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Research paper

In vivo induction of mucosal immune responses by intranasal administration of chitosan microspheres containing *Bordetella bronchiseptica* DNT

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Abstract

In vitro immune-stimulating activities of *Bordetella bronchiseptica* dermonecrotoxin (BBD)-loaded in chitosan microspheres (CMs) were reported with a mouse alveolar macrophage cell line (RAW264.7). Based on the report, in vivo activity of immune-induction was investigated by intranasal administration of the BBD-loaded CMs into mice. BBD was loaded into the CMs prepared by an ionic gelation process with tripolyphosphate. Mice were immunized by direct administration of the BBD-loaded CMs into the nasal cavity. After immunization of the mice, BBD-specific immune responses (IgG and IgA titers) were measured in sera, nasal wash, and saliva by ELISA. BBD-specific IgA titers in the nasal cavity were time- and dose-dependently increased by the administration. Similar phenomena were observed in the analysis of systemic IgA and IgG in sera. However, the antibody in saliva was undetectable by ELISA. These results suggested that direct vaccination via the nasal cavity was effective for targeting nasal-associated lymphoid tissues, and that CMs were an efficient adjuvant in nasal mucosal immunity for atrophic rhinitis vaccine.

Keywords: *Bordetella bronchiseptica*; DNT; Chitosan microspheres; Mucosal immunity; Nasal administration

1. Introduction

Respiratory infection of the nasal cavity of pigs by *Bordetella bronchiseptica* is a common risk factor leading to the disease atrophic rhinitis (AR) [1]. AR has a number of symptoms including sneezing, nasal discharge, epistaxis, staining below the medial canthus of the eye, pneumonia, reduced growth rates, and feed conversion efficiency. Therefore, AR causes vast economic damage to the swine industry worldwide. Several methods have been developed and applied in order to control this disease. However, there are still problems controlling the disease in the swine industry. One of those problems is the low efficiency of commercial vaccines to induce mucosal immunity in the nasal cavity. *B. bronchiseptica* produces several purported virulence factors, including the dermonecrotic toxin (DNT), which has been implicated in the turbinate atrophy, main clinical lesion of AR [2]. Turbinate atrophy of pigs developed only after infection with a DNT producing *B. bronchiseptica* strain and not with an isogenic DNT-deficient strain [2].

Parenterally administered vaccines mainly stimulate systemic responses, whereas vaccines administered by mucosal route can lead to both efficient mucosal and systemic

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immune responses [3]. However, the delivery of antigens by mucosal route frequently results in a poor immune response [4,5]. These results have been attributed to several factors such as the limited diffusion of macromolecules across the mucosal barrier [6], rapid mucociliary clearance of drug formulations [7], and the presence of enzymatic degradation [8]. To overcome these problems, different strategies have been used, such as administration of antigens with mucosal adjuvants and/or entrapment of antigens into biodegradable microspheres/microparticles and liposomes. Chitosan is a biodegradable natural polymer with great potential for biomedical and pharmaceutical applications due to its biocompatibility, high charge density, and non-toxicity [9]. Chitosan has previously been shown to enhance the mucosal absorption of various compounds in a drug delivery system and have adjuvant activity in the mucosal immune response by intranasal administration [10–12]. Microspheres can increase the residence time of drugs in nasal mucosa compared to solutions and exert a direct effect on the nasal mucosa, resulting in the opening of tight junctions between the epithelial cells [13]. Chitosan microspheres (CMs), we used in this study, are the most widely studied drug delivery systems for the controlled release of drugs. There are several reports that demonstrated the efficacy of CMs as a vehicle for the transport of drugs in nasal administration [14,15]. We previously reported in vitro release study that BBD-loaded in CMs had immune-stimulating activities with mouse alveolar macrophages (RAW264.7) [16].

The mucosal immune system exhibits two distinct response patterns: (1) local productive immunity involving T cell-dependent cellular immunity and IgA antibody formulation and (2) the development of systemic and local tolerance. The adaptive humoral immune defense at mucosal surfaces is to a large extent mediated by secretary IgA (sIgA) antibodies. The resistance of sIgA to proteases makes these antibodies uniquely suited for functioning in mucosal secretions. In addition, sIgA antibodies produced by plasma cells in the respiratory mucosa may protect the host from both colonization and disease. The sIgA functions to prevent the adsorption of pathogens or their toxic products at the mucosal epithelium [17]. Based on this knowledge, we evaluated the immuno-stimulating activites of BBD-loaded CMs by measuring the local and mucosal immune response (BBD-specific sIgA) following nasal administration in mice.

2. Materials and methods

2.1. Materials

Three kinds of chitosans [molecular weights (M_w): 10, 100, and 300 K] measured by gel permeation chromatography were kindly provided from Jakwang (Ansung, Korea). Deacetylation of the used chitosan was 90.8%. Sodium tripolyphosphate (TPP) was purchased from Sigma (Missouri, USA). Commercial injectable and mixture vaccine of respiratory diseases in pigs, AR·P·H using for positive control was purchased from DaeSung Microbiological Lab (Uiwang, Korea). All other chemicals were of reagent grade.

2.2. Preparation of BBD

Bordetella bronchiseptica strains were isolated from specimens submitted to the Laboratory of Infectious Diseases, College of Veterinary Medicine, Seoul National University, Korea. Strains were identified by biochemical tests and the Vitek (Hazelwood, MD, USA) automatic bacteria identification system. Gene encoding DNT was detected by PCR amplification and DNT was extracted as described by Shin et al. [18]. Briefly, bacterial cells were cultured in tryptic soy broth (TSB, Difco Co.) at 37 °C for 24 h with shaking. The bacteria were harvested and washed with phosphate-buffered saline (PBS, pH 7.4). It was sonicated for 30 min and then centrifuged at 20,000 rpm for 1 h at 4 °C. Protein concentration was measured with a micro BCA assay kit (Bio-Rad, Co., USA). The supernatants were filtered and analyzed by SDS-PAGE and Western blot. For inactivation of toxicity from DNT, 0.05% formaldehyde (Sigma Co., Missouri, USA) was added to it and kept in 37 °C for 3 days with shaking.

2.3. Preparation of CMs

CMs were prepared according to the procedure previously developed by Park et al. [19], based on the ionotropic gelation of chitosan with tripolyphosphate (TPP) anions. Briefly, chitosan was dissolved in 2% aqueous acetic acid to give a polymer concentration of 0.25 w/v%. Twenty-five milliliter of 0.25 wt% chitosan solution in acetic acid was extruded dropwise through a needle into 5 ml of 15 w/v% TTP under magnetic stirring and sonication (5 W, constant duty cycle). The beads were removed from TTP solution by filtration and washed with distilled water. The CMs were obtained by centrifugation for 15 min at 3000 rpm. The yield of production was calculated as the weight percentage of the final product after drying, with respect to the initial total amount of chitosan salts used for the preparations.

2.4. BBD loading in CMs

BBD (12 mg/ml) dispersed in 0.5 ml PBS (pH 7.4) containing 20 mg CMs were kept at 37 °C for overnight under shaking (speed 5, thermo mixer). After incubation, the suspension was centrifuged at 2500 rpm for 15 min to remove unloaded BBD. Loading contents of BBD in CMs was determined by quantifying unloaded BBD in the supernatant with a Micro BCA protein assay method and with performing chitosan control experiments and calibration curves at each incubation time point. The particle size distributions of the CMs and BBD-loaded CMs were analyzed using an electrophoretic dynamic light-scattering spectrophotometer (ELS 8000). The morphologies of the CMs

and BBD-loaded CMs were observed with scanning electron microscopy (SEM) photographs (JEOL, Tokyo, Japan). The zeta potential was calculated from the mean electrophoretic mobility value, which was determined by laser Doppler anemometry (LDA), using a Zetasizer® III (Malvern Instruments, Malvern, UK). CM suspension was diluted with KCl 0.1 mM and placed in the electrophoretic cell where a potential of ±150 mV was established. Each batch was analyzed in triplicate. The loading content was calculated according to the following equation [20]:

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

2.5. Animals and in vivo studies

Five-week-old BALB/c female mice (Breeding and Research Center, Seoul National University, Korea) were used throughout this study, following the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University, Korea). Each experimental group consisting of 15 mice was immunized at 2 week intervals for 10 weeks. The mice in each group was intranasally immunized by dropping 20 μl PBS containing 1, 10 or 30 μg BBD-loaded CMs or the same concentration of CMs only into the nostrils with inhalation under non-anesthesia. The amount of CMs administered to each mouse in the different formulations was 120 μg. We described the amount of CMs and BBD administered to nasal cavity of mice in each different formulation of BBD-loaded CMs (Table 1). A positive control group was intramuscularly immunized with the commercial injectable vaccine, AR·P·H using the same schedule as that used with nasal administration.

2.6. Sample collection

Saliva was collected using a transfer pipette from mice anesthetized with a mixture of Ketamine hydrochloride and Xylazine (Bayer Korea Co., Seoul, Korea) and administered intraperitoneally with 100 μl of pilocarpine (0.5 mg/ml) (Sigma) in PBS. Blood was collected from orbital veins, clotted at room temperature, and left to stand at 4 °C overnight. Sera were collected by centrifugation at 3000 g for 10 min at 4 °C and stored at −20 °C until use. Nasal fluid was obtained by flushing the nasal cavity from the pharynx through the nostrils two times with 50 μl PBS after the mice were scarified by an overdose of Ketamine hydrochloride (Yu Han Corporation, Korea). Nasal wash and saliva samples were immediately placed in ice and stored at −20 °C for further analysis.

Table 1
The amount of CMs and BBD administered in different formulations of BBD-loaded CMs

Formulations	BBD (μg)	CMs (μg)
BBD-loaded CMs (1)	1	120
BBD-loaded CMs (2)	10	120
BBD-loaded CMs (3)	30	120

2.7. Measurement of BBD-specific antibody responses

The level of antigen-specific antibodies (IgA or IgG) in serum, saliva, and nasal wash samples was determined using enzyme-linked immunoabsorbent assay (ELISA). Ten nanogram of BBD suspended in 100 μl of coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, and 3.1 mM NaN_3, pH 9.6) was added to a microplate for ELISA (Greiner, Australia) and incubated overnight at 4 °C. The plate was washed three times with PBST (0.05% Triton X-100 in PBS) and blocked with PBST containing 1% bovine serum albumin (Amresco Inc., Solon, OH, USA) for 1 h at 37 °C. Primary antibodies collected from the sera, saliva, and nasal wash of immunized mice were used for the analysis of immune response. Primary antibodies (sera, saliva, and nasal wash from mice) were diluted 20-fold, added to the plate and incubated for 1 h at 37 °C. After washing with PBST, 0.1 μl of goat anti-mouse IgG (H + L)–HRP conjugated (Bio-Rad) or anti-mouse IgA (α-chain specific)–HRP conjugated (Sigma) was added to the plate, and incubated for 1 h at 37 °C. Color was developed by adding 100 μl ABST substrate solution (Bio-Rad) to the plate. After 20 min incubation at room temperature, the OD value was measured at 405 nm using an ELISA reader (Molecular Device Corp., Sunnyvale, CA, USA).

2.8. Statistical analysis

Statistical analysis was performed using the computer-based Excel program version 2004. All results were expressed as mean ± standard deviation (SD). Statistical differences between the groups were analyzed with Student's *t*-test. Differences were considered significant if probability values of $p < 0.05$ were obtained.

3. Results

3.1. Characterization of BBD-loaded CMs

Particle size distribution of CMs and BBD-loaded CMs was measured by electrophoretic dynamic light scanning (ELS 8000). CMs and BBD-loaded CMs used in this study had a mean size of 0.29 ± 0.68 and 5.23 ± 0.46 μm, respectively (Table 2). Scanning electron microscopy (SEM) photographs showed the CMs were spherical and smooth-surfaced without cracks or wrinkles. The BBD-loaded CMs, however, were aggregated and became bigger than CMs only (Fig. 1). The surface charge of CMs and BBD-loaded CMs was 9.96 and 18.22 mV, respectively. Average loading efficiency of BBD in CMs was 65–75% (w/w) (Table 2).

Table 2
Physicochemical characteristics of CMs and BBD-loaded CMs

Particles	Size (μm) ± SD	Surface charge (mV)	Loading efficiency (%)	Yield of production
CMs	0.29 ± 0.68	9.96	–	80–90%
BBD-loaded CMs	5.23 ± 0.46	18.22	65–75%	–

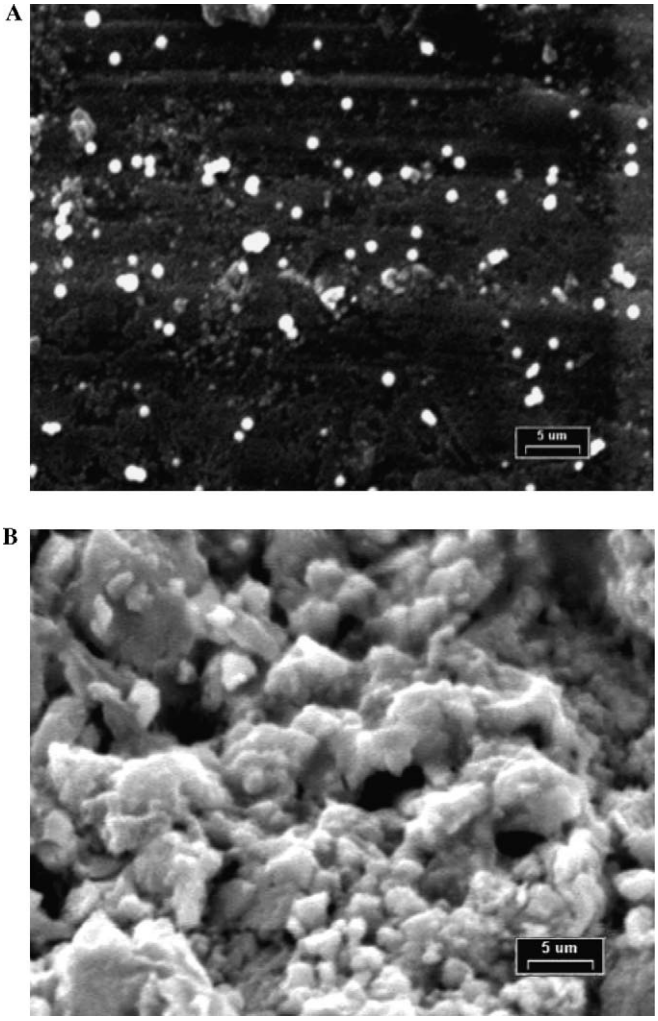

Fig. 1. Scanning electron microscopy (SEM) photographs of CMs (A) and BBD-loaded CMs (B); (5000×).

3.2. BBD-specific antibody in nasal wash

Specific IgA responses to BBD in the nasal wash of mice immunized intranasally with BBD-loaded CMs were higher than those of control groups (Fig. 2). In particular, specific IgA titer of the group intranasally administered with 30 μg BBD-loaded CMs was significantly higher than that of control groups after the third immunization ($p < 0.05$). In other groups receiving 1 and 10 μg BBD-loaded CMs, specific IgA to BBD was increased at the fourth or final immunization but the values were lower than those of the group administered with 30 μg BBD-loaded CMs. Therefore, BBD-specific IgA titers in nasal wash were time- and dose-dependently increased by the administration. However, the AR·P·H intramuscular injected group showed no significant level of IgA antibody titer in their nasal wash from the initial immunization to the final immunization. Also, the group with only administration of CMs did not show BBD-specific immune response in nasal mucus. Specific IgG antibody in the nasal wash was undetectable in all experimental groups.

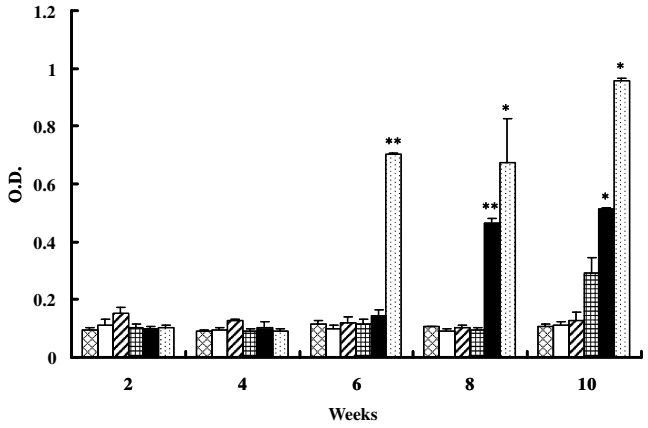

Fig. 2. Anti-BBD IgA levels in nasal wash were determined by ELISA. (⬚) nasal administration with 30 μg BBD-loaded CMs, (■) nasal administration with 10 μg BBD-loaded CMs, (▦) nasal administration with 1 μg BBD-loaded CMs, (▨) intramuscular injection with ARPH, (□) nasal administration with only CM, and (▩) non-treated group (data shown are means ± SD, $n = 3$). Significant differences between control groups and vaccinated groups were expressed as $*p < 0.05$ and $**p < 0.01$, respectively.

3.3. BBD-specific antibody in serum

Systemic anti-BBD IgA antibody titers showed a pattern similar to those from nasal wash, however, the response was lower. High IgA titers in sera were maintained in the vaccinated group with 30 μg BBD-loaded CMs after the third immunization ($p < 0.05$) (Fig. 4). There was no significant increase in the other vaccinated groups with BBD-loaded CMs, but the level of antibodies was slightly higher than that of the group with administration of AR·P·H. The group vaccinated intramuscularly with AR·P·H showed a high level of anti-BBD IgG in sera following the second immunization. However, systemic IgA antibody titer in the AR·P·H group was at a very low level. The groups vaccinated with BBD-loaded CMs showed a high IgG anti-BBD antibody response in serum samples after the third immunization, even though the value was lower than that of the AR·P·H group ($p < 0.05$) (Fig. 3).

3.4. BBD-specific antibody in saliva

In specific antibody responses to BBD in saliva, IgA and IgG antibodies were undetectable in all experimental groups (data not shown).

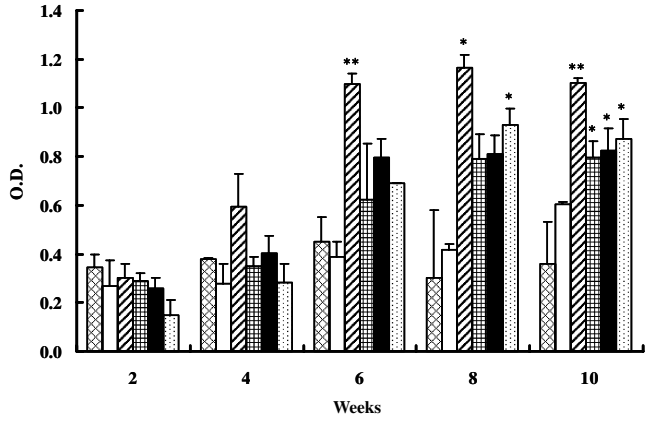

Fig. 3. Anti-BBD IgG levels in serum were determined by ELISA. (▨) nasal administration with 30 μg BBD-loaded CMs, (■) nasal administration with 10 μg BBD-loaded CMs, (▦) nasal administration with 1 μg BBD-loaded CMs, (▨) intramuscular injection with ARPH, (□) nasal administration with only CM, and (▨) non-treated group (data shown are means ± SD, $n = 3$). Significant differences between control groups and vaccinated groups were expressed as $*p < 0.05$ and $**p < 0.01$, respectively.

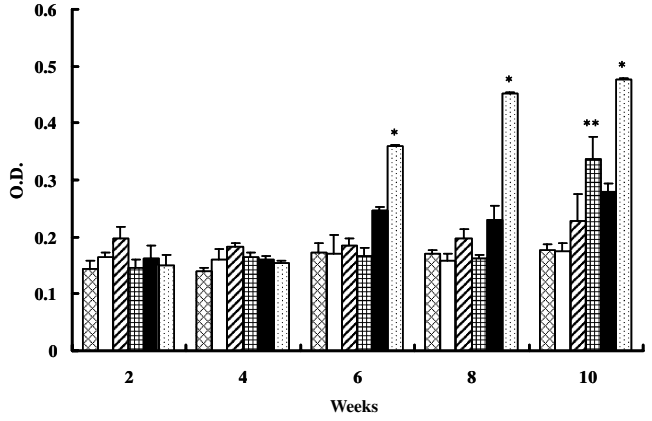

Fig. 4. Anti-BBD IgA levels in serum were determined by ELISA. (▨) nasal administration with 30 μg BBD-loaded CMs, (■) nasal administration with 10 μg BBD-loaded CMs, (▦) nasal administration with 1 μg BBD-loaded CMs, (▨) intramuscular injection with ARPH, (□) nasal administration with only CM, and (▨) non-treated group (data shown are means ± SD, $n = 3$). Significant differences between control groups and vaccinated groups were expressed as $*p < 0.05$ and $**p < 0.01$, respectively.

4. Discussion

Recently, chitosan has received attention as an adjuvant for mucosal delivery by facilitating higher drug and carrier bioavailability [11,12]. Microspheres, recently became advantageous for drug carrier dosage forms, also have a direct effect on the mucosa [13]. In this study, we investigated the effect of CMs as an adjuvant in nasal mucosal immune response for the purpose of preventing AR. CMs containing BBD showed high loading efficiency (65–75%). Many factors may affect the loading efficiency of BBD in the CMs, e.g., nature of BBD, concentrations of BBD and chitosan, BBD polymer ratio, amount of TTP solution, stirring speed, etc. The drug loading was found to be dependent upon the initial drug concentration. A higher initial concentration led to a higher loading efficiency [21]. The initial concentration of BBD before the loading into CMs was 12 mg/ml. The high concentration was obtained from repeated culture and harvesting of *B. bronchiseptica*. Also the high loading efficiency may be due to favorable interactions between the positively charged CMs and the negatively charged BBD. Our result, the surface charge of BBD-loaded CMs became higher than that of CMs themselves, indicates the possibility of the assumption mentioned above (Table 2). Several studies have shown that the roughness on the surface and the mean size of the drugs in CMs increased with increase in loading on SEM micrographs [15,22]. Our results, the SEM micrographs of the microspheres, indicate the morphologies of BBD-loaded CMs became bigger and more aggregated ones than CMs themselves (Fig. 1). For the intranasal delivery of vaccines, the appropriate size of microspheres is closely related to the amount of vaccine uptake into mucosal associated lymphoid tissues (MALTs) [23]. Therefore, the SEM micrographs of the microspheres supported the expectation of effective delivery of BBD-loaded CMs to MALTs.

sIgA is the major immunoglobulin in secretions that bathe mucosal surfaces, and sIgA antibodies have been proposed to play the first line of defense against pathogens in the respiratory tract [17]. Nasal associated lymphoid tissues (NALTs) play an important role in the defense of mucosal surfaces. They may also function as sites for priming the immune response to vaccines and as immune effectors against pathogens [24]. Therefore, an important strategy to prevent AR, swine respiratory disease, is the effective induction of sIgA responses in NALTs located at the base of the nasal cavity. However, the nasal mucosal immunity has a several limitation, such as limited diffusion [6], rapid mucociliary clearance [7], and enzymatic degradation [8]. Therefore, we used CMs as a safe and effective mucosal adjuvant or absorption enhancer for overcoming the limitation of mucosal immunization and induction of effective mucosal immune responses. The effective immune-induction was substantiated by the increase of anti-BBD IgA in both nasal wash and serum after nasal administration of BBD-loaded CM vaccine (Figs. 2 and 4). Our results showed that BBD specific IgA titers of nasal wash in the group with administration of 30 μg BBD-loaded CMs were about fivefold higher than those of other groups. Especially the titers were significantly increased and maintained after third immunization (Fig. 2). As these reasons, our result strongly supports the remarkable efficacy of the chitosan as an adjuvant and microspheres as a drug carrier dosage form in the AR vaccine. Several reports have verified the efficacy of CMs as a vehicle for the transport of drug through the nasal mucosa [14,15]. However, no antibodies were detectable in the saliva of mice immunized intranasally with BBD-loaded CMs. This result was

consistent with a previous study that showed no demonstrable effect on the salivary concentration of *B. bronchiseptica*-reactive IgA or IgG after the intranasal vaccination of dogs [25].

The nasal vaccine induced significantly higher local IgA in nasal washings and local cell-mediated immunity but less high serum antibody titers than the injectable vaccine [17]. The groups intranasally vaccinated with BBD-loaded CMs showed a high IgG anti-BBD antibody response in serum samples, even though the value was lower than that of the AR·P·H group (Fig. 3). Our results agree with those of previous studies in which mucosal immune response was increased following intranasal vaccination for preventing AR [25,26]. We observed no significant response to AR·P·H (a commercial injectable vaccine) in the amount of mucosal IgA in nasal wash (Fig. 2). However, nasal immunization with BBD-loaded CMs resulted in an increase of the anti-BBD IgA titer not only in the nasal mucosa, but also in the serum at a level comparable to that of AR·P·H (Figs. 2 and 4). Thus, intranasal immunization has been shown to be a promising route for the induction of specific antibodies in the nasal cavity. Although proving protective immunitys still remains a further study, our results demonstrated that intranasal immunization of mice with BBD-loaded CMs induced both systemic and mucosal immune responses.

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