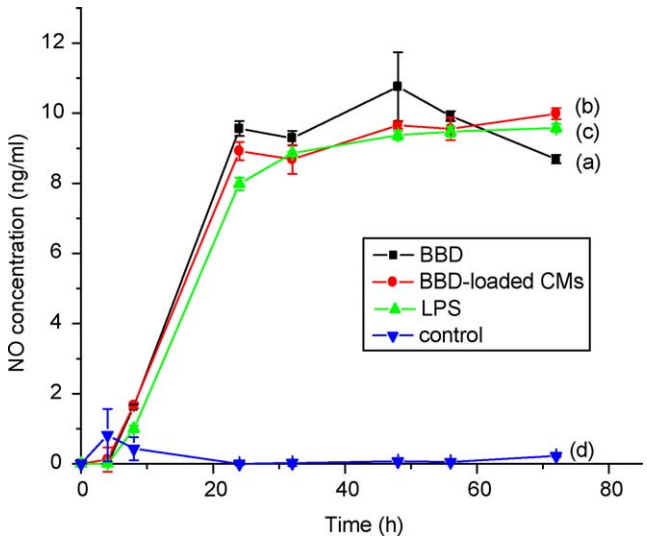

Fig. 6. Secretion of NO from murine macrophage stimulated with BBD (a), BBD-loaded CMs (b), LPS (c) and control (d) ($n = 3$).

the immune stimulating effect. Jeong et al. reported that the capacity of chitosan to increase NO production from IFN-γ-primed RAW264.7 macrophages is the result of chitosan-induced TNF-α secretion via the signal transduction pathway of nuclear factor-κB activation although the chitosan had no effect on NO production without IFN-γ [25]. Our results from this experiment strongly suggest that BBD-loaded CMs will be a new candidate for AR vaccine delivery because Jabbal-Gill et al. reported that *Bordetella pertussis* filamentous haemagglutinin/chitosan formulation induced stimulation of mucosal and systemic antibody responses in mice [26]. BBD-loaded CMs described in the current studies will be evaluated in mouse in the near future.

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