Preventive effects of mycobacteria and their culture supernatants against asthma development in BALB/c mice

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Purpose: Live Mycobacterium bovis Bacille Calmette-Guérin (BCG) has a suppressive effect on asthma, but its use in clinical practice may be limited due to adverse reactions. To develop a product that is effective for suppressing asthma with minimal adverse reactions, we investigated whether the heat-killed body or culture supernatants of mycobacteria could also prevent asthma development. Methods: Female BALB/c mice were treated with live BCG, the heat-killed body, or culture supernatants of BCG or Mycobacterium tuberculosis intraperitoneally, while sensitizing and provoking with ovalbumin. Then they underwent a methacholine bronchoprovocation test, and the peribronchial inflammatory cell numbers and cytokine levels in splenocyte culture supernatants were assessed. Results: The airway sensitivity to methacholine decreased significantly after treatment with not only live BCG (30.8 versus 10.0 mg/mL, P<0.001) but also with the culture supernatant (BCG, 23.0 mg/mL, P<0.05; M. tuberculosis, 20.5 mg/mL, P<0.05). In contrast, heat-killed mycobacteria did not effectively decrease airway sensitivity. The peribronchial eosinophil counts and the goblet cell proportions in total epithelial cells decreased significantly in most of the groups. The interferon-γ/interleukin-5 ratios increased significantly in most of the treatment groups except for the heat-killed groups, and were significantly related to airway sensitivity (r=0.312, P<0.01) and peribronchial eosinophil counts (r=0.416, P<0.001). Interleukin-17A level was inversely related to airway sensitivity (r=-0.212, P<0.05) and was significantly lower in the live BCG group than in the control (137±20 versus 308±57 pg/mL, P<0.05). Conclusions: BCG and mycobacteria culture supernatants may effectively prevent the development of asthma associated with altered Th1/Th2 cytokines and interleukin-17A levels.

Key Words: Asthma; BCG vaccine; interferons; interleukins; mycobacterium

INTRODUCTION

The so-called “hygiene/old friends hypothesis” states that an increase in the prevalence of asthma/allergy is related to diminished exposure to infections and certain relatively harmless environmental organisms or components that the immune system has learned to tolerate.1 In this context, our group2 and other investigators3-7 have shown that Mycobacterium bovis Bacille Calmette-Guérin (BCG) and other mycobacterial infections suppress airway hyperresponsiveness and eosinophilic inflammation, likely through a T-helper 1 (Th1) or regulatory T cell (Treg) response. We also demonstrated that BCG vaccination has therapeutic effects on asthma in adult patients.8 However, Shirtcliffe et al.8 reported that repeated heat-killed BCG vaccinations had no beneficial effects, and they halted the trial early due to severe local reactions in asthma patients. Although the mycobacterial strains,9 route of delivery,10 and other factors might have affected the efficacy of the vaccinations, it is necessary to develop an effective anti-asthmatic agent devoid of adverse reactions.

Mycobacterial components rather than whole mycobacteria may be more promising to avoid untoward vaccination effects. The recognition of mycobacterial components by Toll-like receptor (TLR) 2 associated with TLR1/TLR6 and TLR4 on dendritic cells has been implicated in mycobacteria-induced Th1/Treg cell responses.12 In fact, the mycobacterial cell wall lipoglycans lipoarabinomannan (LAM) and phosphatidylinositol mannos suppress airway eosinophilia in association with increased interleukin (IL)-10 secretion by T cells.13 In addition, mannos-capped LAM from BCG induced Th1 cells,14 and that from M. tuberculosis induced Treg cells from human peripheral blood mononuclear cells.15 Synthetic lipopeptides that stimulate TLR2 decrease airway eosinophilia and IgE levels but in-
crease IL-10 and interferon (IFN)-γ production by T cells. Mycobacterial chaperonin 60.1 also suppresses airway eosinophilia and methacholine airway hypersensitivity. However, only a few studies using mycobacterial components in vaccinations have investigated their effects on asthma, and no study has compared the efficacy of various mycobacterial strains. Therefore, we investigated whether whole heat-killed mycobacteria and their products in the culture supernatants, from both \( M. \) tuberculosis and BCG, are effective for preventing the development of asthma.

**MATERIALS AND METHODS**

**Experimental animals**

Specific pathogen-free, 6-week-old female BALB/c mice (Dae-han BioLink, Eumsung, Korea) were fed standard mouse chow and given water ad libitum in an animal care room at Chonnam National University Medical School, Korea. Animal care and treatment followed the guidelines of the Chonnam National University Research Institute of Medical Sciences.

**Study design**

The mice were divided into 12 groups (n=10 per group): one normal control group, one asthma control group, four \( M. \) tuberculosis groups (heat-killed or culture supernatants), and six BCG groups (live, heat-killed, or culture supernatants; Fig. 1). On the first day of the study, the mice in the treatment groups were given 1×10⁵ or 1×10⁶ colony-forming units (CFUs) of mycobacteria, or 20 or 200 μg of culture supernatant intraperitoneally. Then, the mice were sensitized and provoked using ovalbumin, underwent a methacholine bronchial challenge test, and were sacrificed so that the inflammatory cell numbers in the peribronchial tissue and the cytokine levels in the supernatants of concanavalin A-stimulated splenocytes could be quantified.

**Preparation of mycobacteria and culture supernatants**

Pathogenic \( M. \) tuberculosis H37Rv (ATCC 27294) and \( M. \) bovis BCG Tokyo 172 strain (obtained from the Korean National Tuberculosis Association, Seoul) were cultured as surface pellicles on Sauton’s medium at 37°C. After 6 weeks, the bacilli were removed by filtration through filter paper. The culture supernatants were sequentially sterilized using a membrane filter (1.2- and 0.2-μm pore size) and concentrated by ultrafiltration (Amicon Centriprep-10, Millipore, Bedford, MA, USA). The concentration of culture filtrate antigen was determined using a bicinechonic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

To prepare the live bacterial stock, both mycobacteria were grown in roller bottles containing Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with 0.05% Tween 80 and 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson, Cockeysville, MD, USA) at 37°C until the optical density at 600 nm reached 0.8. The cells were washed three times with phosphate-buffered saline (PBS) and centrifuged at 150×g for 5 minutes to remove any clumps. Aliquots of the upper bacterial suspension were kept at ~80°C until use. The thawed bacterial aliquots used for infection were dispersed using a bath sonicator. The CFUs in the dispersed supernatants were determined by preparing serial dilutions in 7H9 broth and plating them onto Middlebrook 7H10 agar (Difco). To protect the laboratory workers from mycobacterial infection, the \( M. \) tuberculosis inoculum was prepared like those that were heated for 15 minutes. The culture supernatants, which contain most of the secreted antigens, were concentrated 10-fold for administration.

**Ovalbumin sensitization and provocation**

The animals were sensitized using two intraperitoneal injections of 200 μL PBS containing 20 μg ovalbumin (Grade V; Sigma-Aldrich, St. Louis, MO, USA) and 2.25 mg aluminum hydroxide (Imject Alum; Pierce) 2 weeks apart. One week after the second sensitization injection, the mice were provoked with 1% ovalbumin aerosol using an ultrasonic nebulizer (UltraNeb; DeVilbiss, Somersett, PA, USA) in an animal body plethysmograph (OCP3000; All Medicus, Ahnyang, Korea) for 30 minutes per day for three successive days.

**Lung function tests**

Airway responsiveness to methacholine (Sigma) was measured using a OCP3000 body plethysmograph, as described previously. Briefly, 24 hours after the final ovalbumin inhalation challenge, the animals inhaled PBS aerosol and then inhaled progressively doubled concentrations of methacholine, starting from 3.125 mg/mL and increasing to 50 mg/mL. Aerosols were generated using an Ultra-Neb nebulizer, and 3 mL of...
solution was aerosolized for 3 minutes. Enhanced pause (P_{enh}) was used to represent airway resistance, as described previously.\textsuperscript{10} The methacholine concentration required for a 200% increase in P_{enh} from the post-saline value (PC200) was obtained as an index of airway sensitivity.

**Histological analysis**

The left lung was fixed in 4% formalin and embedded in paraffin for histopathological analysis. The embedded tissue was sectioned every 4 \( \mu \text{m} \) and stained with hematoxylin-eosin and periodic acid-Schiff. Small airways (circumference <500 \( \mu \text{m} \)) were selected,\textsuperscript{18} and the numbers of eosinophils, lymphocytes, and neutrophils within the peribronchial area from the basement membrane to a depth of 100 \( \mu \text{m} \) were determined and expressed as the number of cells/mm\(^2\) using a Nikon microscope with a computerized image analyzer program (AnalySIS\textsuperscript{19} Pro; Soft Imaging System GmBH, Münster, Germany).\textsuperscript{19} Goblet cell hyperplasia was evaluated according to a 5-point scoring system (0-4), defined as follows: 0, no goblet cells; 1, <25%; 2, 25-50%; 3, 50-75%; and 4, \( \geq \)75%.\textsuperscript{20}

**Culture supernatant cytokine assays**

Splenocyte culture and cytokine measurements were performed as described previously.\textsuperscript{10} Briefly, splenocytes were cultured in RPMI 1640 (BioWhittaker, Walkersville, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and a 1% penicillin-streptomycin-amphotericin B mixture (BioWhittaker) and were stimulated with 2.5 \( \mu \text{g/mL} \) concanavalin A (Sigma) for 48 hours. The IFN-\( \gamma \), IL-5, and IL-17A concentrations were determined in the supernatant of stimulated splenocytes using commercial enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, Inc., San Diego, CA, USA). The sensitivities of the assays for IFN-\( \gamma \), IL-5, and IL-17A were 4, 8, and 7.8 pg/mL, respectively.

**Statistical analysis**

The PC200 was Log\(_10\) transformed before analysis, and all statistical results are expressed as the mean±SEM. The Kruskal-Wallis test and Mann-Whitney \( U \)-test were used to determine the significant differences between groups. Associations between variables were examined using Spearman’s rank correla-

![Fig. 2. Dose response curves for methacholine (MCh) challenges. n=10 per group. P_{enh}, enhanced pause; BCG, bacille Calmette-Guérin; TB, \textit{Mycobacterium tuberculosis}; Cul-S, culture-supernatant. \*P<0.05 and **P<0.01 for low dose treatments, and †P<0.05 and ††P<0.01 for high dose treatments compared with the asthma control.](http://e-aair.org)
**RESULTS**

**Airway responsiveness**

The percent increases in Penh relative to baseline after methacholine inhalation were significantly higher in the asthma group than the normal control group at each concentration of methacholine (P<0.01; Fig. 2). Compared to the asthma group, the Penh values were significantly lower in response to 3.125, 6.25, 12.5, and 25 mg/mL methacholine in mice treated with 1×10⁵ CFUs of live BCG. Similarly, 1×10⁶ CFUs of live BCG significantly decreased the values obtained after 3.125, 6.25, and 12.5 mg/mL methacholine treatment. Moreover, 20 μg BCG culture supernatant significantly decreased the Penh values at 3.125 and 12.5 mg/mL methacholine treatment while 200 μg BCG culture supernatant decreased the value at 12.5 mg/mL methacholine treatment. Both 20 and 200 μg *M. tuberculosis* mycobacteria did not significantly suppress airway responsiveness.

**Inflammatory cells in peribronchial tissue and epithelial goblet cells**

The asthma control group had significantly more peribronchial eosinophils than the normal control group (Fig. 4). All treatments except 1×10⁶ CFUs of heat-killed *M. tuberculosis* and 1×10⁵ CFUs of heat-killed BCG significantly suppressed peribronchial eosinophilia. The peribronchial neutrophil counts were also significantly higher in the asthma control group than in the normal control group (251±64 versus 57±19/mm², P<0.01), and were lower in all of the treatment groups but only significantly lower in those treated with 200 μg BCG (85±22/mm²), 200 μg *M. tuberculosis* (150±25/mm²), and 200 μg BCG culture supernatant (145±25/mm², P<0.001).

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Fig. 3. Comparisons of the airway responsiveness to methacholine (PC200) between the asthma control group and the other groups. n=10 per group. TB, *Mycobacterium tuberculosis*; BCG, bacille Calmette-Guérin; HK, heat-killed; Cul-S, culture-supernatant. *P<0.05, **P<0.01, and ***P<0.001 compared with the asthma control.

Fig. 4. Comparisons of the peribronchial eosinophil numbers and goblet cell hyperplasia in the bronchial epithelium. n=10 per group. TB, *Mycobacterium tuberculosis*; BCG, bacille Calmette-Guérin; HK, heat-killed; Cul-S, culture-supernatant. *P<0.05, **P<0.01, and ***P<0.001 compared with the asthma control.
P<0.05) or M. tuberculosis (93±12/mm², P<0.05) culture supernatant. Furthermore, the proportion of goblet cells in the epithelial cells was significantly lower in all of the treated groups than in the asthma control group (Fig. 4).

**Cytokine concentrations in the cultured splenocyte supernatants**

In the supernatants of concanavalin A-stimulated splenocytes, the IL-5 level was significantly higher, and the IFN-γ concentration and the IFN-γ/IL-5 ratio were significantly lower, in the asthma control group than in the normal control group (Fig. 5). Compared to the asthma group, the IFN-γ/IL-5 ratio was significantly higher in all treated groups except those treated with heat-killed mycobacteria. The IL-17A level was significantly higher in the asthma control group than in the normal control group (Fig. 4). We also found that vaccination with heat-killed mycobacteria was ineffective for airway responsiveness even though it significantly suppressed airway eosinophilia and goblet cell hyperplasia. Major et al.23 also showed that the efficacy of heat-killed BCG against the development of airway eosinophilia was lower than that of live BCG. The lower effectiveness of the culture supernatants and heat-killed preparations used in this study may be explained at least in part as follows: a) live BCG may not only release an effective factor after inoculation, but it also has cell wall components such as LAM and phosphatidylinositol that increase IL-10 secretion;14 b) the effects are dose-dependent3 and live BCG must work longer in vivo due to its proliferation potential; c) some components that actively suppress allergic responses are heat-labile. Means et al.25 showed that a heat-sensitive cell-associated mycobacterial factor distinct from LAM mediated TLR4-dependent activation. However, Zuany-Amorim et al.1 demonstrated that treating mice with heat-killed M. vaccae induces allergen-specific Treg cells and effectively suppresses methacholine airway responsiveness and airway eosinophilia. Therefore, if these less effective preparations are used instead of live mycobacteria as an asthma vaccine to avoid adverse effects, there may be a need to increase the vaccine dose or manipulate the vaccine content to increase their efficacy.

**DISCUSSION**

Treatment of mice with live BCG significantly suppressed airway responsiveness, eosinophilia, and goblet cell hyperplasia. These results are consistent with those of previous studies showing that BCG2-4,10,11,21 and M. vaccae18 have suppressive effects on asthma.

Furthermore, we found that the BCG and M. tuberculosis culture supernatants also effectively suppressed asthmatic reactions, although the effectiveness for suppressing airway responsiveness was somewhat lower in the culture supernatant groups than in the live BCG group. Means et al.22 demonstrated that M. tuberculosis culture media induce TLR2-dependent cellular activation, and suggested that TLR2 ligand was the non-proteinaceous heat-stable factor. Because TLR2 stimulation suppresses airway eosinophilia,26 a soluble factor released by cultured mycobacteria might have acted as a TLR2 ligand in the present study.

We also found that vaccination with heat-killed mycobacteria is 4-fold greater and M. vaccae18 have suppressive effects on asthma.
*Mycobacterium tuberculosis* culture supernatants or heat-killed preparations would be more effective for suppressing asthmatic reactions than those of BCG. However, this was not the case in the present study, and there were no differences between them. A large epidemiological study by von Mutius et al. showed that an increase in the tuberculosis notification rates of 25 per 100,000 was associated with a decrease in the prevalence of asthma by 4.7%, while another large epidemiological study by Grüber et al. reported that BCG vaccination had only a weak protective effect against asthma. Thus, the difference in IFN-γ levels between BCG and *M. tuberculosis* and its effect on asthma may be related to any difference in a heat-sensitive cell-associated mycobacterial factor. Further studies are needed to investigate this possibility.

The effects of the mycobacteria treatments, including the mycobacteria culture supernatants, on asthmatic reactions were associated with increases in the IFN-γ/IL-5 ratio. These results are consistent with those of previous studies showing that mycobacterial infections or administering mycobacterial components shift the Th1/Th2 balance toward Th1. IFN-γ seems to play a key role in the mycobacterial effects on Th2 cytokines and airway eosinophilia, because these effects are strongly impaired in IFN-γ-deficient mice. In addition, provocation via allergen inhalation induces IL-17 mRNA expression and neutrophilic influx in the airways of mice. Recently, McKinley et al. showed that transferring Th17 cells that produce IL-17 into mice results in neutrophilic airway inflammation and methacholine airway hyperresponsiveness. Therefore, the inverse relationship between PC200 and IL-17A in the present study may be attributable to neutrophils despite the lack of a significant relationship between IL-17A and neutrophils. However, IL-17 decreases airway eosinophilia and may reduce matrix deposition by inducing metalloproteinases. The complex action of IL-17 on asthma needs further investigation.

Because the airway cytokines and the ovalbumin-specific cytokines in the splenocyte culture supernatants were almost undetectable in a preliminary study, we did not check them in the present study. Future studies should consider airway cytokine profiles, especially for allergen-specific cytokines, using other methods such as Western blotting. Furthermore, lung function measurements should be validated using more rigorous methods, even though several previous studies used Penh values as an airway hyperresponsiveness measure.

Taken together, our results show that both live BCG and mycobacteria culture supernatants are effective for suppressing the development of asthmatic reactions. Therefore, the use of soluble culture supernatant components is promising for the development of asthma treatments without the adverse effects associated with whole live BCG. Heat-killed preparations were also effective for suppressing airway eosinophilia and remodeling. However, the loss of an effect on airway hyperresponsiveness suggests that further studies of mycobacterial cell wall components are needed to develop more effective vaccines to treat asthma. Finally, the effects of mycobacteria and their components were associated with altered levels of Th1/Th2 cytokines and interleukin-17A.

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