J. Microbiol. Biotechnol. (2008), **18**(6), 1179–1185

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Chitosan Microspheres Containing *Bordetella bronchiseptica* Antigens as Novel Vaccine Against Atrophic Rhinitis in Pigs

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Received: October 15, 2007 / Accepted: December 15, 2007

The immune-stimulating activities of *Bordetella bronchiseptica* antigens containing dermonecrotoxin (BBD) loaded in chitosan microspheres (CMs) have already been reported *in vitro* and *in vivo* with a mouse alveolar macrophage cell line (RAW264.7) and mice. Therefore, this study attempted to demonstrate the successful induction of mucosal immune responses after the intranasal administration of BBD loaded in CMs (BBD-CMs) in colostrum-deprived pigs. The BBD was introduced to the CMs using an ionic gelation process involving tripolyphosphate (TPP). Colostrum-deprived pigs were then directly immunized through intranasal administration of the BBD-CMs. A challenge with a field isolate of *B. bronchiseptica* was performed ten days following the final immunization. The BBD-specific IgG and IgA titers, evident in the nasal wash and serum from the vaccinated pigs, increased with time ($p<0.05$). Following the challenge, the clinical signs of infection were about 6-fold lower in the vaccinated pigs compared with the nonvaccinated pigs. The grades for gross morphological changes in the turbinate bones from the vaccinated pigs were also significantly lower than the grades recorded for the nonvaccinated pigs ($p<0.001$). Therefore, the mucosal and systemic immune responses induced in the current study would seem to indicate that the intranasal administration of BBD-CMs may be an effective vaccine against atrophic rhinitis in pigs.

Keywords: Atrophic rhinitis, intranasal administration, chitosan microspheres, *Bordetella bronchiseptica*, pigs

Two infectious agents, toxigenic strains of *Bordetella bronchiseptica* and *Pasteurella multocida*, are associated with the etiology of atrophy rhinitis (AR) in swine. However, whereas infection with toxigenic *P. multocida* results in severe, growth-retarding, and progressive AR, infection with *B. bronchiseptica* only leads to mild nonprogressive forms of AR that do not affect the growth rate [8]. Moreover, *B. bronchiseptica* adheres well to epithelial cells, whereas *P. multocida* adheres poorly to an intact mucus membrane and requires predisposing conditions to colonize the mucosa [13, 21]. Several studies have already demonstrated that infection with *B. bronchiseptica* permits colonization by toxigenic *P. multocida* [4, 24, 26]. Therefore, infection with *B. bronchiseptica* would seem to be an essential condition for the development of progressive AR.

The nasal mucosa is an important arm of the mucosal immune system, since it is often the first portal of entry for inhaled antigens. When compared with other routes of drug administration, nasal delivery offers many advantages, including a large epithelial surface with the presence of numerous microvilli, a porous endothelial membrane, and highly vascularized mucosa facilitating absorption [23]. Parenterally administered vaccines mainly stimulate systemic responses, whereas vaccines administered by nasal delivery can lead to both efficient mucosal and systemic immune responses [5, 23, 34]. Several AR vaccines have already been investigated using *B. bronchiseptica* or *P. multocida* with attenuation or genetical modification [6, 17, 27, 33]. However, none have been proven to induce specific immune responses in the nasal mucosa and no vaccine delivery system has been considered. Successful nasal mucosal immunization of pigs will most likely require an antigen delivery system or adjuvant that adheres to the mucosa at the time of administration, as nasal mucosal immunization is often followed by rapid systemic drug absorption or sneezing.

Chitosan microspheres (CMs), as used in the present study, have already been extensively studied as a drug delivery system. The positive charge on chitosan generated under physiological conditions has been found to be responsible for its enhanced bioadhesivity and site-specific applications in controlled delivery systems [1, 12, 31]. Thus, microspheres

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can increase the residence time of drugs in the nasal mucosa compared with solutions, and exert a direct effect on the nasal mucosa, resulting in the opening of tight junctions between the epithelial cells [16, 25]. Several reports have already demonstrated the efficacy of CMs as a vehicle for the transport of drugs in nasal administration [11, 28, 35].

In previous *in vitro* and *in vivo* studies, the current authors reported that *Bordetella bronchiseptica* antigens containing dermonecrotoxin (BBD) loaded in chitosan microspheres (CMs) produced immune-stimulating activities in mouse alveolar macrophage cells and mice [14, 15]. Accordingly, this study evaluated the intranasal administration of BBD-CMs as regards inducing a mucosal immune response in colostrum-deprived pigs. For comparison with the *in vivo* immune-stimulating activity, the mucosal immune response (BBD-specific sIgA) and systemic immune response (BBD-specific IgG) were observed in an enzyme-linked immunoabsorbent assay (ELISA). In addition, protective immunity comparisons were made based on an analysis of the clinical signs and gross morphological changes in the turbinate bones after a challenge with a field isolate of *B. bronchiseptica via* the nasal cavity.

Materials and Methods

Materials and Experimental Animals

The chitosan (molecular weight, 10,000; deacetylation degree, 80.4%) was kindly supplied by Jakwang (An-sung, Kyunggi, Korea), and sodium tripolyphosphate (TPP) was purchased from Sigma (St. Louis, MO, U.S.A.). Six-week-old colostrum-deprived pigs (XP-bio, An-sung, Kyunggi, Korea) were used throughout the study, following the policies and regulations for the care and use of laboratory animals set by the Laboratory Animal Center, Seoul National University, Seoul, Korea. All other chemicals were of reagent grade.

Preparation of Antigens

The *B. bronchiseptica* strains were isolated from specimens submitted to the Laboratory of Infectious Diseases, College of Veterinary Medicine, Seoul National University, Seoul, Korea. A virulent (*Bvg*+) *B. bronchiseptica* strain was confirmed by hemolysis production on a sheep blood agar. The strains were identified using biochemical tests and Vitek (Hazelwood, MD, U.S.A.), an automatic bacteria identification system. The bacterial cells were cultured in a tryptic soy broth (TSB, Difco Co., Franklin Lakes, NJ, U.S.A.) at 37°C for 24 h under shaking conditions, and then harvested and washed with phosphate-buffered saline (PBS, pH 7.4). Thereafter, the compound was sonicated for 1 h and centrifuged at 20,000 rpm for 1 h at 4°C. The protein concentration was measured using a micro BCA assay kit (Bio-Rad Co., Hercules, CA, U.S.A.), and the supernatants were filtrated and analyzed by SDS-PAGE and a Western blot. To determine the presence of multiple *B. bronchiseptica* antigens, a polyclonal antibody against *B. bronchiseptica* was used that had been produced in a previous study [32]. The genes encoding the dermonecrotoxin (DNT) were detected by PCR amplification and the presence of DNT in the supernatant was confirmed by the inoculation of multiple *B. bronchiseptica* antigens as a lethal test in mice. To inactivate the toxicity of the prepared antigens, 0.05% formaldehyde (Sigma Co.) was added, and the solution maintained at 37°C for three days with shaking.

Preparation of Chitosan Microspheres

The CMs were prepared according to the procedure from a previous study [22], 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%. Twenty-four ml of the 0.25 wt% chitosan solution in acetic acid was dropped through a needle into 5 ml of 15 w/v% TPP under magnetic stirring and sonication (5 W, constant duty cycle). The beads were removed from the TPP solution by filtration and then washed with distilled water. The CMs were obtained by centrifugation for 15 min at 3,000 rpm.

Antigen Loading in Chitosan Microspheres

The BBD was loaded into the CMs according to a previous study (14). Briefly, BBD (12 mg/ml) dispersed in 0.5 ml of PBS (pH 7.4) containing 20 mg of CMs was maintained at 37°C overnight under shaking conditions (speed 5, thermo mixer). After incubation, the suspension was centrifuged to remove the unloaded BBD.

Selection of Experimental Pigs

To prevent any transfer of maternal antibodies, all the experimental pigs were deprived of colostrum from birth. The pigs were subsequently raised in isolation units where they received feed and water. Before the vaccination, all the pigs tested negative for the isolation of *B. bronchiseptica* in nasal swabs using Vitek, and serum antibodies against BBD using ELISA.

Immunization of Pigs

Six-week-old colostrum-deprived pigs were used throughout the study. Each experimental group consisted of 7 pigs. The pigs in the vaccinated group were directly immunized *via* the nasal cavity at ten-day intervals for 40 days, where 1 ml of PBS containing BBD-CMs was sprayed into the nostrils during inhalation under non-anesthetic conditions. The amount of BBD and CMs in the BBD-CM vaccine administered to each pig was 3 mg and 10 mg, respectively.

Sample Collection

Serum, nasal wash, and saliva samples were collected from the pigs at ten-day intervals during the experimental period to determine the BBD-specific antibody response. The nasal washes were collected as previously described [18]. Briefly, 10 ml of sterile PBS with 1% bovine serum albumin (BSA), penicillin (300 U/ml), and streptomycin (300 mg/ml) were injected into the nasal passages. The head was moved gently, and the fluid drained from the nasal passages into a collection cup. The saliva samples were collected using a round-tip, stainless steel, oral gavage needle attached to a syringe. The nasal wash and saliva samples were immediately placed in ice and stored at −20°C until further analysis.

ELISA

To measure the total amount of BBD-specific IgA and IgG in the nasal washes, saliva, and serum samples, an ELISA plate (Greiner, Australia) was coated with BBD (10 ng per well) in a coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.1 mM NaN_3, pH 9.6) and incubated overnight at 4°C. The plate was washed three times with

PBS containing 0.05% Triton X-100 and blocked with PBS containing 1% bovine serum albumin (Amresco Inc., Solon, OH, U.S.A.) and 0.05% Triton X-100 for 1 h at 37°C. The plate was then washed again three times. The nasal wash, saliva, and serum samples from the pigs, 2-fold serially diluted (serum diluted beginning at 1:100), were added to each well (100 μl per well) in triplicate and incubated for 1 h at 37°C. After washing three times with PBS containing 0.05% Triton X-100, horseradish peroxidase-conjugated goat anti-pig IgG (Serotec, Oxford, OX5 1JE, U.K.) or IgA (Serotec) (1:1,000 in PBS containing 1% BSA) was used as a secondary antibody. Color was developed by adding an ABTS substrate solution (Bio-Rad Co.) to the wells. The absorbency at 405 nm was then measured using an ELISA reader (Molecular Device Co., Sunnyvale, CA, U.S.A.). The specific IgG and IgA titers were presented as the reciprocal of the highest dilutions that yielded a 4-fold absorbency compared with that of the nonimmune samples.

Challenge with *B. bronchiseptica*

A challenge with *B. bronchiseptica* was performed 10 days after the final immunization. One pig per group was euthanized for necropsy prior to the challenge. This specimen was used as the negative control to compare any gross pathological changes in the turbinate bone. The remaining pigs in the experimental groups were all challenged intranasally with a dose (1.5×10^9 CFU/ml) of a *B. bronchiseptica* field isolate obtained from a pig with AR. The pigs were monitored for one week thereafter.

Clinical Evaluation

The pigs were evaluated for seven days to assess any respiratory disease after the challenge with *B. bronchiseptica*. Various clinical signs were marked as 1 if present and 0 if absent, including sneezing, epistaxis, nasal discharge, and discolored patches around the eyes due to ocular discharge.

Turbinate Score

On day 7 following the challenge, the pigs were humanely euthanized and all the snouts transversely sectioned in the first premolar tooth region. Any turbinate lesions in the rostral face were subjectively scored through gross examination by a group of collaborative investigators. Each snout was visually scored based on a previously reported method [10]. Left and right turbinate atrophy and deviation of the nasal septum were all graded separately on a scale of 0 (normal) to 3 (complete atrophy). Normal turbinates received a grade of 0; slight, yet obvious atrophy was graded as 1; moderate atrophy of not less than half the turbinates, especially the dorsal and ventral scrolls, was graded as 2; and severe atrophy of the dorsal and ventral scrolls was graded as 3. No, slight, moderate, and severe deviations of the septum were graded from 0 to 3, respectively. The three scores for each snout were then added together and divided by 3 to determine the final visual score for each pig, ranging from 0 to 3. The group means were also calculated.

Statistical Analysis

Statistical differences in specific immune responses between the groups were analyzed using a two-tailed, non-paired Student's *t* test, assuming an unequal variance. Differences in the post challenge clinical evaluation between the groups were evaluated using a two-tailed Fisher's exact test. Differences were considered significant if probability values of $p<0.05$ were obtained. (SPSS software; SPSS Inc., Chicago, IL, U.S.A.).

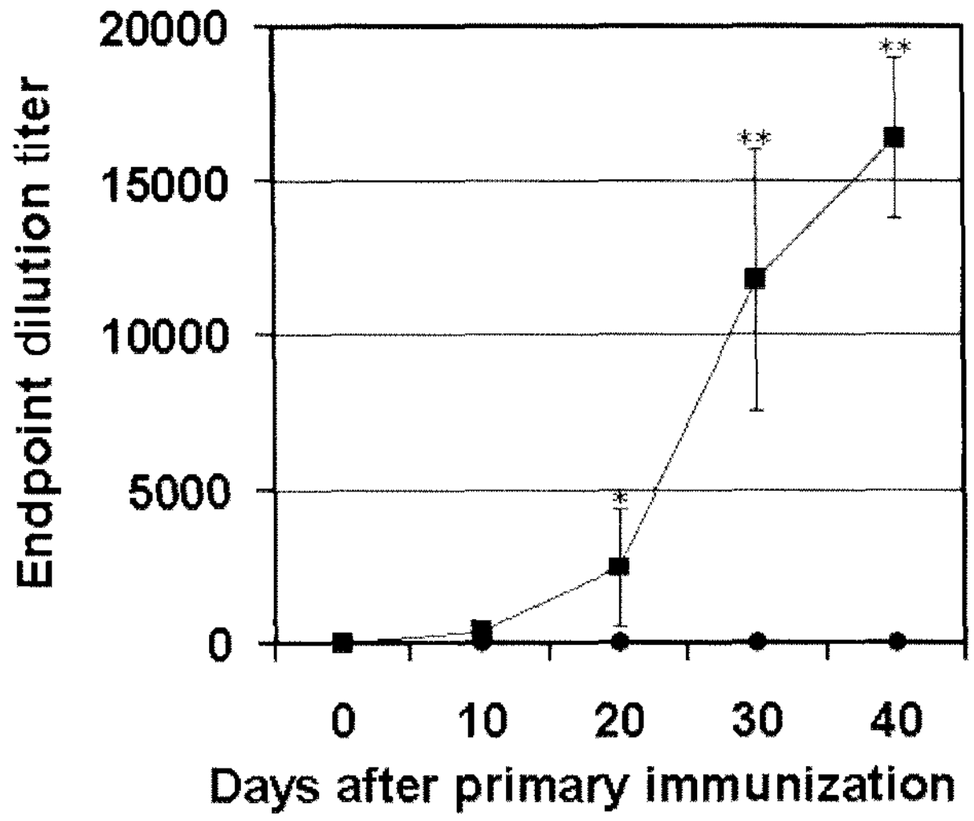

Fig. 1. Anti-BBD IgA levels in nasal wash.
Nasal administration of BBD-CMs (■) and nontreated control group (●). The significant difference between the control group and the vaccinated group was expressed as * $p<0.001$ and ** $p<0.05$.

RESULTS

BBD-Specific IgA in Mucosal Secretion

Pigs immunized intranasally with BBD-CMs showed a higher BBD-specific IgA response in the nasal wash than the control group (Fig. 1). The specific IgA antibody responses in the nasal secretions from the vaccinated pigs increased after the third immunization and showed a

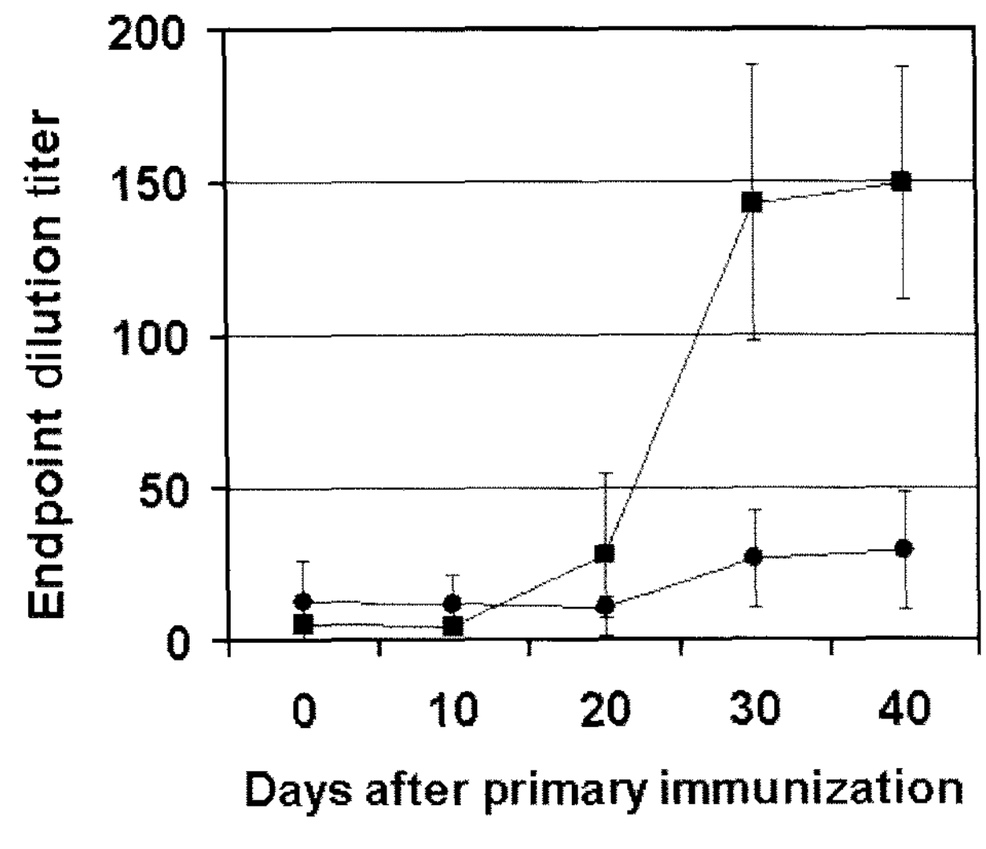

Fig. 2. Anti-BBD IgA levels in saliva.
Nasal administration of BBD-CMs (■) and nontreated control group (●). No significant difference was expressed between the control group and the vaccinated group.

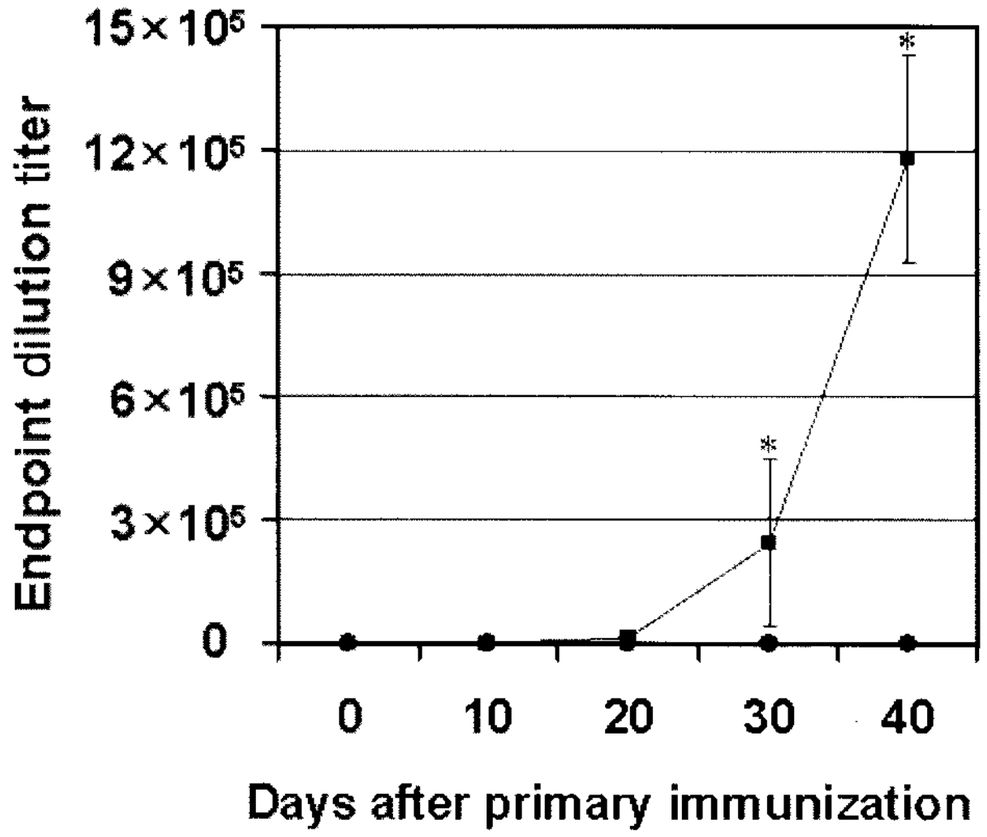

Fig. 3. Anti-BBD IgG levels in serum.
Nasal administration of BBD-CMs (■) and nontreated control group (●). The significant difference between the control group and the vaccinated group was expressed as * $p<0.05$.

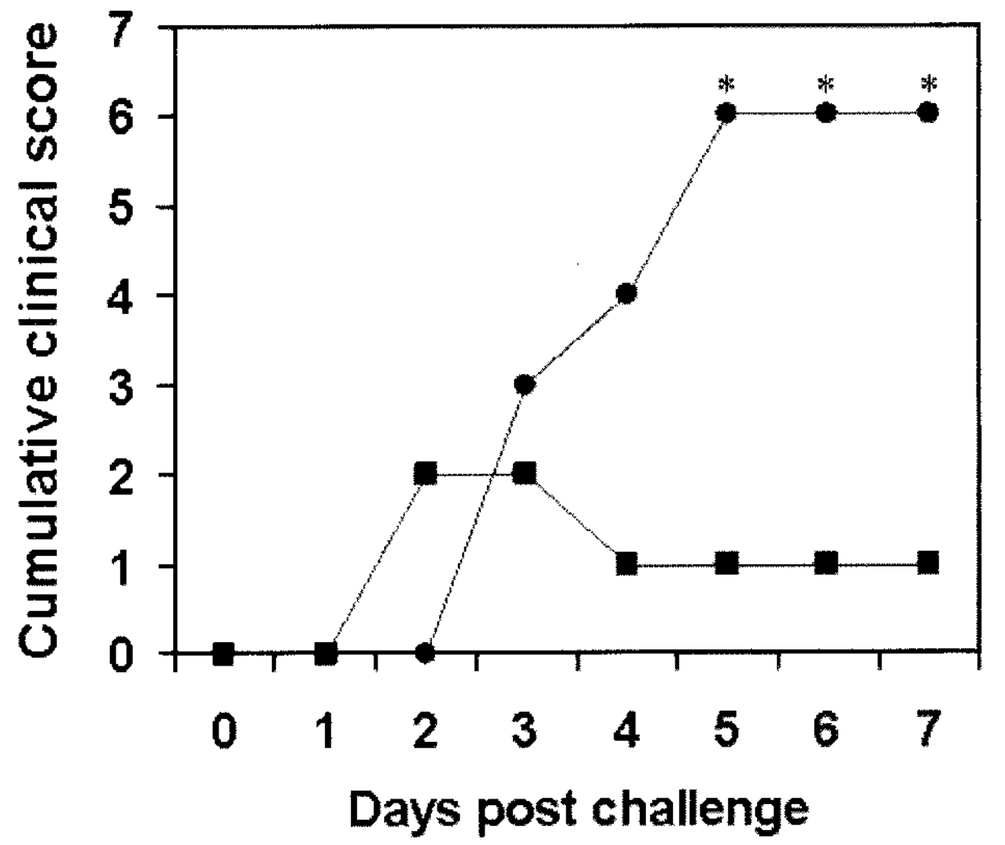

Fig. 4. Clinical scores for experimental pigs.
Clinical scores after intranasal challenge with a field isolate of *B. bronchiseptica* estimated according to reference scores in Table 1. Nasal administration of BBD-CMs (■) and nontreated control group (●). The significant difference between the clinical scores for the vaccinated pigs and nonvaccinated pigs was expressed as * $p<0.001$ (using two-tailed Fisher's exact test).

significant difference with those from the control pigs ($p<0.05$). No specific IgG antibody was detected in the nasal wash from any of the experimental groups. The salivary IgA responses to BBD in the pigs immunized intranasally with the BBD-CMs increased after the fourth immunization (Fig. 2). However, the specific IgA titers in the saliva samples from the control and vaccinated groups showed no significant difference, even after the final immunization.

BBD-Specific Antibody in Serum

The systemic anti-BBD IgG antibody titers for the group receiving the BBD-CMs increased in a time-dependent manner (Fig. 3). After the fourth immunization, the highest anti-BBD IgG titers were observed in the sera from the vaccinated pigs. In the serum samples following the fourth immunization, the specific IgG titers for the group that received the BBD-CMs were significantly higher than those for the control group ($p<0.05$). No anti-BBD IgA antibody responses were detected in the sera from any of the experimental pigs from the initial immunization to the final immunization.

Clinical Evaluation

A summary of the clinical scores is presented in Fig. 4. The group immunized with the BBD-CMs showed lower clinical scores than the control group ($p<0.001$). The health condition of the pigs in the control group gradually declined following the challenge, whereas the vaccinated pigs remained healthy. In the group immunized with the BBD-CMs, sneezing was rare and only detected in one pig two days after the challenge. However, three pigs in the nontreated control group continued sneezing for three to seven days following the challenge. One of these pigs also had a nasal hemorrhage that was discharged five days after the challenge. No epistaxis or nasal discharge was observed in any of the pigs that received intranasal administration of the BBD-CMs.

Turbinate Scores

The mean atrophy scores recorded for the nasal turbinates are shown in Fig. 5. The scores for the vaccinated pigs were lower than that for the nonvaccinated pigs. The average score for turbinate atrophy in the vaccinated group was 0.50±0.28. In contrast, the nonvaccinated pigs showed mild to severe turbinate atrophy with an average score of 1.39±0.82, which was significantly different from that for the BBD-CM vaccinated group ($p<0.001$). Some pigs in the control group showed severe atrophy of the turbinate, especially the dorsal and ventral scrolls, but not the septum (Fig. 5C).

DISCUSSION

The induction of immune responses in the nasal mucosa, the first site of respiratory tract infection, is a crucial step for a swine AR vaccine. Although several reports have already investigated the immunogenicity of intranasally administered AR vaccines in pigs [6, 27], the induction of a specific immune response in the nasal mucosa has not been demonstrated, as the delivery of antigens by the mucosal route invariably results in a poor immune response. Such results have been attributed to several factors, including the limited diffusion of macromolecules across

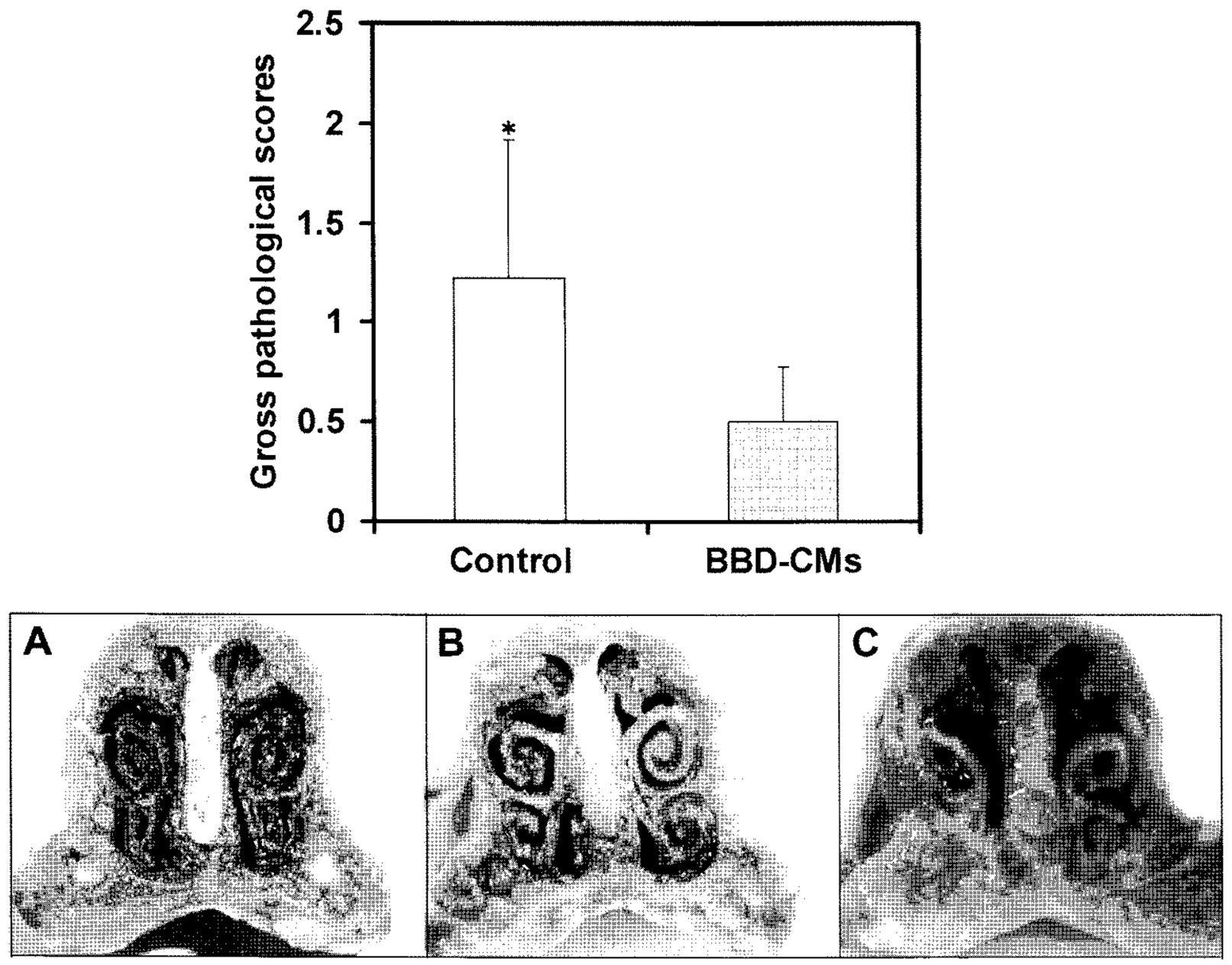

Fig. 5. Gross pathological scores for turbinate from experimental pigs.
The grade scores for gross morphological changes in the turbinate bones after the intranasal challenge with *B. bronchiseptica* were estimated according to a grade from 0 (normal) to 3 (complete atrophy). Left and right turbinate atrophy and deviation of the nasal septum were all graded separately. The graph indicates the mean of the three scores for the turbinate bone from each pig. The significant difference between the control group and the vaccinated group was expressed as * $p<0.001$. The photographs are cross-sections of turbinate bone tissue from (**A**) a nonchallenged pig, (**B**) vaccinated/challenged pig, and (**C**) nonvaccinated/challenged pig.

the mucosal barrier [7], rapid mucociliary clearance of drug formulations [30], and the presence of enzymatic activity [29]. Thus, to overcome this problem, the present study used CMs as an adjuvant-carrier system for the intranasal administration of BBD in pigs.

As mucosally induced antibodies are specifically transported through epithelial cells into bodily secretions, including mucus, tears, and saliva, the mucosal immune response was determined based on measurements of these bodily secretions [20]. Thus, the induction of specific antibodies into the turbinate mucosa would seem to be an essential step for protection against AR. The present results showed a significant induction of BBD-specific IgA in the nasal wash after the intranasal administration of BBD-CMs (Fig. 1), demonstrating that the intranasal administration of BBD-CMs was able to induce mucosal immune responses in the nasal cavity of pigs. However, no significant salivary antibodies were induced by the same vaccination schedule (Fig. 2). Nonetheless, the measurement of antibodies in secretions can be difficult to standardize and assay owing to discrepancies in the sample processing, methods of collection, stimulation of secretion, varying viscosities, and presence of contaminants, including enzymes and desquamated cells [2, 3]. The present experiment also had many variables, including high dilution factors, rough sample collection, no stimulation, and a diversity in the origin of the saliva, which may have affected the measurements.

Whereas parenterally administered vaccines mainly stimulate systemic responses, vaccines administered by a mucosal route can lead to both efficient mucosal and systemic immune responses [5, 19, 23, 34], as observed in the present experiment based on a significant induction of specific IgG antibody responses (Fig. 3).

The turbinate atrophy concomitant with AR has been suggested as a good indicator of vaccine efficacy against AR infection [9, 10]. Therefore, this study evaluated the gross morphological changes in the turbinates from the pigs immunized with BBD-CMs after being challenged with a field isolate of *B. bronchiseptica.* The turbinate conchal atrophy after the challenge with *B. bronchiseptica* was reduced by the BBD-CM vaccination (Fig. 5) ($p<0.001$). A similar reduction was also observed when comparing the clinical signs of AR after the challenge with *B. bronchiseptica* (Fig. 4). Protective antibodies in the mucosal tissue are usually only generated following mucosal immunization. Mucosal tissues act as the common gate of

entry for most pathogenic organisms [19]. Therefore, it is suggested that intranasal vaccination is appropriate for inducing protective immunity in the nasal mucosa, especially in the case of *B. bronchiseptica* infection.

In conclusion, the successful induction of mucosal immune responses was observed in the nasal mucosa, the main site of AR infection, after intranasal administration of BBD-CMs in pigs. Therefore, the present results demonstrated that BBD-CMs can induce significant mucosal immune responses following intranasal vaccination in pigs. In future research, larger numbers of experimental pigs, the addition of other control groups, especially commercial AR vaccines, larger scale production, and experimentation on field conditions will all contribute to a more effective evaluation of this AR vaccine.

Acknowledgments

This study was supported by MAFF special grants (202129-3), KRF (2006-005-J02901), BK21, and the Research Institute for Veterinary Science, Seoul National University, Korea.

References

1. Aksungur, P., A. Sungur, S. Unal, A. B. Iskit, C. A. Squier, and S. Senel. 2004. Chitosan delivery systems for the treatment of oral mucositis: *In vitro* and *in vivo* studies. *J. Control. Release* **98:** 269–279.
2. Bokor-Bratic, M. 2000. Clinical significance of analysis of immunoglobulin A levels in saliva. *Med. Pregl.* **53:** 164–168.
3. Challacombe, S. J. 1995. Assessing mucosal humoral immunity. *Clin. Exp. Immunol.* **100:** 181–182.
4. Chanter, N., T. Magyar, and J. M. Rutter. 1989. Interactions between *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs. *Res. Vet. Sci.* **47:** 48–53.
5. Davis, S. S. 2001. Nasal vaccines. *Adv. Drug Deliv. Rev.* **51:** 21–42.
6. de Jong, M. F. 1987. Prevention of atrophic rhinitis in piglets by means of intranasal administration of a live non-AR-pathogenic *Bordetella bronchiseptica* vaccine. *Vet. Q.* **9:** 123–133.
7. Donovan, M. D., G. L. Flynn, and G. L. Amidon. 1990. Absorption of polyethylene glycols 600 through 2000: The molecular weight dependence of gastrointestinal and nasal absorption. *Pharm. Res.* **7:** 863–868.
8. Foged, N. T. 1992. *Pasteurella multocida* toxin. The characterization of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. *APMIS Suppl.* **25:** 1–56.
9. Francisco, C. J., T. R. Shryock, D. P. Bane, and L. Unverzagt. 1996. Serum haptoglobin concentration in growing swine after intranasal challenge with *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* type D. *Can. J. Vet. Res.* **60:** 222–227.
10. Gatlin, C. L., W. H. Jordan, T. R. Shryock, and W. C. Smith. 1996. The quantitation of turbinate atrophy in pigs to measure the severity of induced atrophic rhinitis. *Can. J. Vet. Res.* **60:** 121–126.
11. Gavini, E., A. B. Hegge, G. Rassu, V. Sanna, C. Testa, G. Pirisino, J. Karlsen, and P. Giunchedi. 2005. Nasal administration of carbamazepine using chitosan microspheres: *In vitro/in vivo* studies. *Int. J. Pharm.* **307:** 9–15.
12. He, P., S. S. Davis, and L. Illum. 1998. *In vitro* evaluation of the mucoadhesive properties of chitosan microspheres. *Int. J. Pharm.* **166:** 75–88.
13. Jacques, M., N. Parent, and B. Foiry. 1988. Adherence of *Bordetella bronchiseptica* and *Pasteurella multocida* to porcine nasal epithelial cells. *Can. J. Vet. Res.* **52:** 283–285.
14. Jiang, H. L., I. K. Park, N. R. Shin, S. G. Kang, H. S. Yoo, S. I. Kim, S. B. Suh, T. Akaike, and C. S. Cho. 2004. *In vitro* study of the immune stimulating activity of an atrophic rhinitis vaccine associated to chitosan microspheres. *Eur. J. Pharm. Biopharm.* **58:** 471–476.
15. Kang, M. L., S. G. Kang, H. L. Jiang, S. W. Shin, D. Y. Lee, J. M. Ahn, N. Rayamahji, I. K. Park, S. J. Shin, C. S. Cho and H. S. Yoo. 2006. *In vivo* induction of mucosal immune responses by intranasal administration of chitosan microspheres containing *Bordetella bronchiseptica* DNT. *Eur. J. Pharm. Biopharm.* **63:** 215–220.
16. Kang, S. W., E. R. Cho, and B. S. Kim. 2005. PLGA microspheres in hyaluronic acid gel as a potential bulking agent for urologic and dermatologic injection therapies. *J. Microbiol. Biotechnol.* **15:** 510–518.
17. Kim, Y. H., K. Y. Cheong, W. S. Shin, S. Y. Hong, H. J. Woo, and M. S. Kwon. 2006. Immunological characterization of full and truncated recombinant clones of *ompH(D:4)* obtained from *Pasteurella multocida* (D:4) in Korea. *J. Microbiol. Biotechnol.* **16:** 1529–1536.
18. Larsen, D. L., A. Karasin, F. Zuckermann, and C. W. Olsen. 2000. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. *Vet. Microbiol.* **74:** 117–131.
19. McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: From fundamental concepts to vaccine development. *Vaccine* **10:** 75–88.
20. Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.* **7:** 265–276.
21. Nakai, T., K. Kume, H. Yoshikawa, T. Oyamada, and T. Yoshikawa. 1988. Adherence of *Pasteurella multocida* or *Bordetella bronchiseptica* to the swine nasal epithelial cell *in vitro*. *Infect. Immun.* **56:** 234–240.
22. Park, I. K., H. L. Jiang, C. H. Yun, Y. J. Choi, S. J. Kim, T. Akaike, S. I. Kim, and C. S. Cho. 2004. Release of Newcastle disease virus vaccine from chitosan microspheres *in vitro* and *in vivo*. *Asian Austral. J. Anim. Sci.* **17:** 543–547.
23. Partidos, C. D. 2000. Intranasal vaccines: Forthcoming challenges. *Pharm. Sci. Technolo. Today* **3:** 273–281.
24. Pedersen, K. B. and F. Elling. 1984. The pathogenesis of atrophic rhinitis in pigs induced by toxigenic *Pasteurella multocida*. *J. Comp. Pathol.* **94:** 203–214.
25. Pereswetoff-Morath, L. 1998. Microspheres as nasal drug delivery system. *Adv. Drug Deliv. Rev.* **29:** 185–194
26. Rutter, J. M. 1985. Atrophic rhinitis in swine. *Adv. Vet. Sci. Comp. Med.* **29:** 239–279.

27. Sakano, T., K. Sakurai, T. Furutani, and T. Shimizu. 1984. Immunogenicity and safety of an attenuated *Bordetella bronchiseptica* vaccine in pigs. *Am. J. Vet. Res.* **45:** 1814–1817.
28. Sankar, C., M. Rani, A. K. Srivastava, and B. Mishra. 2001. Chitosan based pentazocine microspheres for intranasal systemic delivery: Development and biopharmaceutical evaluation. *Pharmazie* **56:** 223–226.
29. Sarkar, M. A. 1992. Drug metabolism in the nasal mucosa. *Pharm. Res.* **9:** 1–9.
30. Schipper, N. G. M., J. C. Verhoef, and F. Merkus. 1991. The nasal mucociliary clearance: Relevance to nasal drug delivery. *Pharm. Res.* **8:** 807–814.
31. Senel, S., M. J. Kremer, S. Kas, P. W. Wertz, A. A. Hincal, and C. A. Squier. 2000. Enhancing effect of chitosan on peptide drug delivery across buccal mucosa. *Biomaterials* **21:** 2067–2071.
32. Shin, N. R., I. S. Choi, J. M. Kim, W. Hur, and H. S. Yoo. 2002. Effective methods for the production of immunoglobulin Y using immunogens of *Bordetella bronchiseptica*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. *J. Vet. Sci.* **3:** 47–57.
33. To, H., S. Someno, and S. Nagai. 2005. Development of a genetically modified nontoxigenic *Pasteurella multocida* toxin as a candidate for use in vaccines against progressive atrophic rhinitis in pigs. *Am. J. Vet. Res.* **66:** 113–118.
34. Ugwoke, M. I., R. U. Agu, N. Verbeke, and R. Kinget. 2005. Nasal mucoadhesive drug delivery: Background, applications, trends and future perspectives. *Adv. Drug Deliv. Rev.* **57:** 1640–1665.
35. Varshosaz, J., H. Sadrai, and R. Alinagari. 2004. Nasal delivery of insulin using chitosan microspheres. *J. Microencapsul.* **21:** 761–774.