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Controlled Release of *Bordetella Bronchiseptica* Dermonecrotoxin (BBD) Vaccine from BBD-Loaded Chitosan Microspheres *In Vitro*

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Chitosan microspheres were prepared by ionic gelation process with sodium sulfate for nasal vaccine delivery. *Bordetella Bronchiseptica* Dermonecrotoxin (BBD) as a major virulence factor of a causative agent of atrophic rhinitis (AR) was loaded to the chitosan microspheres for vaccination. Morphology of BBD-loaded chitosan microspheres was observed as spherical shapes. The average particle sizes of the BBD-loaded chitosan microspheres were about 2.69 μm. More BBD was released with an increase of molecular weight of chitosan and with an increase of medium pH *in vitro* due to weaker intermolecular interaction between chitosan and BBD. Tumor necrosis factor-α (TNFα) and nitric oxide (NO) from RAW264.7 cells stimulated with BBD-loaded chitosan microspheres were gradually secreted, suggesting that released BBD from chitosan microspheres had immune stimulating activity of AR vaccine.

Key words: Chitosan microspheres, *Bordetella Bronchiseptica* Dermonecrotoxin, Nasal vaccine delivery, Tumor necrosis factor-α, Nitric oxide

INTRODUCTION

Polymeric microspheres have received much attention to deliver therapeutic peptide, protein, oligonucleotide and gene by intravenous, oral, and mucosal administrations (Janes *et al.*, 2001). The polymeric microspheres have been demonstrated to significantly enhance the systemic and/or mucosal immune responses after mucosal vaccination (Eyles *et al.*, 1998). Adjuvants are required to be co-delivered with the antigens to enhance the immune responses because mucosally delivered antigens are not much immunogenic. The microspheres were one of the most common forms used for the controlled-vaccine delivery formulations. 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 (Mi *et al.*, 1999).

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 (Artursson *et al.*, 1994). Molecular weight and degree of acetylation 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 (Artursson *et al.*, 1994 and Illum *et al.*, 1994). Generally after nasal administration of the chitosan-antigen nasal vaccines, the nasal formulation induced significant serum IgG responses and secretory IgA levels (Illum *et al.*, 2001). Chitosan has been found to induce a wide range of antigens from bacteria, viruses and tumors. And with respect to mucosal drug delivery, chitosan showed strong mucoadhesive properties (Suzuki *et al.*, 1984; Saiki *et al.*, 1990; Lika *et al.*, 1987; Kreuter *et al.*, 1976,1981,1988; He *et al.*, 1998). For this reason chitosan has been used as an immunological adjuvant or a vaccine carrier for mucosal vaccination. Also, advantage of chitosan microspheres is that the loading of vaccine is performed by incorporation in an aqueous solution without exposition to organic solvents (Van der Lubben *et al.*, 2003).

Swine respiratory diseases have induced severe economic losses in swine industry in the world. Atrophic rhinitis (AR) is a serious and widely prevalent infectious

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disease of swine. The signs of AR usually appear by 812 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 (Hasebe *et al.*, 1971). Two infectious agents, *Bordetella bronchiseptica* and *Pasteurella multocida* type D, are associated with the etiology of AR. Toxigenic strains of *Bordetella bronchiseptica* vaccination is widely used to reduce the incidence of AR (Shin *et al.*, 2000).

In this study, chitosan microspheres were prepared for the controlled release of the antigen of the AR vaccine. Release of *Bordetella bronchiseptica* Dermonecrotoxin (BBD) from BBD-loaded chitosan microspheres was performed *in vitro*. And immune stimulating activity of BBD-loaded chitosan microspheres as AR vaccine was checked in murine macrophage cell-line.

MATERIALS AND METHODS

Materials

Chitosans with molecular weights of 10 K, 100 K and 300K were kindly provided by Jakwang (Ansung, Korea). Deacetylation of the used chitosan was 90.8%. Sodium sulfate was supplied by Sigma (St Louis, MO, USA). All other chemicals were reagent grade chemicals.

AR vaccine

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).

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.* (Shin *et al.*, 2000). DNT was extracted as described by Shin *et al* (Shin *et al.*, 2000). 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.

Preparation of chitosan microspheres

Chitosan was dissolved in 2% aqueous acetic acid to give a polymer concentration of 0.25 wt-%. Five mL of 15 wt-% sodium sulfate was dropped into 25 mL of 0.25 wt-% chitosan solution under magnetic stirring and sonication (5 W, constant duty cycle). The chitosan microspheres were obtained by centrifugation for 15 min at 3000 rpm.

Loading of AR vaccine

BBD (0.5 mL) in phosphate buffered saline (PBS, pH 7.4) containing 20 mg of chitosan microspheres 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 chitosan microspheres was determined by quantifying unloaded BBD in the supernatant with Lowry protein assay method (Lowry *et al.*, 1951). The loading content was calculated according to following equation:

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

Observation of chitosan microspheres by scanning electron microscopy (SEM)

The chitosan microspheres were gold coated using coating chamber (CT 1500HF, Oxford Instruments, Oxfordshire, UK). The coated samples were observed using JSM 5410LV field emission SEM (Tokyo, Japan).

Measurement of dynamic light scattering (DLS)

DLS was measured with a DLS-7000 (Otsuka Electronics. Ltd., Tokyo, Japan) with an argon laser beam at a wavelength of 488 nm at 20°C. The value is expressed in weight-averaged scales as unimode. The scattering angle of 90° was used.

Release of BBD from chitosan microspheres *in vitro*

The *in vitro* BBD release from chitosan microspheres was carried out by filling 20 mg of BBD-loaded chitosan microspheres into 1.5 mL microtubes and was performed at 37°C and pH 7.4 of PBS using shaking water bath. One mL of aliquot was withdrawn from the release medium and replaced by an equal volume at each sampling time. The amount of BBD released was determined by the Lowry protein assay method (Lowry *et al.*, 1951).

Measurement of secreted tumor necrosis factor-α (TNFα) and nitric oxide (NO)

Secreted TNFα from PAW264.7 cells [murine macrophage cell-line suspended in modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS)] was analyzed by enzyme-linked immunosorbent assay (ELISA) kit (Endogen co.). Lipopolysaccharide (LPS) was used as a positive control.

Production of NO in culture medium was measured by nitrite accumulation with the Griess reaction (Green *et al.*,

1992). Briefly 100 aliquots of the culture supernatants were incubated with same volume of the solution containing 1% sulfanilamide and 0.1% naphthllethyl enediamine dihydrochloride in 2.5% phosphoric acid at room temperature. After 10 min, absorbance was measured at 540 nm using spectrometer, and nitrite concentrations were calculated based on the standard curve generated with nitrite.

RESULTS AND DISCUSSION

Characterization of chitosan microspheres

SEM micrographs of chitosan microspheres and BBD-loaded chitosan microspheres were shown in Fig. 1. The morphologies of chitosan and BBD-loaded chitosan microspheres were observed as spherical shapes.

Particle size distribution of chitosan microspheres (a) and BBD-loaded chitosan microspheres (b) measured by DLS were shown in Fig. 2. The results indicated that the average particle sizes of chitosan itself and BBD-loaded chitosan were 503.5 nm and 2.69 nm, respectively, indicating that the particle sizes of chitosan became bigger after loading of BBD. It was already reported that 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

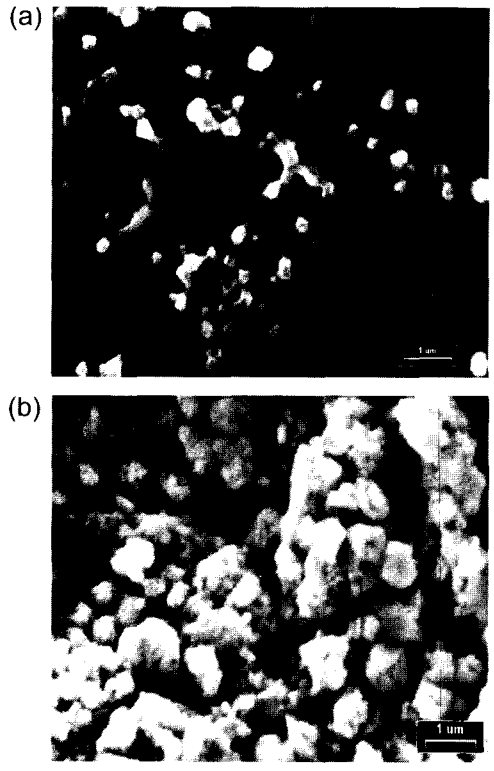

Fig. 1. SEM photographs of chitosan microspheres (a) and BBD-loaded chitosan microspheres (b).

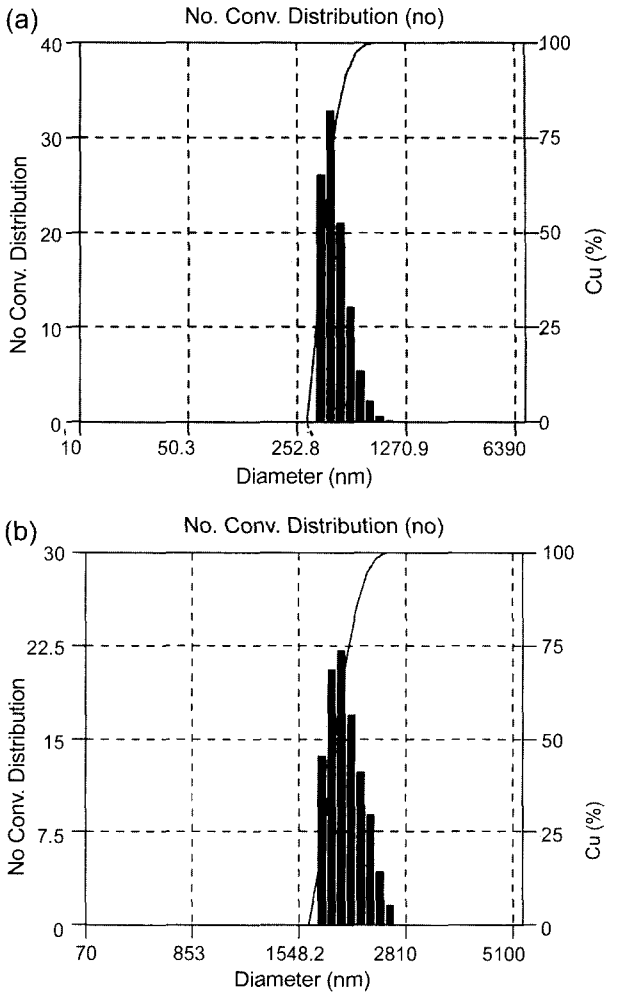

Fig. 2. Particle size distribution of chitosan microspheres (a) and BBD-loaded chitosan microspheres (b).

applied intranasally for the delivery of vaccine (Eldridge *et al.*, 1990; Lydyard *et al.*, 1998). Therefore, it is possible that the size of BBD-loaded chitosan microspheres will be effective for delivery of vaccine to the MALTs of the respiratory tract.

Release of BBD from BBD-loaded chitosan microspheres *in vitro*

Fig. 3 shows release of BBD from BBD-loaded chitosan microspheres in PBS (pH 7.4) at 37°C according to molecular weight of chitosan. The results indicated that more BBD was released with an decrease of molecular weight of chitosan without much differences of loading contents of BBD (85~94 wt-%) according to the molecular weight of chitosan. It suggests that higher molecular weight of chitosan could form more stronger intermolecular interaction with the BBD than low molecular weight of chitosan, resulting in a very slow release rate of BBD.

Fig. 4 shows release of BBD from BBD-loaded chitosan

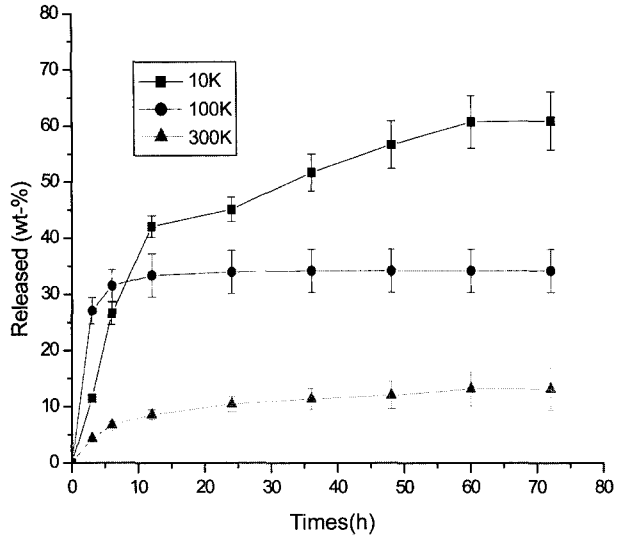

Fig. 3. Release of BBD from BBD-loaded chitosan microspheres *in vitro* according to molecular weight of chitosan (pH 7.4 and n=3).

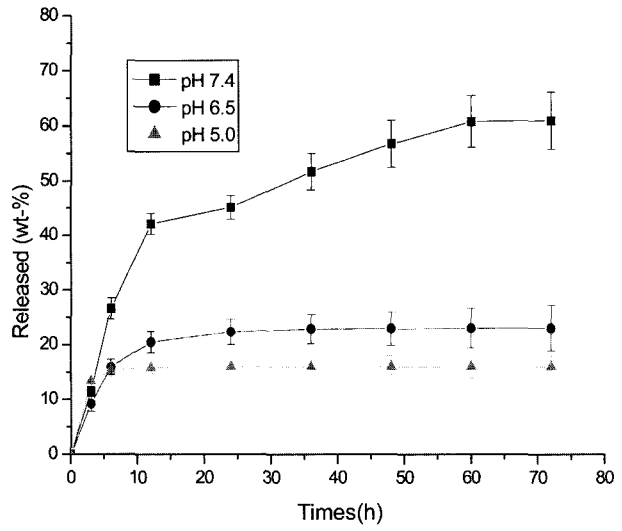

Fig. 4. Release of BBD from BBD-loaded chitosan microspheres *in vitro* according to medium pH (molecular weight of chitosan: 10k and n=3).

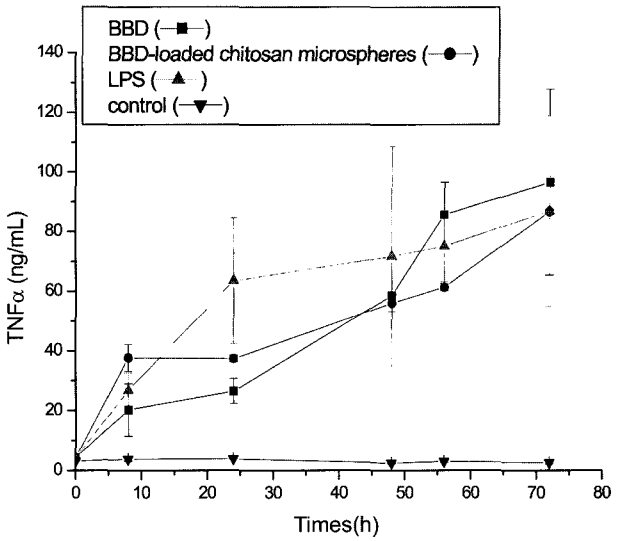

Fig. 5. Secretion of TNFα from murine macrophage stimulated with BBD, BBD-loaded chitosan microspheres, LPS and control (n=3).

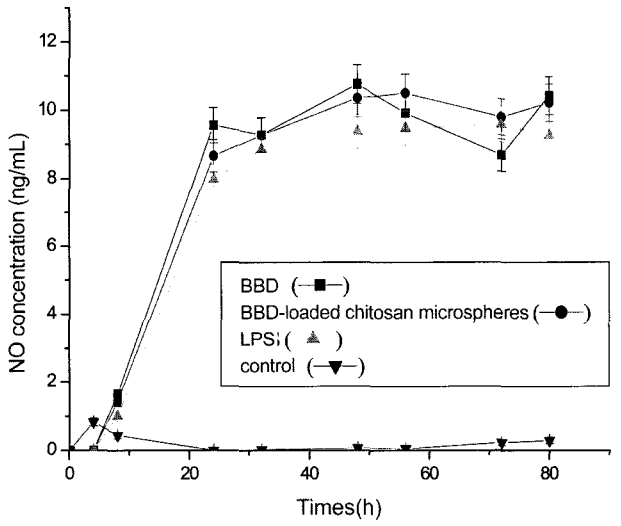

Fig. 6. Secretion of NO from murine macrophage stimulatted with BBD, BBD-loaded chitosan microspheres, LPS and control (n=3).

microspheres 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 charge of chitosan is increased with a decrease of medium pH because pKa value of chitosan is about 6.5, and can strongly interact with the BBD, resulting in a slow release of BBD at low pH.

Secretion of TNFα and NO from RAW264.7 cells

The profiles of proinflammatory mediators including TNFα and NO are shown in Figs. 5 and 6, respectively. Production of TNFα and NO from RAW264.7 cells were increased in time-dependent manner after stimulated with BBD-loaded chitosan microspheres, BBD, or LPS. The production patterns of TNFα and NO were similar between BBD-loaded chitosan microspheres and BBD itself in time-course. It indicates that BBD hold the ability to stimulate immune system in the similar level with BBD itself after loading of vaccine into chitosan microspheres. Also, BBD-loaded chitosan microspheres continuously stimulated TNFα secretion from RAW264.7 cells whereas concentration of TNFα secreted by LPS began to decrease at 72 h post-stimulation. It indicates that BBD-loaded chitosan microspheres can maintain steadily the immune stimulating effect. Our results from this experiment strongly suggest that BBD-loaded chitosan microspheres could be a new candidate for AR vaccine delivery. It is planned that

BBD-loaded chitosan microspheres described in the current studies will be evaluated in mouse in the near future.

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REFERENCES

Artursson, P., Lindmark, T., Davis, S. S., and Illum, L., Effect of chitosan on permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm. Res.*, 11, 1358-1361 (1994).

Eldridge, J. H., Hammond, C. J., Meulbrock, J. A., Staas, J. K., Gilley, R. M., and Rice, T. R., Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the peyer's patches. *J. Cont. Rel.*, 11, 205-214 (1990).

Eyles, J., Sharp, G., Williamson, E., Spiers, I., and Alpar, H., Intranasal administration of poly-lactic acid microsphere co-encapsulated *Yersinia pestis* subunits confers protection from pneumonic plague in the mouse. *Vaccine*, 16, 698-707 (1998).

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., J. Wishnok, S., and Tannenbaum, S. R., Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Anal. Biochem.*, 126, 131-138 (1992).

Hasebe, H., Occurrence and epizootiological surveys of infectious atrophic rhinitis in swine. *Nippon Vet. Zootech. Col. Bull.*, 19, 92-102 (1971).

He, P., Davis, S. S, and Illum, L., *In vitro* evaluation of the mucoadhesive properties of chitosan microspheres. *Int. J. Pharm.*, 166, 75-88 (1998).

Illum, L., Farraj, N. F., and Davis, S. S., Chitosan as a novel nasal delivery system for peptide drugs. *Pharm. Res.*, 11, 1186-1189 (1994).

Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A. N., and Davis, S. S., Chitosan as a novel nasal delivery system for vaccines. *Adv. Drug Deliv. Rev.*, 51, 81-96 (2001).

Janes, K. A., Calvo. P., and Alonso, M. J., Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv. Drug Deliv. Rev.*, 47, 83-97 (2001).

Kreuter, J. and Speiser, P. P., New adjutants on poly (methylmethacrylate) base. *Infect Immunol.*, 13, 204-210 (1976).

Kreuter, J. and Speiser, P. P., *In vitro* studies of poly (methylmethacrylate) adjutants. *J. Pharm. Sci.*, 65, 1624-1627 (1976).

Kreuter, J. and Liehl, E., Long-term studies of microencapsulated and adsorbed influenza vaccine nanoparticles. *J. Pharm. Sci.*, 70, 367-371 (1981).

Kreuter, J., Liehl, E., and Berg, V., Influence of hydrophobicity on the adjuvant effect of particlate polymeric adjutants. *Vaccine*, 6, 253-256 (1988).

Lika, I., Vine, T., Nishimura, K., and Azuma, I., Simulation of non-specific host resistance against Sendai virus and *E. coli* infection by chitin in mice. *Vaccine*, 5, 270-274 (1987).

Lowry, O. and Rosebrough, N., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193-265 (1951).

Lydyard, P. and Grossi, C., The lymphoid system. *Immunology*, 5, 31-42 (1998).

Mi, F. L., Shyu, S. S., Chen, C. T., and Schoung, J. Y., Porous chitosan microsphere for controlling the antigen release of Newcastle disease vaccine: preparation of antigen-adsorbed microsphere and *in vitro* release, *Biomaterials,* 20, 1603-1612 (1999).

Saiki, I., Murata, J., Tokura, S., and Azuma, I., Inhibition by sulfated chitin derivatives of advasion through extracellular matrix and enzymatic degradation metastatic melanoma cells. *Cancer Res.*, 50, 3631-3637 (1990).

Shin, N. R., Kim, J. M., and Yoo, H. S., *Korean J. Vet. Res.*, 40, 551-561 (2000).

Suzuki, K., Ogawa, Y., Hashimoto, K., Suzuki, S., and Suzuki, M., Protecting e!ect of chitin and chitosan on experimentally induced murine candidasis. *Microbiol. Immunol.*, 28, 903-904 (1984).

Van der Lubben, I. M., Kersten, G., Fretz, M. M., Beuvery, C., Verhoef, J. C., and Junginger, H. E., Chitosan microparticles for mucosal vaccination against diphtheria:oral and nasal efficacy studies in mice. *Vaccine*, 21, 1400-1408 (2003).