Original Article
J Vet Sci 2017, 18(3), 327-332 · https://doi.org/10.4142/jvs.2017.18.3.327

Bordetella bronchiseptica antigen enhances the production of *Mycoplasma hyopneumoniae* antigen-specific immunoglobulin G in mice

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We previously demonstrated that *Bordetella* (*B.*) *bronchiseptica* antigen (Ag) showed high immunostimulatory effects on mouse bone marrow cells (BMs) while *Mycoplasma* (*M.*) *hyopneumoniae* Ag showed low effects. The focus of this study was to determine if *B. bronchiseptica* Ag can enhance the *M. hyopneumoniae* Ag-specific immune response and whether the host's immune system can recognize both Ags. MTT assay results revealed that each or both Ags did not significantly change BM metabolic activity. Flow cytometry analysis using carboxyfluorescein succinimidyl ester showed that *B. bronchiseptica* Ag can promote the division of BMs. In cytokine and nitric oxide (NO) assays, *B. bronchiseptica* Ag boosted production of tumor necrosis factor-alpha in *M. hyopneumoniae* Ag-treated BMs, and combined treatment with both Ags elevated the level of NO in BMs compared to that from treatment of *M. hyopneumoniae* Ag alone. Immunoglobulin (Ig)G enzyme-linked immunosorbent assay using the sera of Ag-injected mice clearly indicated that *B. bronchiseptica* Ag can increase the production of *M. hyopneumoniae* Ag-specific IgG. This study provided information valuable in the development of *M. hyopneumoniae* vaccines and showed that *B. bronchiseptica* Ag can be used both as a vaccine adjuvant and as a vaccine Ag.

Keywords: *Bordetella bronchiseptica*, *Mycoplasma hyopneumoniae*, antigen-specific immune response, bone marrow cells, vaccine adjuvant

Introduction

Mycoplasma (*M.*) *hyopneumoniae* is a causative pathogen of swine enzootic pneumonia, leading to significant economic loss in the swine industry [11]. It is widely distributed and occurs in a range of 38%–100% of pigs in swine farms. Although a variety of *Mycoplasma* vaccines have been developed for disease prevention, their effects are not fully satisfactory [6]. *Bordetella* (*B.*) *bronchiseptica* is another main causative pathogen of the swine respiratory disease atrophic rhinitis [7]. Infection with *B. bronchiseptica* alone does not seriously damage of respiratory system; however, a combined infection with other pathogens, such as *Pasteurella multocida*, does result in serious respiratory damage [4], leading to loss of weight and associated economic loss [15]. Thus, a vaccine for *B. bronchiseptica* was developed [5].

Our previous report demonstrated that *M. hyopneumoniae* antigen (Ag) has low immunostimulatory activity, although it did induce a T helper 1-type immune response [13]. In contrast, *B. bronchiseptica* Ag had high immunostimulatory activity and increased cytokine production [14]. These results led us to speculate that both Ags could be combined into a single vaccine if the *B. bronchiseptica* Ag was able to enhance the production of *M. hyopneumoniae* Ag-specific immunoglobulin (Ig)G. It has been widely reported that adjuvants, such as alum and MF59, can enhance the immunogenicity of vaccine Ag [1,3]. Generally, bacteria-derived Ags are not used as a vaccine adjuvant, and their toxic effects have limited their use in the development of vaccines; one example of a bacterial adjuvant is Bacillus Calmette-Guérin (BCG) [12].

In this study, we investigated whether *B. bronchiseptica* Ag can enhance the *M. hyopneumoniae* Ag-specific immune responses and if the host's immune system can recognize both Ags.

Received 4 May 2016, Revised 5 Aug. 2016, Accepted 22 Sep. 2016
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pISSN 1229-845X
eISSN 1976-555X

Materials and Methods

Animals and reagents

C57BL/6 and BALB/c mice were purchased from ORIENT BIO (Korea) and were maintained at our animal facility. Female mice of both strains, 7- to 12-weeks-old were used in all experiments. Animal experiments were performed in accordance with the Institutional Guideline for Animal Use and Care of Jeju National University (approval No. 2013-0008). The *M. hyopneumoniae* and *B. bronchiseptica* were obtained from CAVAC and KBNP (both in Korea), respectively. Both bacteria were inactivated by treating with formalin and were used as Ags. Following inactivation, they were washed with phosphate-buffered saline (PBS) before use. The amount of protein was measured by performing protein assays (Bio-Rad Laboratories, USA) with the results based on a standard curve derived using bovine serum albumin (Sigma-Aldrich, USA).

Preparation of bone marrow cells

Bone marrow cells (BMs) were obtained from the femur and tibia of mice by flushing. To remove red blood cells, BMs were treated with a hypotonic lysis buffer (ammonium chloride-potassium lysis buffer containing 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA). The cells were filtered through a cell strainer (cut-off: 40 μm) to obtain single cells. The single BMs were then cultured in 5% complete medium (RPMI 1640 medium containing 5% fetal bovine serum, 100 IU/mL penicillin/streptomycin, 2 mM L-glutamine).

Measurement of the BM metabolic activity

For measurement of BM metabolic activity, BMs were cultured in 96-well culture plates at a concentration of 1×10^6 cells/mL (200 μL/well) and treated with the indicated concentrations of Ags. After treatment, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) solution was added at a concentration of 0.5 mg/mL. Subsequently, viable cells generated purple-colored crystals in proportion to their metabolic activity, and 10% (w/v) sodium dodecyl sulfate (Sigma-Aldrich) solution was used to dissolve the crystals. The optical density of the solution was measured at 570 nm by a microplate reader (Multiskan FC; Thermo Fisher Scientific, USA). To investigate whether two Ags affected cellular proliferation, we undertook a proliferation assay using carboxyfluorescein succinimidyl ester (CFSE). The cells were stained 5 μM CFSE and incubated with *M. hyopneumoniae* Ag and *bronchiseptica* Ag. After incubation, the cells were analyzed by using FACSCalibur (Beckton Dickinson, USA) and Flowing Software (Finland).

Determination of cytokines and nitric oxide (NO) production

BMs were cultured in 96-well culture plates at a concentration of 1×10^6 cells/mL (200 μL/well) for cytokine or 2×10^6 cells/mL (200 μL/well) for NO assay. Culture supernatants of the Ag-treated cells were harvested and used in both enzyme-linked immunosorbent assay (ELISA) and NO assay. The amount of tumor necrosis factor (TNF)-α in the culture supernatants was measured by using CytoSet kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Optical density was measured at 450 nm by using a microplate reader. The amount of NO in the culture supernatants was measured by using an NO detection kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Optical density was measured at 570 nm by using a microplate reader.

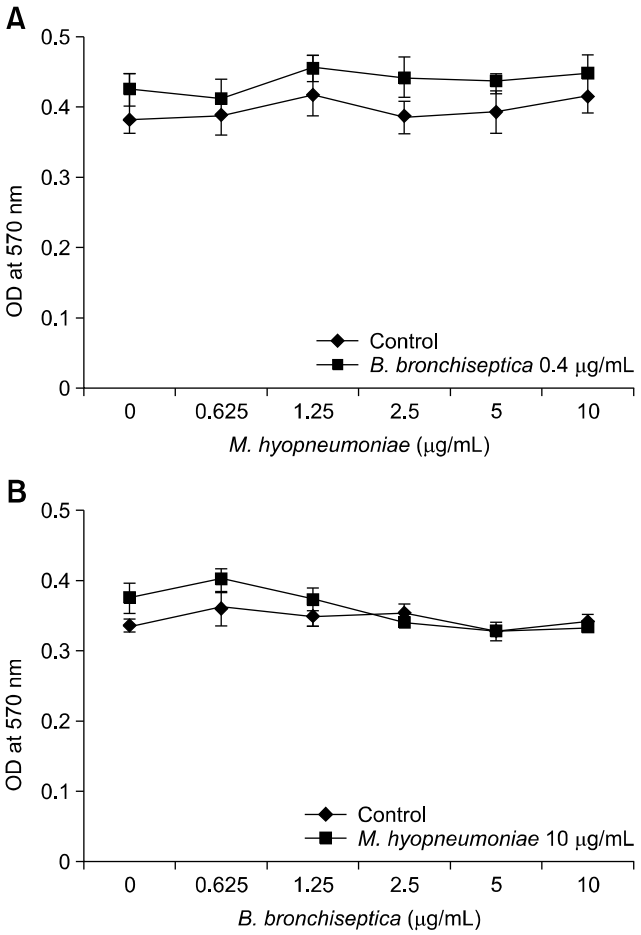

Fig. 1. The effect of *Bordetella* (*B.*) *bronchiseptica* Ag and *Mycoplasma* (*M.*) *hyopneumoniae* antigen (Ag) on the cell metabolic activity of bone marrow cells (BMs). BMs were cultured for 4 days with 0 to 10 μg/mL *M. hyopneumoniae* Ag and 0.4 μg/mL *B. bronchiseptica* Ag (A) or with 0 to 10 μg/mL *B. bronchiseptica* Ag and 10 μg/mL *M. hyopneumoniae* Ag (B). MTT solution was treated at a concentration of 0.5 mg/mL. The optical density (OD) was measured at 570 nm by using a microplate reader. All values are presented as mean ± SD values from four individual wells.

Injection of *M. hyopneumoniae* Ag and *B. bronchiseptica* Ag into mice

To measure the effects of *M. hyopneumoniae* Ag and *B. bronchiseptica* Ag, mice were divided into three groups (each n = 5): PBS, *M. hyopneumoniae* Ag only, and *M. hyopneumoniae* Ag + *B. bronchiseptica* Ag groups. Mice were intraperitoneally injected twice with an interval of 2 weeks between injections. The amounts injected were 10 μg/mouse of *M. hyopneumoniae* Ag and 5 μg/mouse of *B. bronchiseptica* Ag. Two weeks after the second injection, mice were sacrificed and used in subsequent experiments.

Determination of Ag-specific IgG in mouse sera

Mouse blood was collected by cardiac puncture after CO_2 euthanasia. After generation of a blood clot, serum was prepared by using a microcentrifuge and serial 5-fold dilution. The detection of Ag-specific serum IgG was carried out by ELISA. Briefly, *M. hyopneumoniae* Ag or *B. bronchiseptica* Ag was coated on an ELISA module at a concentration of 5 μg/mL. An assay buffer (Thermo Fisher Scientific) for blocking, as well as goat anti-mouse IgG-horseradish peroxidase and tetramethylbenzidine solution (both from SouthernBiotech, USA) were sequentially used in the ELISA detection of IgG. Optical density was measured at 450 nm by using a microplate reader.

Statistical analysis

Data in graphs are presented as mean ± SD values. Statistical analysis was performed by performing one-way ANOVA followed by Tukey-Kramer multiple comparisons test in InStat software (V2, GraphPad Software, USA). A p value of < 0.05 was considered significant.

Results

Effect of *B. bronchiseptica* Ag on BM metabolic activity and proliferation

In order to determine whether *B. bronchiseptica* Ag or *M. hyopneumoniae* Ag affects the cell metabolic activity of BMs, we performed MTT assays. After 4 days of treatment with 0 to 10 μg/mL *M. hyopneumoniae* Ag and 0.4 μg/mL *B. bronchiseptica* Ag, BM metabolic activity was measured (panel A in Fig. 1). In another treatment set, BMs were treated with 0 to 10 μg/mL *B. bronchiseptica* Ag and 10 μg/mL *M. hyopneumoniae* Ag (panel B in Fig. 1). The results showed that the concentration or presence of *M. hyopneumoniae* Ag and *B. bronchiseptica* Ag did not markedly alter the cell metabolic activity of BMs. Flow

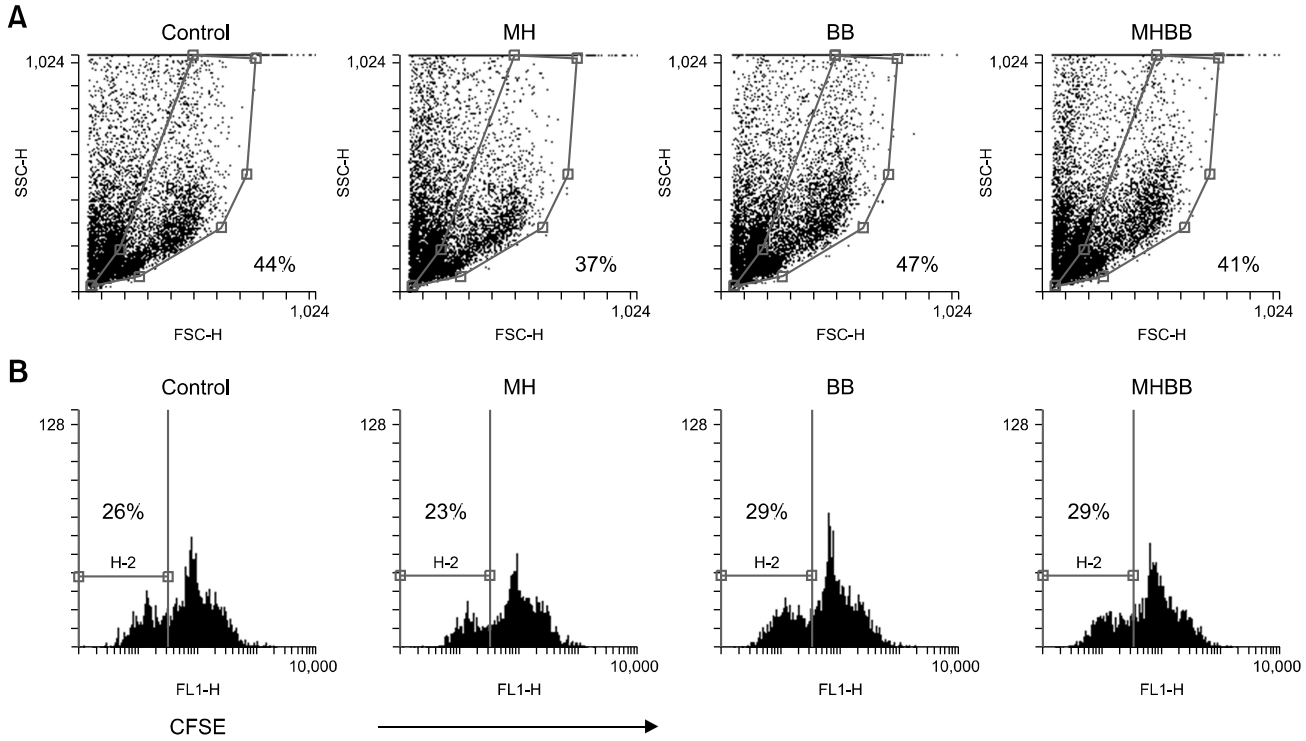

Fig. 2. *Bordetella* (*B.*) *bronchiseptica* antigen (Ag) enhances the proliferation of bone marrow cells (BMs). Carboxyfluorescein succinimidyl ester (CFSE)-stained BMs were treated with 5 μg/mL *Mycoplasma* (*M.*) *hyopneumoniae* Ag and 1 μg/mL *B. bronchiseptica* Ag for 4 days. Stained cells were analyzed by flow cytometry as described in Materials and Methods. The number in each dot plot or histogram indicates the percentage of normal cell size (A) or the percentage proliferating cells with low fluorescence intensity (B). MH, *M. hyopneumoniae* Ag-treated BMs; BB, *B. bronchiseptica* Ag-treated BMs; MHBB, both MH and BB Ags-treated BMs.

cytometry analysis revealed that *B. bronchiseptica* Ag marginally enhanced cell size (forward scatter/side scatter), but *M. hyopneumoniae* Ag did not (panel A in Fig. 2). In addition, *B. bronchiseptica* Ag increased the percentage of cells with low CFSE fluorescence intensity, indicating the presence of proliferating cells, whereas *M. hyopneumoniae* Ag did not show such an increase (panel B in Fig. 2).

B. bronchiseptica Ag enhances the production of TNF-α and NO in BMs

To investigate the functional immune effects of *B. bronchiseptica* Ag, TNF-α and NO production was examined. After 3 days of treatment with 0 to 10 μg/mL *M. hyopneumoniae* Ag with or without 1 μg/mL *B. bronchiseptica* Ag, the amount of TNF-α was measured. *M. hyopneumoniae* Ag treatment without *B. bronchiseptica* Ag did not produce a detectable amount of TNF-α, except at the 10 μg/mL treatment level. The presence of *B. bronchiseptica* Ag markedly increased the production of TNF-α, whereas *M. hyopneumoniae* Ag did not significantly alter the production level, except at the 2.5 μg/mL treatment level (Fig. 3). On the other hand, *M. hyopneumoniae* Ag enhanced NO production in a concentration-dependent manner. *M. hyopneumoniae* Ag (1.25−10 μg/mL) significantly enhanced NO production in the presence of *B. bronchiseptica* Ag. In addition, *B. bronchiseptica* Ag enhanced NO production in 0 to 2.5 μg/mL *M. hyopneumoniae* Ag treatments to a greater extent than with treatment by *M. hyopneumoniae* Ag alone (Fig. 4).

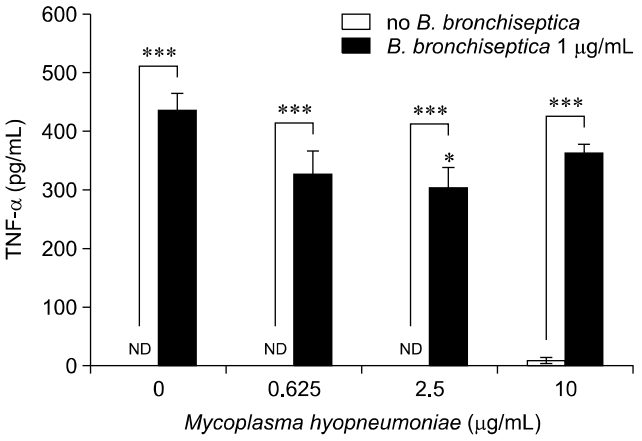

Fig. 3. *Bordetella* (*B.*) *bronchiseptica* antigen (Ag) promotes the production of tumor necrosis factor alpha (TNF-α) in bone marrow cells (BMs). After 3 days of treatment with 0 to 10 μg/mL *Mycoplasma* (*M.*) *hyopneumoniae* Ag and 1 μg/mL *B. bronchiseptica* Ag, supernatants of the treated BMs were collected and used for measurement of TNF-α. Optical density was measured at 450 nm by a microplate reader. All values are presented as mean ± SD values from four individual wells. ND, not detectable. $*p < 0.05$ compared to control (0 μg/mL *M. hyopneumoniae* Ag), $***p < 0.001$ compared between two treatments (with or without *B. bronchiseptica* Ag).

B. bronchiseptica Ag increases the level of *M. hyopneumoniae* Ag-specific serum IgG

To measure the level of *M. hyopneumoniae* Ag-specific IgG, 5 μg/mL *M. hyopneumoniae* Ag were coated on ELISA plates. Serum was 5-fold serially diluted and the level of Ag-specific IgG was detected by ELISA (panel A in Fig. 5). No detectable amount of *M. hyopneumoniae* Ag-specific IgG was measured in the serum of mice injected with PBS only. A significantly higher amount of *M. hyopneumoniae* Ag-specific IgG was detected in sera of mice injected with *M. hyopneumoniae* Ag (MH) or injected with *M. hyopneumoniae* Ag + *B. bronchiseptica* Ag (MHBB). Furthermore, the level of Ag-specific IgG with the MHBB injection was higher than that from the MH treatment. In addition, the level of *B. bronchiseptica* Ag-specific IgG was also measured in similar sera samples (panel B in Fig. 5B). A significant amount of *B. bronchiseptica* Ag-specific IgG was detected in MHBB-injected mice, but not in PBS- and MH-injected mice.

Discussion

To improve the immunogenicity of Ag, a variety of adjuvants can be used in the process of developing vaccines, and new vaccine adjuvants with high efficiency are in high demand. Bone marrow is the primary organ that produces premature lymphocytes and harbors antibody-producing cells with

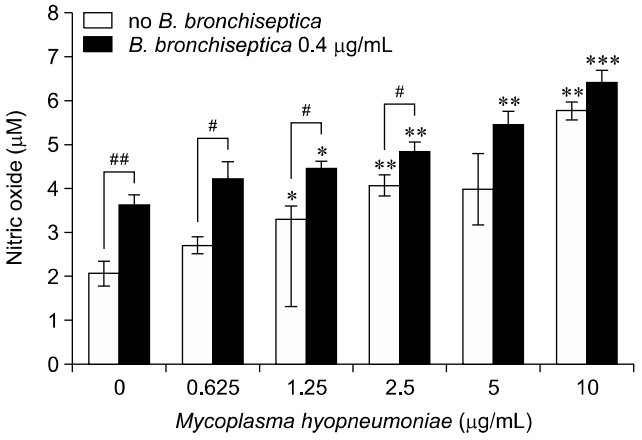

Fig. 4. *Mycoplasma* (*M.*) *hyopneumoniae* antigen (Ag) and *Bordetella* (*B.*) *bronchiseptica* Ag promote the production of nitric oxide (NO) in bone marrow cells (BMs). After 3 days of treatment with 0 to 10 μg/mL *M. hyopneumoniae* Ag and 0.4 μg/mL *B. bronchiseptica* Ag, supernatants of the treated BMs were collected and used to measure NO level. Optical density was measured at 570 nm by a microplate reader. All values are presented as mean ± SD values from four individual wells. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, respectively, compared to control (0 μg/mL *M. hyopneumoniae* Ag). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, respectively, compared between two treatments (with and without *B. bronchiseptica* Ag).

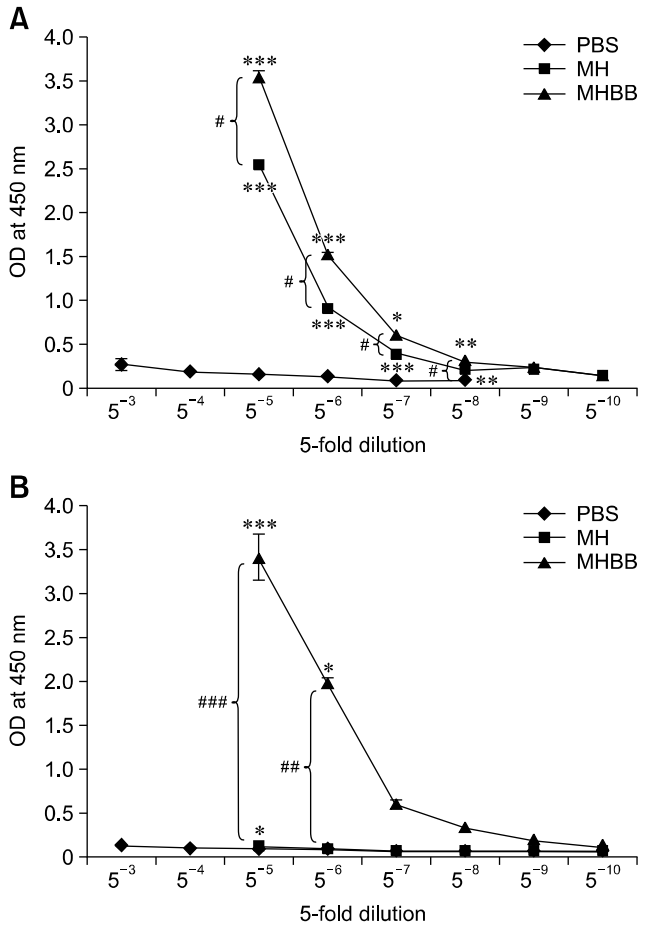

Fig. 5. *Bordetella* (*B.*) *bronchiseptica* antigen (Ag) treatment increases *Mycoplasma* (*M.*) *hyopneumoniae* Ag-specific immunoglobulin (Ig)G. Mice were injected with phosphate-buffered saline (PBS), *M. hyopneumoniae* Ag (10 μg/mouse), or *M. hyopneumoniae* Ag + *B. bronchiseptica* Ag (5 μg/mouse). After 4 weeks, blood was collected and sera were used for measuring Ag-specific IgG via enzyme-linked immunosorbent assay (ELISA). For this assay, the ELISA module was coated with 5 μg/mL *M. hyopneumoniae* Ag (A) or *B. bronchiseptica* Ag (B). The antibody titer was determined at an optical density (OD) of 450 nm. MH, *M. hyopneumoniae* Ag-treated BMs; BB, *B. bronchiseptica* Ag-treated BMs; MHBB, both MH and BB Ags-treated BMs. All values are presented as mean ± SD values from four individual wells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively, compared to the lowest serum concentration (5^{-10} dilution). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, respectively, compared between two treatments (MH and MHBB).

Ag-specific memory. Immune memory is a critical defense mechanism used to induce more powerful immune responses when the host's immune system is attacked a second time by the same pathogen. Long-lived memory B cells and plasma cells are essential to remove pathogens and act by producing high titers of Ag-specific antibodies [9], a major indicator of vaccine efficacy. For these reasons, we used BMs of mice in this study.

The MTT assay results revealed that treatment of *M. hyopneumoniae* Ag and/or *B. bronchiseptica* Ag did not significantly affect the cell metabolic activity of BMs. It is thus likely that these Ags are not significantly cytotoxic to BMs.

TNF-α is a major inflammatory cytokine secreted from macrophages that induces essential immune responses related to vaccine efficacy. Some Ags that induce a low production of TNF-α need adjuvants to induce a high level of immunostimulatory activity. ELISA revealed that *M. hyopneumoniae* Ag alone produced minimal amount of TNF-α, whereas *B. bronchiseptica* Ag increased the level when in a combination of *M. hyopneumoniae* Ag, suggesting that *B. bronchiseptica* Ag can boost the vaccine efficacy of *M. hyopneumoniae* Ag.

NO is an intracellular killing factor in cell-mediated-immunity and eliminates pathogenic microorganisms invading host cells [2,8]. To estimate the intracellular cytotoxic ability of BMs treated by both Ags, the amount of NO was determined in the culture supernatants. NO assay results revealed that the combined treatment of both Ags elevated the level of NO in BMs compared to that after treatment with *M. hyopneumoniae* Ag alone. Importantly, the effect of *B. bronchiseptica* Ag was high when it was combined with 0 to 2.5 μg/mL of *M. hyopneumoniae* Ag. The optimal concentration of *M. hyopneumoniae* Ag for NO production was 2.5 μg/mL.

CFSE is a cell-permeable, non-fluorescent pro-dye that is cleaved by intracellular esterase in living cells, and a decrease of intracellular CFSE fluorescence intensity indicates cell division [10]. In this study, the CFSE-stained cells were analyzed by flow cytometry, which revealed that the percentage of BMs with a regular cell size decreased following *M. hyopneumoniae* Ag treatment, but increased with *B. bronchiseptica* Ag treatment. In addition, the ratio of highly proliferating cells (*i.e.*, showing low fluorescence intensity) showed a similar pattern. Thus, it appears that *B. bronchiseptica* Ag can promote cell division in BMs.

Plasma cells differentiated from B lymphocytes produce IgG. Bone marrow contains both types of cells and is thus critical in maintaining humoral immunity. In particular, antibody-producing cells with long-term memory are essential for vaccine efficacy. To evaluate the effects of *B. bronchiseptica* Ag on the *M. hyopneumoniae* Ag-specific immune response, IgG ELISA was performed using the sera of Ag-injected mice. The level of *M. hyopneumoniae* Ag-specific IgG was enhanced by *B. bronchiseptica* Ag. In similar samples, *B. bronchiseptica* Ag-specific IgG was detected only in *B. bronchiseptica* Ag + *M. hyopneumoniae* Ag-injected mice. These results indicate that the IgG ELISA used in the present study was very Ag-specific.

In conclusion, neither *M. hyopneumoniae* Ag nor *B. bronchiseptica* Ag had cytotoxic effects on BMs. *M. hyopneumoniae* Ag slightly decreased the cell size and division, whereas *B. bronchiseptica* Ag reversed those decreases. *M.*

hyopneumoniae Ag-specific immune response was increased by *B*. *bronchiseptica* Ag, which was reflected by an increase in IgG production. This study provides valuable information related to developing vaccines for respiratory diseases, especially *M*. *hyopneumoniae*-involved diseases, and shows that *B*. *bronchiseptica* Ag can be used as both a vaccine adjuvant and a vaccine Ag. Further study on the development of a new vaccine adjuvant using *B*. *bronchiseptica* Ag is needed.

Acknowledgments

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (IPET) through Agri-Bio Industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (grant Nos. 111065-3 and 109014-03-1-CG000).

Conflict of Interest

The authors declare no conflict of interests.

References

1. **Audibert FM, Lise LD.** Adjuvants: current status, clinical perspectives and future prospects. Immunol Today 1993, **14**, 281-284.
2. **Avron A, Gallily R.** Mycoplasma stimulates the production of oxidative radicals by murine peritoneal macrophages. J Leukoc Biol 1995, **57**, 264-268.
3. **Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, De Gregorio E, Seubert A, Wack A.** Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine 2011, **29**, 1812-1823.
4. **Chanter N, Magyar T, Rutter JM.** Interactions between *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs. Res Vet Sci 1989, **47**, 48-53.
5. **Chi Y, Lu C, Han JH, Hahn TW.** [Efficacy of atropic rhinitis vaccine in pigs]. Korea J Vet Res 2004, **40**, 707-717. Korean.
6. **Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A.** Efficacy of vaccines against bacterial diseases in swine: what can we expect? Vet microbial 2004, **100**, 255-268.
7. **Horiguchi Y.** Swine atrophic rhinitis caused by pasteurella multocida toxin and bordetella dermonecrotic toxin. Curr Top Microbiol Immunol 2012, **361**, 113-129.
8. **MacMicking J, Xie QW, Nathan C.** Nitric oxide and macrophage function. Annu Rev Immunol 1997, **15**, 323-350.
9. **O'Connor BP, Gleeson MW, Noelle RJ, Erickson LD.** The rise and fall of long-lived humoral immunity: terminal differentiation of plasma cells in health and disease. Immunol Rev 2003, **194**, 61-76.
10. **Parish CR.** Fluorescent dyes for lymphocyte migration and proliferation studies. Immunol Cell Biol 1999, **77**, 499-508.
11. **Simionatto S, Marchioro SB, Maes D, Dellagostin OA.** *Mycoplasma hyopneumoniae*: from disease to vaccine development. Vet Microbiol 2013, **165**, 234-242.
12. **Wangoo A, Brown IN, Marshall BG, Cook HT, Young DB, Shaw RJ.** Bacille Calmette-Guérin (BCG)-associated inflammation and fibrosis: modulation by recombinant BCG expressing interferon-gamma (IFN-γ). Clin Exp Immunol 2000, **119**, 92-98.
13. **Yim SH, Joo HG.** [T helper 1-type immunogenicity of *Mycoplasma hyopneumoniae* antigen on mouse spleen cells]. J Biomed Res 2013, **14**, 55-59. Korean.
14. **Yim SH, Joo HG.** [Stimulatory effects of *Bordetella bronchiseptica* antigen on bone marrow cells and immune memory responses]. Korean J Vet Res 2014, **54**, 203-208. Korean.
15. **Zhao Z, Wang C, Xue Y, Tang X, Wu B, Cheng X, He Q, Chen H.** The occurrence of *Bordetella bronchiseptica* in pigs with clinical respiratory disease. Vet J 2011, **188**, 337-340.